MINISTÉRIO DA SAÚDE
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO OSWALDO CRUZ
PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR
DOUTORADO EM BIOLOGIA CELULAR E MOLECULAR

Triagem de extratos vegetais e fúngicos de diferentes biomas para identificação de antagonistas do receptor P2X7

RÔMULO JOSÉ SOARES BEZERRA

Orientadores: Prof. Dr. Luiz Anastacio Alves
Prof. Dr. Valber da Silva Frutuoso

Rio de Janeiro
2012

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

INSTITUTO OSWALDO CRUZ
Pós-Graduação em Biologia Celular e Molecular

RÔMULO JOSÉ SOARES BEZERRA

Triagem de extratos vegetais e fungicos de diferentes biomas brasileiros para identificação de antagonistas de receptores P2 com foco no receptor P2X7

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Ciências. Área de concentração: Farmacologia e Imunologia.

ORIENTADORES: Prof. Dr. Luiz Anastacio Alves
Prof. Dr. Valber da Silva Frutuoso

Rio de Janeiro
2012

Ficha catalográfica elaborada pela
Biblioteca de Ciências Biomédicas/ ICICT / FIOCRUZ - RJ

B574 Bezerra, Rômulo José Soares

Triagem de extratos vegetais e fúngicos de diferentes biomas para identificação de antagonistas do receptor P2X7 / Rômulo José Soares Bezerra. – Rio de Janeiro, 2012.

xix, 199 f. il. ; 30 cm.

Tese (Doutorado) – Instituto Oswaldo Cruz, Pós-Graduação em Biologia Celular e Molecular, 2012.
Bibliografia: f. 131 - 142

1. Receptores P2. 2. Screening. 3. Antagonistas. 4. Produtos naturais. I. Título.

CDD 615.32

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

INSTITUTO OSWALDO CRUZ
Pós-Graduação em Biologia Celular e Molecular

RÔMULO JOSÉ SOARES BEZERRA

Triagem de extratos vegetais e fungicos de diferentes biomas brasileiros para identificação de antagonistas de receptores P2 com foco no receptor P2X7

ORIENTADORES: Prof. Dr. Luiz Anastacio Alves
Prof. Dr. Valber da Silva Frutuoso

Aprovada em: 29/10/2012

EXAMINADORES:

Prof. Dr. Marco Aurélio Martins – Presidente

IOC – FIOCRUZ - RJ

Prof. Dr. Pedro Muanis Persechini

Deptº. Biofísica – UFRJ - RJ

Prof. Dr. Eduardo Caio Torres dos Santos

IOC – FIOCRUZ - RJ

SUPLENTES:

Prof. Dra. Patrícia Machado Rodrigues e Silva
IOC – FIOCRUZ – RJ

Prof. Dr. Vinícius Cotta de Almeida
IOC – FIOCRUZ - RJ

Rio de Janeiro, 29 de Outubro de 2012.

DEDICATÓRIA

Dedico este trabalho a Deus,
a toda minha família (especialmente ao meu pai, minha mãe (*in memoriam*), minha irmã (*in memoriam*) e ao meu irmão, ao meu eterno pai científico Dr. Marcelo Genestra (*in memoriam*) e aos meus Orientadores pela oportunidade de concluir essa etapa na minha vida.

AGRADECIMENTOS

Primeiramente agradeço a DEUS todo poderoso e ao seu Filho amado JESUS CRISTO, pela graça a mim concedida de chegar até aqui vivo e com saúde;

Ao meu pai, por ter acreditado em mim e apoiado desde a faculdade e agora no doutorado, sendo compreensivo com meus ataques de estress, não deixando que eu nunca desistisse dos estudos, por mais árduos que os períodos fossem e por todo apoio financeiro (espero retribuir em dobro quando for pesquisador IA do CNPq);

Ao meu mano querido Rafael Antônio pela força, pelas broncas, pelo apoio nos momentos em que precisei e pelas “injeções de ânimo” quando este se fez ausente em mim;

A minha querida Mãezinha que já não se encontra mais entre nós e sei que está olhando por mim lá de cima e junto com a minha também já falecida Irmãzinha, estão torcendo e zelando por mim;

Ao meu caro e para sempre orientador Marcelo da Silva Genestra (*In memoriam*), que foi um pai, irmão, amigo, mestre e companheiro. Por ter concedido a mim a chance de fazer parte da comunidade científica desta renomada instituição de pesquisa. Espero me tornar um futuro Professor Doutor com ética, postura, conhecimento e humildade semelhantes aos seus;

Felizmente Deus, em sua infinita sabedoria, não me deixou desamparado, ao ter levado meu querido “pai científico”, ele me concedeu dois “pais” do mesmo quilate do que me foi tomado. Por isso venho aqui prestar os meus agradecimentos a eles, primeiramente ao Dr. Luiz Anastacio Alves, que me abrigou em seu laboratório e assumiu a minha “arriscada” orientação, devido ao curto período de tempo que restava. Obrigado pela dedicação nas horas de estudo, discussão dos dados, sugestões, madrugadas escrevendo e discutindo artigos, assim como

pelos momentos de “puxões de orelha”, por acreditar em mim. Enfim um verdadeiro orientador-educador, ou melhor, um “Pai educador”;

Ao estimado Orientador Dr. Valber da Silva Frutuoso pelo apoio, dedicação, por também ter aberto as portas do seu laboratório para que eu executasse parte da minha tese, inclusive os experimentos *in vivo*, pelos momentos de discussão de resultados e sugestões, também pelos necessários “puxões de orelha”.....Obrigado por também acreditar no doutor que esta formando e espero ser um orientador como você no futuro...Obrigado por tudo...serei eternamente grato!

A toda equipe do Laboratório de Comunicação Celular/IOC/ FIOCRUZ: Dra. Cristina Souza (Vice-chefe) pelas conversas, orientações e sugestões, um verdadeiro “anjo-amigo”; Dr. Robson Xavier pelos conselhos e sugestões sempre úteis para o aprimoramento do meu trabalho, aos Drs. Pedro Celso e Renato Mattos também pela presteza nos momentos que precisei de sua ajuda;

A toda equipe do LCC e da Imunofarmacologia que me ajudou quando precisei e pelos momentos de descontração, quando o trabalho se tornava bastante exaustivo;

As minhas “irmãzinhas” da Imunofarmacologia: Zanon, Surrage e Juliana....e ao meu “irmãozinho” Diogo;

A minha “pequena notável”, Natiele Ferreira, minha aluna de iniciação científica que compartilhou comigo os momentos de bancada, aprendizado e superação....Agradeço também pelo companheirismo e pela presteza sempre ofertados a mim.

Ao meu “irmãozinho” de doutorado Anael Viana, pelas dicas, conselhos, sugestões e pelo apoio que recebi desde que entrei no laboratório, o qual foi de suma importância para que eu me adaptasse no laboratório e no meu novo projeto;A minha “irmãzinha” doutoranda Kátia Quaresma, que também foi peça

fundamental para a minha adaptação no meu “novo lar”, obrigado pelos conselhos, pelas sugestões valiosas, pelos momentos de descontração quando o trabalho pesava, pela amizade valiosa que nutrimos nesse período de tese.

A “irmãzinha olho junto” doutoranda Carla Santos, pela amizade, conselhos, ajuda e principalmente pelas valiosas caronas para Niterói...rs!

Aos alunos de iniciação científica: Raquel, Liana, Tiago, Simone e Rafael.

Aos Mestrandos Leonardo Braga, Dinarte Neto e Paulo Furtado pelos conhecimentos trocados.

A nossa “preciosa” Ivone Luci, por me ajudar nos trâmites burocráticos inerentes a qualquer pesquisa.

À Dra. Tânia Alves e a sua Tecnologista Fernanda Francisco pelo carisma, pela presteza e por terem cedido os extratos testados nesse trabalho.

A todos os meus colegas de pós-graduação do IOC.

Aos Funcionários do setor de ensino, especialmente a Daniele, secretária da pós em Biologia Molecular e celular, pela paciência, presteza e compreensão.

Aos coordenadores Alexandre Peixoto e Leila Mendonça.

Ao Coordenador anterior, Milton Ozório, por ter acreditado em mim no momento que mais precisei da sua ajuda....Essa vitória devo a você também!

Aos membros da banca: Dr. Pedro Persechini, Dr. Marco Aurélio Martins, Dr. Eduardo Caio Torres, Dr. Vinícius Cotta e Dra. Patrícia Silva. Por estarem contribuindo de forma decisiva na minha formação.

As agências de fomento: CAPES, CNPq, FAPERJ e FIOCRUZ.

Obrigado!

“O gênio é composto por 2% de talento e de 98% de perseverante aplicação.” (Ludwig Van Beethoven)

“Há pessoas que desejam saber só por saber, e isso é curiosidade; outras, para alcançarem fama, e isso é vaidade; outras, para enriquecerem com a sua ciência, e isso é um negócio torpe: outras, para serem edificadas, e isso é prudência; outras, para edificarem os outros, e isso é caridade”
(S. Tomás de Aquino)

SUMÁRIO

LISTA DE ABREVIATURAS

2-meSATP: 2-metiltio- adenosina 5’-trifosfato

A438079: 3-[[5-(2,3-diclorofenil)-1H-tetrazol-1-il] metil] piridina hidroclorídica

A740003: N-(1-([(cianoamino)(5-quinolinilo amino) metil] amino)-2,2-dimetilpropil)-2-(3,4- dimetóxifenil)acetamida

A804598: 2-ciano-1-[(1S)-1-feniletil]-3-quinolina-5-il-guanidina

ADO – Adenosina

ADP: Adenosina 5’-difosfato

ALT – Alanina Aminotransferase ou TGP – Transaminase Glutâmico Pirúvica

AMP: Adenosina 5’-monofosfato

AST – Aspartato Aminotransferase ou TGO – Transaminase Glutâmico Oxaloacética

ATP: Adenosina 5’-trifosfato

AZ10606120: N-[2-[[2-[(2-hidróxietil)amino]etil]amino]-5-quinolinil]-2-triciclo[3.3.1.13,7]dec-1-il-acetamida dihidroclorida

AZ11645373: 3- [1- [[(3’-nitro [1,1’-bifenil]-4 -il) oxi] metil]-3-(4-piridinil) propil]-2,4-tiazolidinadiona

BBG: Coomassie Azul Brilhante G (Coomassie Brilliant Blue G)

bis-1,3,5-naftaleno ácido trisulfônico

BOD – “Biochemical Oxygen Demand” (Demanda bioquímica de oxigênio)

BzATP: 2’(3’)-O-(4-Benzoilbenzoil) adenosina 5’-trifosfato

Ca2+: Cálcio (íon)

CaCl2: Cloreto de cálcio

CD 14- Co-receptor expresso na membrana de macrófagos e células dendriticas

CRAMP: Peptídeo antimicrobiano relacionado à catelina

DMSO – Dimetil Sulfóxido

DNA – “Desoxirribonucleic acid” (Ácido desoxirribonucléico)

DO – Densidade óptica

EC50: Concentração eficaz em 50% da população (effective concentration)

FRET: transferência de energia por ressonância

HEK293: Célula de rim de embrião humano 293

HEPES: 4-(2-hidróxietil)-1-ácido piperazinaetanosulfônico

IC50: Concentração que causa inibição em 50% da população (inhibitory concentration)

IL (2,3,4,5,10,12,13) – Interleucina do tipo 2,3,4,5,10,12,13

IL-18: Interleucina-18

IL-1β: Interleucina-1 beta

IL-6: Interleucina-6

K+: Potássio (íon)

KCl: Cloreto de potássio

KN-62: 1-[N,O-bis(5-isoquinolinasulfonil)-N-metil-L-tirosil]-4-fenilpiperazina

LPS- Lipopolisacarídeo

MAPK- “Mitogen-activated Protein Kinase” (Proteína quinase ativada por mitógeno)

MgCl2: Cloreto de magnésio

MRS2159:4-[(4-formil-5-hidróxi-6-methil-3-[(fosfonooxi)metil}-2-piridinil)azo]-ácido benzóico

MTT – Brometo de 3-[4,5-dimetiltiazol-2-il]-2,5-difenil-tetrazol

Na+: Sódio (íon)

NaCl: Cloreto de sódio

NF279: 8,8'-[carbonilbis(amino-4,1-fenilenocarbonilamino-4,1-fenilenocarbonilamino)]

NK – "Natural Killer"

NO – Óxido nítrico

NOS – Òxido Nítrico Sintase

O2- – Superóxido

OATP – ATP oxidado

OMS – Organização Mundial de Saúde

ONOO- – Ânion peroxinitrito

P1= Receptor purinérgico do tipo 1

P2 = Receptor purinérgico do tipo 2

P2X = Receptores Purinérgicos metabotrópicos da família X

P2X7 = Receptor Purinérgico Ionotrópico da subclasse X do tipo 7

P2X7R = Receptor Purinérgico Ionotrópico P2X7

P2XR: receptores P2X

P2Y = Receptores Purinérgicos Metabotrópicos da família Y

PBS – "Phosphate Buffer Saline" (Tampão Salina Fosfatado)

PKC – Proteína Quinase C

PPADS: Piridoxal 5-fosfato 6-azofenil-2'-, 4'- ácido disulfônico

PPNDS: Piridoxal-5’-fosfato-6-(2’-naftilazo-6’-nitro-4’-,8’-disulfonato

PTN G- Proteína G.

RFU: Unidade de fluorescência relativa

ROS- “Reactive Oxygen Species” (Espécie reativas de oxigênio).

RPMI 1640: Instituto Memorial Parque Roswell 1640

SFB – Soro Fetal Bovino

SFM – Sistema Fagocítico Mononuclear

TGF-β–“Transforming Growth Factor beta” (Fator de crescimento de transformação beta)

TLR-4 – Toll like receptor 4

TNF-α: fator de necrose tumoral

UDP: Uridina 5’-difosfato

UDP-glicose: Uridina 5’-difosfato-glicose

UTP: Uridina 5-trifosfato

αβ-meATP: αβ- metileno-adenosina 5’-trifosfato

βγ-meATP: βγ-metileno-adenosina 5′-trifosfato

RESUMO

O P2X7 é um receptor purinérgico que está envolvido em importantes funções fisiológicas e metabólicas, mas também tem participação em diversas patologias, principalmente aquelas de caráter inflamatório. Apesar de sua relevância, ainda não se têm disponíveis antagonistas específicos que possam ser utilizados na clínica para o tratamento de doenças relacionadas à ativação deste receptor. Muitos fármacos comercializados nos dias de hoje apresentam estruturas químicas relacionadas a um produto natural extraído de alguma espécie botânica de uso consagrado na medicina popular. Baseia-se nisso a relevância do estudo de produtos naturais para obtenção de uma atividade específica sobre alvos celulares. Logo, esse trabalho teve como interesse o estudo de extratos vegetais e fúngicos obtidos de espécies dos diferentes biomas, cedidos pelo LQPN do Centro de Pesquisas René Rachou – Fiocruz-MG, visando á identificação de antagonistas para o receptor P2X7. Nosso primeiro passo foi à padronização de uma metodologia que permitiu a triagem de cerca de 60 extratos ao mesmo tempo, através da utilização de um espectrofotômetro de placas. Depois de padronizada, promovemos a aplicação dessa metodologia na triagem de 1800 extratos, dos quais apenas três extratos (8067,8549 e 8568) apresentaram atividade antagonista na faixa de corte pré-estabelecida [100 µg/mL], com perfis de inibição de 65 %, 62 % e 61 % respectivamente, sobre o P2X7R. Destes extratos foram determinados os $IC´s_{50}$ tanto em células de linhagem murina (2,1 µg/mL; 2.6 µg/mL e 3.8 µg/mL) quanto em células de linhagem humana (0.69, 0.92 e 1.5 µg/mL), sendo possível verificar maior atividade quando testados em células de linhagem humana. Posteriormente avaliamos a ação destes sobre funções fisiológicas relacionadas à ativação do P2X7R, nessa etapa, observamos o efeito inibitório destes extratos sobre a liberação de IL-1beta, ROS e NO, onde os três compostos foram capazes de inibir estas funções numa faixa entre 50 % a 60 %. Para obtermos uma caracterização do efeito farmacológico destes extratos sobre o receptor P2X7, realizamos experimentos de eletrofisiologia, caracterizando assim uma ação inibitória dose-dependente destes, sendo que nos respectivos $IC´s_{50}$ os perfis de inibição da corrente foram de: 76 %, 47 % e 75 %. Também avaliamos a citotoxicidade *in vitro* utilizando as células de ambas às linhagens e verificamos que não apresentaram significativa toxicidade quando tratadas por 24 h. em doses até quatro vezes maiores que o IC_{50}, visto que os resultados foram semelhantes ao controle não tratado. Depois de avaliarmos a atividade antagonista destes extratos *in vitro*, partimos para os experimentos *in vivo* utilizando os modelos de úlcera induzida por etanol e de dor neuropática e inflamatória, pois existem trabalhos previamente descritos na literatura que correlacionam a atividade do P2X7R com a evolução dessas patologias. No ensaio de dor neuropática, apenas dois extratos mostraram atividade analgésica (8067 e 8549) inibindo o estímulo de dor em 68 % e 66 %, porém no contexto da dor inflamatória os três extratos mostraram efeito analgésico, inibindo o estímulo em: 8067 = 48%, 8549 = 50% e 8568 = 44 %. Os resultados obtidos do experimento de úlcera induzida por etanol demonstraram o efeito inibitório sobre a formação de úlceras desses extratos em: 88 %, 84 % e 51 %, inclusive foram mais efetivos que o BBG (antagonista reversível deste receptor) e que o medicamento utilizado na clínica (Lansoprazol), os quais inibiram a formação de úlceras em 43 % e 46% respectivamente. Nosso conjunto de resultados apontam extratos com significativa atividade antagonista sobre o P2X7R, com potencial para o desenvolvimento de novos fármacos com grande interesse para a indústria farmacêutica, além de contribuir para o conhecimento acerca de propriedades medicinais presentes na biodiversidade.

Palavras-chave: Receptores P2; Screening; Antagonistas; Produtos Naturais.

ABSTRACT

The purinergic receptor P2X7 is involved in important physiological and metabolic functions, but it also participates in pathology, especially when inflammatory in character. Despite the importance of P2X7, it has no specific antagonists yet available for use in clinical treatment of diseases related to the receptor's activation. Today, many drugs on the market have chemical structures related to natural products obtained from botanical species with traditional use in indigenous medicine, forming the basis for studying natural products to obtain specific activity on cellular targets. This work focused primarily on the study of plant and fungal species extracts obtained from different biomes provided by LQPN of the Research Center René Rachou - Fiocruz-MG, aiming to identify antagonists for the P2X7 receptor. The first step was to standardize a method that allowed the screening of approximately 60 extracts at the same time through the use of a plate spectrophotometer. Once standardized, the application of this methodology was promoted in the screening of 1800 extracts. Of these, only three extracts (8067, 8549 and 8568) showed antagonistic activity in the pre-established cut range [100 mg/mL], with inhibition profiles of 65%, 62%, and 61% respectively, on the P2X7R. The IC's50 of them were determinate in murine (2.1, 2.6, and 3.8 mg/mL) and human (0.69, 0.92, and 1.5 mg/mL) cell lines. Which an increased activity was possible to verify when they were tested in human cells. Consequent evaluation of action on physiological functions related to the activation of P2X7R revealed an inhibitory effect of these extracts on the release of IL-1beta, NO, and ROS. The three tested compounds were able to inhibit these functions in a range between 50% and 60%. To obtain a pharmacological characterization of these extracts on the P2X7 receptor, electrophysiological experiments were conducted, which characterized the dose-dependent inhibitory effects, exhibiting inhibitory current profiles of 76%, 47%, and 75%, respectively. In vitro cytotoxicity was also evaluated, using both cell strains, showing no significant toxicity after 24 hours of treatment at doses of up to four times that of the IC50; the results were similar to the untreated control. After evaluating the antagonistic activity of these extracts in vitro, experiments using the in vivo models of ethanol-induced ulcers and inflammatory and neuropathic pain were performed. Previous studies correlate the activity of the P2X7R with the evolution of these pathologies. In the neuropathic pain protocol experiment, only two extracts showed analgesic activity (8067 and 8549) by inhibiting the pain stimulation by 68% and 66%, but in the context of inflammatory pain, the three tested extracts showed analgesic effects by inhibiting the stimulus by the following percentages: 8067 = 48 %, 8549 = 50%, and 8568 = 44%. The results of the ethanol-induced ulcer demonstrated an inhibitory effect on the ulcer's development of 88%, 84%, and 51% by these extracts, and found that the extracts were more effective than BBG (reversible antagonist of this receptor) and the medicine used clinically (Lansoprazole), which inhibited the formation of ulcers by 43% and 46%, respectively. The data set links to extracts with significant antagonist activity on the P2X7R, and potential to the development

of new medicines of great interest to the pharmaceutical industry and important contributions to the knowledge of medicinal properties present in biodiversity.

Keywords: P2 receptors, screening, antagonists, natural products.

1. INTRODUÇÃO

1. INTRODUÇÃO

1.1 UM BREVE HISTÓRICO

As purinas e pirimidinas extracelulares são moléculas importantes na sinalização de diversos processos biológicos como: resposta imune, neurotransmissão, contração do músculo liso, inflamação, dor, entre outros. Estas moléculas atuam via receptores de superfície de membrana denominados receptores purinérgicos. Entretanto, essa descoberta iniciou-se em 1929, quando Drury e Szent-Györgyi demonstraram que a adenosina e a adenosina 5'-monofosfato (AMP) extraídas do músculo cardíaco eram capazes de promover efeitos biológicos que incluíam a dilatação arterial e a diminuição da pressão sanguínea (Drury et al. 1929). Seguindo essa mesma lógica, Gillespie, em 1934, em outro estudo realizado com gatos e coelhos, relatou que o ATP e a adenosina possuíam ações funcionais distintas. Logo, ele observou a estrutura da adenosina e percebeu que a sua desaminação era capaz de reduzir a atividade farmacológica, enquanto que a remoção do fosfato influenciava tanto na sua potência quanto no tipo de resposta, o que resultou na hipótese de que haveria diferentes tipos de receptores de adenosina (Gillespie 1934).

Em 1935, Katashi Makino propôs a primeira conformação estrutural da molécula de ATP, a qual foi confirmada em 1945 (apud Ralevic & Burnstock 1998). A partir de então diversos grupos de pesquisa dedicaram-se ao estudo da função da adenosina e do ATP *in vitro* e *in vivo*, bem como suas ações em diferentes tipos de tecidos, observando seus efeitos e respostas. Porém, de todos os

resultados obtidos, destaca-se um de grande importância: a descoberta do papel do ATP na neurotransmissão, atuando possivelmente como um neurotransmissor ou co-transmissor. Holton e Holton (1953; 1959 apud Ralevic & Burnstock 1998) observaram que o ATP era liberado de nervos sensoriais na orelha de coelhos enquanto eles estimulavam a sua vasodilatação.

Sabendo-se dessa diferença de ação entre a adenosina e o ATP, Burnstock em 1972 postulou a existência dos "nervos purinérgicos" que utilizavam a molécula de ATP como neurotransmissor. O que norteou os estudos acerca da existência de receptores para purinas e pirimidinas, posteriormente em 1978, Burnstock propôs uma primeira classificação formal para os receptores destas moléculas, isto é, os receptores purinérgicos. Estes foram divididos em duas famílias: receptores P1, os quais tem a adenosina como principal ligante natural, e os receptores P2, nos quais esses ligantes são o ADP e o ATP. Entretanto, na medida em que os estudos avançavam, foram descobertos novos subtipos em ambas as classes, bem como diferenças estruturais dentro do grupo dos receptores P2, o que levou a sua reclassificação em duas classes de receptores: os P2XR (ionotrópicos) e os P2YR (metabotrópicos) (Apud Ralevic & Burnstock 1998).

1.2. RECEPTORES PURINÉRGICOS

Os receptores purinérgicos se dividem em receptores P1 e P2. Os receptores P1 possuem quatro subtipos: A1, A2A, A2B e A3. Esses receptores já foram clonados a partir de células de várias espécies, entretanto sua

caracterização está de acordo com as propriedades moleculares, bioquímicas e farmacológicas presentes em células de mamíferos ou em oócitos de *Xenopus*. Eles são receptores metabotrópicos, isto é, estão associados à proteína G, sendo ativados por adenosina e possuindo por antagonistas não-seletivos as xantinas e derivados, incluindo a teofilina e a cafeína, que são produtos naturais (Ralevic & Burnstock 1998).

Já o grupo dos receptores P2 é dividido em duas classes segundo Abbracchio e Burnstock (1994): receptores P2X que são ionotrópicos e os receptores P2Y, os quais assim como os receptores P1 são metabotrópicos, como pode ser observado na fig.1.

1.3. RECEPTORES P2

A distinção de dois tipos de receptores purinérgicos P2, definidos como P2XR e P2YR ocorreu em 1985 (Chang et al. 1985), porém somente a partir da clonagem destes receptores, nos anos 90, foi possível uma classificação mais detalhada dos seus subtipos (Ralevic & Burnstock 1998). Os P2XR são receptores ionotrópicos, ou seja, permitem a passagem de íons de acordo com o gradiente eletroquímico quando ativados (fig.1). Existem sete subtipos de P2XR caracterizados em mamíferos até o momento, como demonstrado na tabela 1.

Os P2YR, por sua vez, são metabotrópicos, ou seja, receptores acoplados a uma proteína G, que ativam segundos mensageiros de diversos processos bioquímicos e fisiológicos, como por exemplo, a ativação da fosfolipase C, MAPK

e inibição de adenilato ciclase. Existem oito subtipos caracterizados em mamíferos como observamos na tabela 1.

Tabela 1– Distribuição dos P2R

Receptor	Distribuição principal	Vias de Transdução
P2X		
$P2X_1$	Músculo liso, plaquetas, cerebelo, corno dorsal, neurônios espinhais	Canal iônico intrínseco com preferência por cátions (especialmente Ca^{2+})
$P2X_2$	Músculo liso, SNC, retina, células cromafins, gânglios autônomos e sensoriais	
$P2X_3$	Neurônios sensoriais, núcleo do trato solitário. alguns neurônios simpáticos	
$P2X_4$	SNC, testículo, cólon	
$P2X_5$	Células proliferativas: na pele, intestino, bexiga, timo, medula espinhal	
$P2X_6$	SNC, neurônios motores na medula espinhal	
$P2X_7$	Células do sistema imunológico, do sistema gastrointestinal, do sistema reprodutor e no sistema nervoso central e periférico.	Canal iônico intrínseco com preferência por cátions e formação de um canal de larga condutância através da ativação prolongada
P2Y		
$P2Y_1$	Células epiteliais , plaquetas, células do sistema imunológico, osteoclastos	Gq/G_{11}; PLC-β ativação
$P2Y_2$	Células do sistema imunológico, células epiteliais , túbulos renais, osteoclastos	Gq/G_{11} e possivelmente Gi/Go; ativação PLC-β
$P2Y_4$	Células endoteliais	Gq/G_{11} e possivelmente Gi, ativação PLC-β
$P2Y_6$	Algumas células epiteliais, placenta, células T, timo	Gq/G_{11}; ativação de PLC-β
$P2Y_{11}$	Baço, intestino, granulócitos	Gq/G_{11} e G_S; ativação PLC-β
$P2Y_{12}$	Plaquetas, células da glia	$G\alpha_i$, inibição da adenilato ciclase
$P2Y_{13}$	Baço, cérebro, linfonodos, medula óssea	Gi/Go
$P2Y_{14}$	Placenta, tecido adiposo, estômago, intestino e na neuroglia	

(modificado de Burnstock et.al. 2007)

Os P2YR e P2XR possuem mecanismos de ativação diferenciados de acordo com a fig.1:

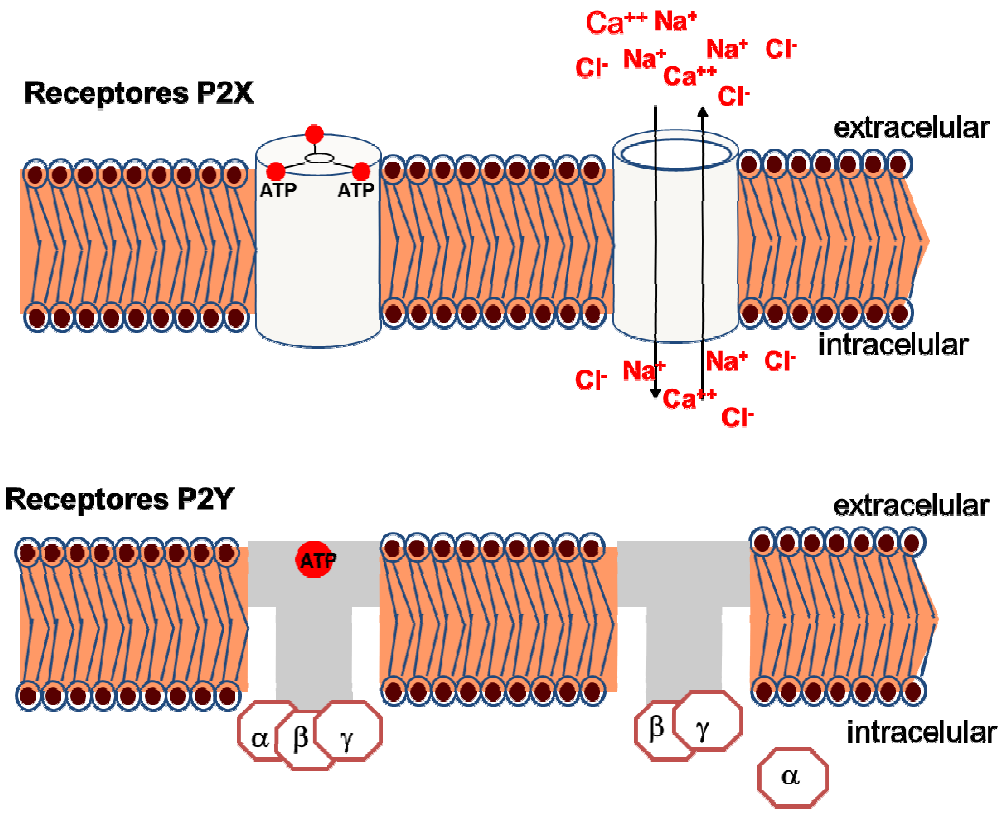


Fig.1: Mecanismos de ativação dos receptores (modificado de Burnstock et al. 2009). Os P2XR são ativados pelo ATP levando à abertura de um canal cátion seletivo na membrana. Já os P2YR, quando ativados pelo ATP, levam à ativação de vias metabólicas intracelulares, como por exemplo, a ativação de uma proteína G.

1.4. RECEPTORES P2X

Os P2XR, quando ativados, formam um canal catiônico não seletivo na membrana celular de diversas espécies, desde organismos unicelulares até humanos, porém a sua filogenia ainda não foi completamente estabelecida. Dentre os organismos que apresentam esses tipos de receptores em suas membranas, o

mais simples é um tipo de alga verde, um organismo eucariótico denominado *Ostreococcus tauri* (Fountain et al. 2008).

Os P2XRs se diferenciam estruturalmente de outros tipos de receptores iônicos, como por exemplo, os da família de receptores para glutamato, ou acetilcolina e nicotinamida, por serem triméricos, já que os outros receptores são tetraméricos, pentaméricos ou hexaméricos, com sítio para ação de uma proteína quinase e são acoplados a uma proteína G (Vial et al. 2004). A região do "loop" extracelular possui aproximadamente 30-353 aminoácidos, sendo 93 desses conservados em cerca de seis membros dessa família de receptores, podendo então estar envolvidos com a ligação com o ATP. Este "loop" extracelular é glicosilado, possuindo 10 resíduos de cisteína conservados, os quais formam pontes de dissulfeto para formarem a estrutura secundária do receptor (Vial et al. 2004).

Os subtipos de receptores que constituem essa família possuem dois domínios transmembranares (TM1 e TM2), com duas terminações N e C intracelulares e um longo "loop" extracelular. (figura 2). O domínio TM1 tem sido relacionado com a função de abertura e fechamento do canal catiônico e o domínio TM2 faz parte do canal de larga condutância. Além disso, os perfis de fases de sensibilização e dessensibilização desses poros são distintos, na presença do ATP, para cada tipo de receptor (figura 3) (Khakh and North 2012).

Os P2XR são distintos tanto farmacologicamente como quanto à atividade fisiológica exercida através da sua ativação, quanto da cinética de ativação e inativação. Por exemplo, alguns receptores dessa família abrem canais mais

permeáveis aos íons cálcio do que outros, uma possível explicação para as diferenças de funções existentes (Burnstock et al. 2004). Coddou et al., 2011, denominou como “sítios ortostéricos” os sítios de ligação do ATP com esses receptores, devido à necessidade destes para a ligação e consequente mudança conformacional em sua estrutura promovendo assim a abertura do canal catiônico não-seletivo.

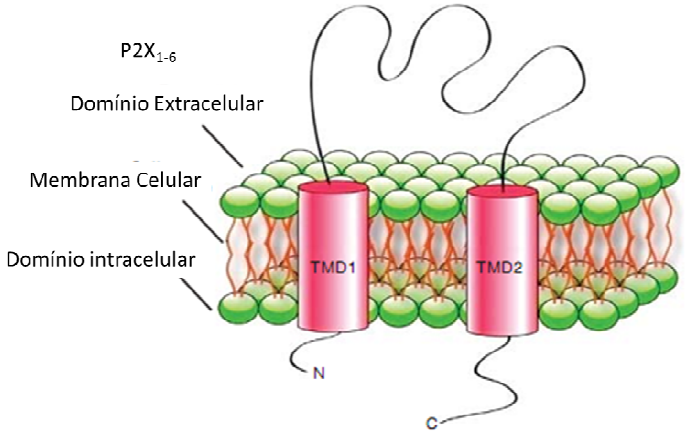


Fig.2: Estrutura do P2XR (Gunosewoyo et al. 2010). Os $P2X_{1-6}$Rs possuem dois domínios transmembranares (TM1 e TM2), com duas terminações N e C intracelulares e um longo “loop” extracelular.

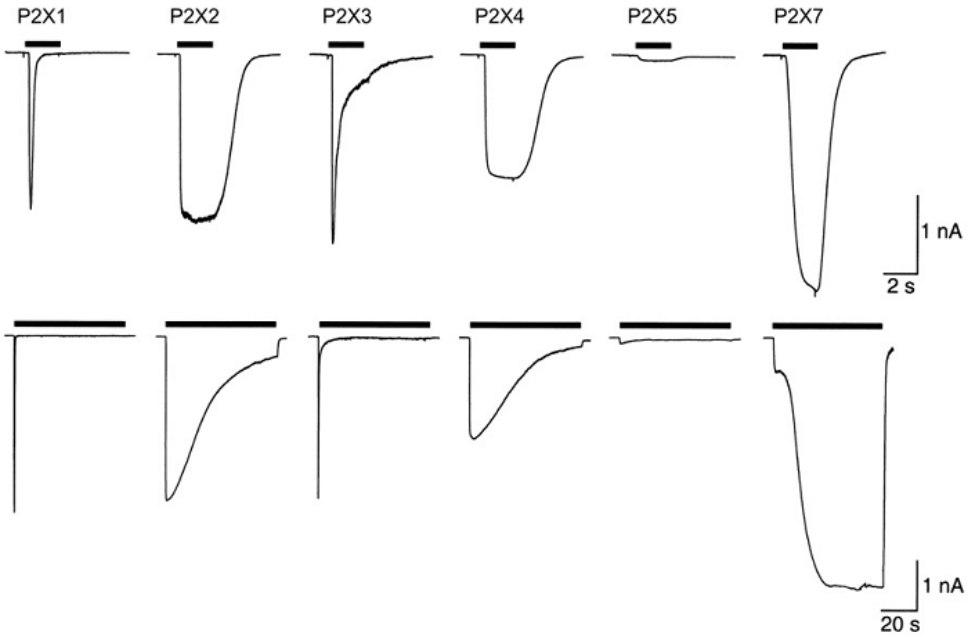


Fig.3: Caracterização eletrofisiológica dos P2XRs através da técnica de "whole-cell" em células HEK-293 transfectadas com cada um dos receptores na presença do ATP [30 μM] para os receptores P2X1, P2X2, P2X3, P2X4 e P2X5 e [1 mM] para o P2X7R. Os registros do tempo de dessensibilização de cada receptor na presença do agonista foram feitos 2 e 20 segundos após o tratamento. Como observado, o P2X7R possui uma dessensibilização lenta, quase inexistente em relação aos outros receptores. (khakh and North 2012).

A caracterização biofísica dos P2XRs nativos ou recombinados foi fundamental para formação da hipótese sobre a qual os canais iônicos formados por estes receptores são homotrímeros ou heterotrímeros através de experimentos de microscopia de força atômica (Khakh and North 2012). A participação de três moléculas de ATP para abertura do canal foi observada através da análise da ativação dos P2XR de forma dependente da concentração do agonista, por técnicas de "patch-clamp" nas modalidades "whole-cell" (análise

de todos os receptores expressos na membrana da célula) ou “single-cell” (análise de um único receptor). Outras técnicas como eletroforese em gel de poliacrilamida, microscopia de força atômica e microscopia eletrônica, confirmaram a natureza trimérica dos P2XR. Uma série de 11 homotrímeros foi descrita através de experimentos de imunopreciptação. Porém, até agora foram caracterizados seis heterotrímeros funcionais de P2XRs: P2X1/2R, P2X1/4R, P2X1/5R, P2X2/3R, P2X2/6R e P2X4/6R (Coddou et al. 2011).

Os homotrímeros e heterotrímeros dos P2XRs respondem de forma específica aos estímulos dos agonistas e à ação de antagonistas. Por isso, uma das formas de caracterização farmacológica de cada um desses receptores é a utilização da ordem de potência dos agonistas e antagonistas e de características cinéticas na eletrofisiologia, nos seus respectivos EC´s_{50} e IC´s_{50}, disponíveis em relação a cada receptor (tabela 3):

Tabela 3 - Caracterização farmacológica dos P2Rs

	P2X1	P2X2	P2X3	P2X4	P2X5	P2X6	P2X7
Gene name	*P2RX1*	*P2RX2*	*P2RX3*	*P2RX4*	*P2RX5*	*P2RX6*	*P2RX7*
Desensitization (Complete in)	Fast (<1 s)	Slow (>20 s)	Fast (<1 s)	Slow (>20 s)	Slow (>20 s)	—	Slow (>20 s)
Pore "dilation"	No	Yes	No	Yes	—	—	Yes
P_{Ca}/P_{Na}	4.8	2.8	1.2	4.2	1.5	—	—
Fractional Ca^{2+} current (%)	12.4	5.7	2.7	11	4.5	—	4.6
KO made	Yes	Yes	Yes	Yes	—	—	Yes
Agonist EC_{50} Values (μM)							
ATP	0.07	1.2	0.5	10	10	12	100
2MeSATP	0.07	1.2	0.3	10	10	9	100
αβmeATP	0.3	>300	0.8	>300	>300	>100	>300
BzATP	0.003	0.75	0.08	7	>500	—	20
Antagonist IC_{50} Values (μM)							
Suramin	1	10	3	>500	4	>100	500
PPADS	1	1	1	>500	3	>100	50
TNP-ATP	0.006	1	0.001	15	—	—	>30
IP_5I	0.003	>300	2.8	Potentiation	—	—	
A-317491	>10	>100	0.10	>100	—	>100	>100
RO-3	>100	>100	0.10	>100	>100		>100
A-740003	>100	>100	>100	>100	—	>100	0.05
AF-353	>10	>10	0.01	>10	>10		>10
A-438079	>100	>100	>100	>100	—	>100	0.06
A-804598	>100	>100	>100	>100	—	>100	0.01
MRS2179	80	>100	>100		—	—	>100
NF279	9	30	50	>100	—	—	20
NF449	0.7	>100	>100	>100	—	—	>100
Modulator EC_{50} Values (μM)							
Ivermectin	—	>30	>30	0.25	—	—	>30
Zn^{2+}	—	Increase EC_{50} = 7 μM	—	Increase 2 μM	—	—	Decrease IC_{50} = 10 μM
H^+	Decrease pKa 6.3	Increase pKa 7.3	Decrease pKa 6.0	Decrease pKa 6.8	—	—	Decrease pKa 6.1

Tabela 3: Caracterização farmacológica dos P2Rs com base no perfil eletrofisiológico e o perfil farmacológico dos agonistas e antagonistas para cada subtipo (khakh and North 2012).

Recentemente, tem aumentado de forma significativa a pesquisa para entendimento das características farmacológicas e fisiológicas dos P2Rs, visando, dessa forma, buscar a correlação destes com diversos processos patológicos e, consequentemente, a descoberta de moléculas candidatas à terapia clínica. No campo da indústria farmacêutica, tem havido uma "corrida farmacológica" pelos compostos que possuam atividade antagonista e seletividade sobre um

determinado P2XR, devido à ausência de agonistas e antagonistas seletivos para cada receptor. A descoberta de agonistas e antagonistas seletivos pode auxiliar no tratamento de desordens fisiopatológicas relacionadas a um receptor específico, como por exemplo, Disfunção Erétil (receptor P2X1); Epilepsia (P2X2/4R); Cistite Intersticial (P2X2/3R); Dor neuropática (P2X4R); Esclerose Muscular (P2X5/6R); Falência Coronariana Crônica (P2X6R); Doenças Inflamatórias, como, por exemplo, a Artrite Reumatóide, Leucemia Mielóide Aguda, Alzheimer, Diabetes entre outras e o P2X7R. Dessa forma esses receptores têm sido alvo de estudo de diversos grupos de pesquisa visando à sua ação como alvo terapêutico, principalmente o P2X7R, devido a sua característica única de formar um canal de larga condutância quando ativado, sendo este relacionado com a progressão de diversas enfermidades, como por exemplo, as doenças neurodegenerativas e a inflamação (Li et al. 2008). Por estes motivos, o enfoque deste trabalho esta relacionado com o P2X7R, o qual será estudado com mais detalhes.

1.5. RECEPTORES P2X7R

O P2X7R é expresso principalmente em células do sistema imune, tais como macrófagos, monócitos, células dendríticas, linfócitos, mastócitos e uma variedade de células da glia no sistema nervoso central e periférico, incluindo microglia, astrócitos, oligodendrócitos e células de Schwann (Burnstock et al. 2009 & Di Virgilio et al. 1997). Em macrófagos peritoneais e esplênicos, assim como em

células de linhagem macrofágica J774, foi identificada a presença predominante de P2X4R e P2X7R, e dos P2Y1, P2Y2 e P2Y4Rs (Coutinho-Silva et al. 2005).

Além disso, o P2X7R possui 595 aminoácidos, apresentando uma homologia de 35 a 40% com relação aos outros seis subtipos. (Evans et al. 1996). Sua topologia é basicamente semelhante aos demais subtipos, possuindo dois domínios transmembranas (TM1 e TM2) e uma grande alça extracelular. Entretanto, seu domínio C-terminal é o maior em comprimento (239 aminoácidos). (fig. 4).

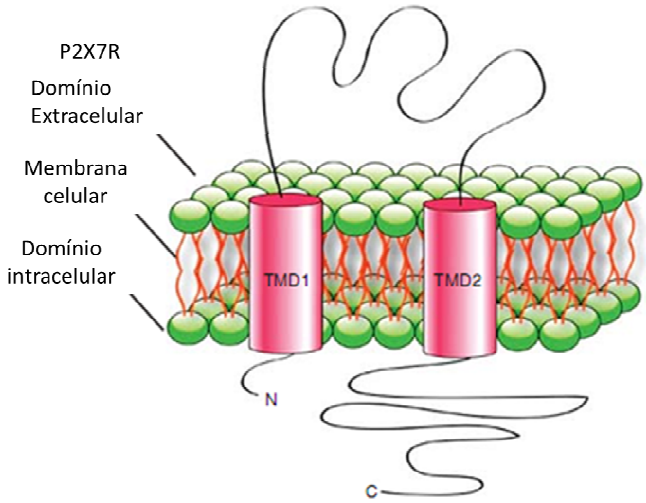


Fig.4: Estrutura do $P2X_7R$. Os P2X7Rs possuem dois domínios transmembranas (TM1 e TM2) e uma grande alça extracelular, com o maior domínio C-terminal.

Alguns estudos vem sendo conduzidos para identificar os resíduos que seriam importantes na formação do canal/poro do P2X7R. Principalmente em relação ao seu processo de conversão rápida de canal catiônico de baixa condutância (~10 pS) para um poro não-seletivo de alta condutância (~400 pS)

(Coutinho-Silva et al. 1997; Persechini et al. 1998; Faria et al. 2005). Esses estudos tem como objetivo compreender melhor a estrutura/função do receptor, dessa forma, favorecendo ao desenho racional de antagonistas e agonistas. Devido ao controle diferenciado da expressão do P2X7R e da sua localização em vários tipos celulares e estágios de diferenciação, é provável que existam múltiplos domínios de controle do processo de formação de canais e poros (canal de alta condutância). O poro formado pela ativação desse receptor na presença da concentração acima de 100 μM de ATP extracelular permite a passagem de moléculas de alto peso molecular de acordo com um gradiente eletroquímico, como íons de brometo de etídio, iodeto de propídio e YOPRO-1 (Evans 1996). Com relação a esse poro não-seletivo de alta condutância, ainda não é sabido se ele deriva da dilatação do canal iônico, ou, se é uma proteína isolada ou se resulta da integração com outros canais ou transportadores (Cankurtaran-Sayar et al. 2002). Inclusive há uma teoria sobre a existência de dois poros seletivos, um para moléculas aniônicas e outro para moléculas catiônicas (Schachter et al. 2008). Esse processo gera alterações na morfologia da célula, tais como, aumento do volume celular e formação de bolhas na membrana (Egan et al. 2006).

O papel funcional do P2X7R foi melhor caracterizado como sendo um importante mediador da regulação da expressão e liberação de citocinas, mediadores inflamatórios e metabólitos inflamatórios incluindo IL-1β (North et al. 2002 & Labasi et al. 2002), IL-1α (Smith et al. 2001), IL-2 (Mehta et al. 2001), IL-4, IL-6, IL-13, IL-18 (Gudipaty et al. 2003 & Wilson et al. 2004), TNF-α (Chessel et al. 2005), óxido nítrico (NO) (Loomis et al. 2003; Sluyter et al. 2004) e ânions

superóxidos (Bulanova et al. 2005). A correlação entre o P2X7R e o processo de liberação de IL-1β está bem descrita na literatura, mostrando o envolvimento da rápida ativação da caspase-1 através de mecanismos dependentes do complexo inflamassoma, conforme observado na fig.5 . Também podemos destacar a formação de células gigantes multinucleares (Hu Y et al.1998 & Sperlagh et al. 1998), indução da morte do *Mycobacterium tuberculosis* (Saunders et al. 2003), reorganização do citoesqueleto (Sikora et al. 1999) e ativação de caspases (Friedle et al. 2010). As atividades biológicas do P2X7R têm sido relacionadas com o domínio C-terminal (resíduos 353 a 595)(Watters et al. 2001).

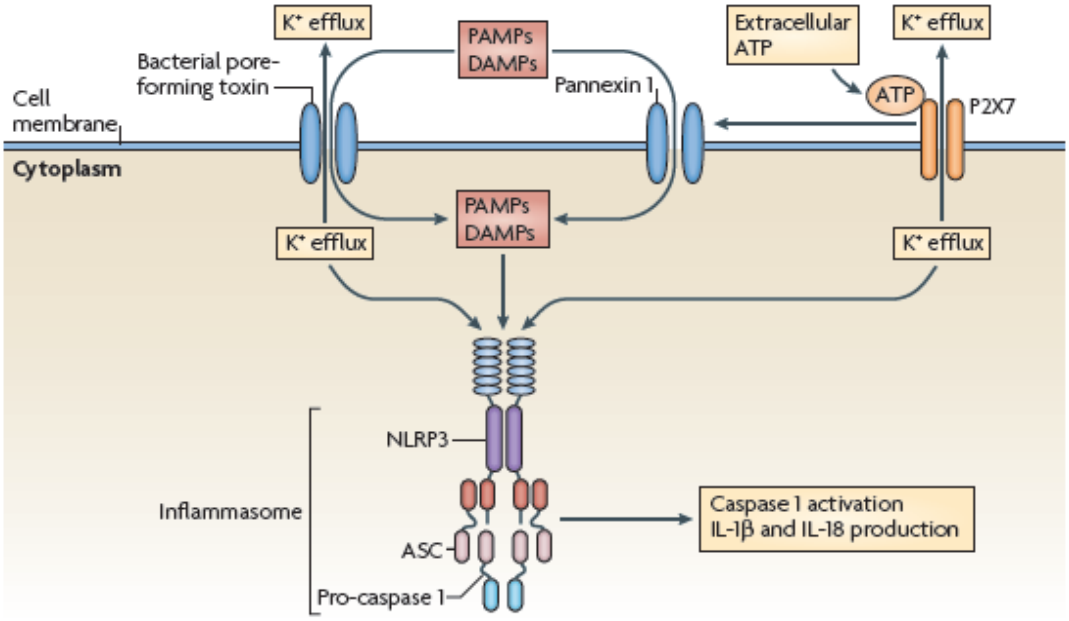


Fig.5: Via de liberação da IL-1β através da ativação do P2X7R. O P2X7R ao ser ativado pelo ATP, permite a passagem de íons potássio através do canal iônico ativado que leva a ativação do NLRP3 e consequentemente da Caspase 1. Esse processo induz a clivagem de pró-IL1β em IL-1β madura, uma potente citocina pró-inflamatória, sendo esta

secretada pela célula para ativar a resposta imune adaptativa (Tschopp & Schroder et al. 2010).

1.6. DOENÇAS RELACIONADAS AO RECEPTOR P2X7

A artrite reumatoide é uma doença inflamatória crônica que apresenta prevalência mundial de 0,4 a 1,9 %, sendo que no Brasil essa característica se encontra entre 0,2 e 1 %. Sua evolução ocorre em graus variáveis, os quais podem levar os pacientes, muitas vezes, a um quadro de incapacidade funcional (Torigoe 2006).

As principais células envolvidas no desenvolvimento das lesões cartilaginosas são os fibroblastos, os macrófagos e os linfócitos ativados. Eles expressam receptores purinérgicos, incluindo o P2X7R, que sofrem ativação em decorrência do aumento de nucleotídeos pirimidínicos e purínicos no meio extracelular, como o ATP, que são encontrados no líquido sinovial de pacientes portadores da artrite reumatoide (Toulme et al. 2010).

Uma vez ativado, esse receptor medeia a produção e consequentemente a liberação de citocinas pró-inflamatórias, como a IL-6, IL-18 e TNF-α, as quais possuem a capacidade de induzir e perpetuar a inflamação. Além disso, devido à presença de macrófagos no líquido sinovial, como demonstrado na fig. 6, ocorre a liberação da citocina IL-1β, que está envolvida na infiltração de leucócitos produzindo assim, hiperplasia sinovial, além da promoção da ativação celular e da degradação ou inibição da síntese de cartilagem (Lister et al. 2007; Al-Shukaili 2008).

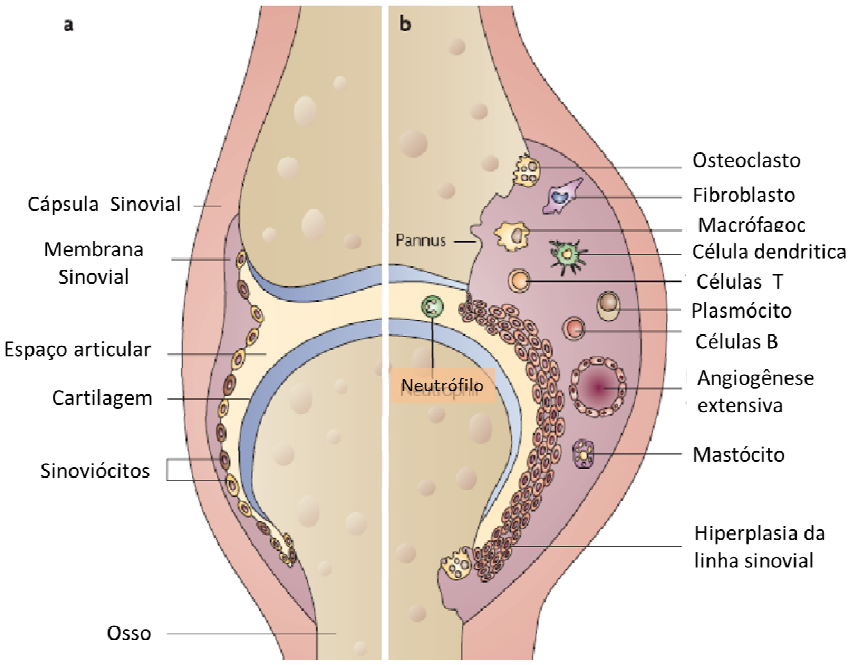


Fig.5: Comparação entre uma articulação normal (a) e uma com artrite reumatoide (b). A destruição do tecido cartilaginoso devido à ação de células do sistema imune.
Imagem disponível em: <http://dc229.4shared.com/doc/BS2Ax0ic/preview.html>.

O P2X7R também já foi observado como participante da dor neuropática. Essa é definida por Kobayashi et al. 2011, como uma "dor iniciada por lesão ou disfunção do sistema nervoso, sendo melhor compreendida como resultado da ativação anormal da via nociceptiva (fibras de pequeno calibre e trato espinotalâmico)". Além disso, é sabido que o ATP é um mediador importante na cotransmissão da informação sensorial do sistema nervoso periférico para o sistema nervoso central, o que revela um papel-chave do P2X7R nessa doença (Skaper et al. 2009).

Dessa forma, em virtude das lesões nas células neuronais periféricas participantes da via nociceptiva, ocorre a liberação do ATP e, consequentemente, a ativação do P2X7R expresso nas células da micróglia. Esse, por sua vez,

promove entre outros efeitos, a liberação da citocina IL-1β, a qual além de ser uma potente citocina pró-inflamatória, também promove uma cascata de eventos que irão gerar produtos de ânions superóxidos, como o peróxido de hidrogênio, que participam ativamente na manutenção da dor (Toulme et al. 2010).

Assim, além de promover a dor e a inflamação, esses receptores podem ainda induzir estados crônicos, como pode ser observado devido a sua ativação excessiva que pode estimular a reorganização do citoesqueleto e, consequentemente, a abertura do poro membranar, o que eventualmente leva à morte das células imunes (Toulme et al, 2010). Nessa perspectiva, diversos trabalhos, *in vivo*, como o de Kobayashi et al. 2011, têm demonstrado que o uso de antagonistas para esse receptor promove um efeito antinociceptivo.

1.7. P2X7R E APLICAÇÕES CLÍNICAS

No contexto que relaciona o P2X7R com suas aplicações clínicas, estudos ainda em fase experimental, relatam o uso do ATP, *in vitro*, para aumentar a capacidade microbicida de macrófagos infectados com *Mycobacterium tuberculosis* (Fernando et al. 2007). Em 2006, Yoon e colaboradores demonstraram que o tratamento de células humanas de leucemia mieloide, *in vitro,* com diferentes concentrações de ATP induziu apoptose e permitiu o controle do crescimento celular destas células. Por esses resultados, os autores sugeriram que o tratamento com nucleotídeos extracelulares pode significar uma via terapêutica alternativa e potente para a leucemia mieloide (Yoon et al. 2006). Outros autores demonstraram que o antibiótico natural polimixina B não

apresentou efeitos sobre a viabilidade de linfócitos de pacientes com leucemia linfocítica crônica, mesmo durante incubações prolongadas. Entretanto, esse efeito foi fortemente sinergizado em presença de ATP. A polimixina B, em concentrações ótimas e dependendo do tipo celular, potenciou fortemente o efeito da estimulação do P2X7R mediado por nucleotídeos. Com esses resultados, os autores sugeriram que a potenciação do efeito da polimixina B, quando usada sinergicamente com o ATP, pode ter aplicações práticas no desenvolvimento de uma terapia baseada no uso de ATP (Ferrari et al. 2004).

Visando à busca de compostos que possam ser candidatos a um potencial antagonista para o P2X7R, com seletividade de ação e possível aplicabilidade clínica (Gunosewoyo et al. 2010), vários compostos têm sido descobertos como, por exemplo, os tetrazoles disubstituídos, as cianoguanidinas (Nelson et al. 2006), a A-438079, 3-((5-(2,3-diclorofenil)-1H-tetrazol-1-il)metil piridina, um antagonista seletivo e competitivo com perfil de inibição em concentrações submicromolares para os receptores rP2X7R (Rato), hP2X7R (humano) e mP2X7R (camundongo) (Nelson et al. 2006). Assim como outro composto com ação antagonista seletiva, o A-740003 (N-cianoguanidina) (5 -quinolinilamino) metil] amino} - 2,2 - dimetilpropil) – 2 - (3,4 - dimetoxifenil) acetamida) com IC´s_{50} = 18 nM para o rP2X7R e 40 nM para o hP2X7R e submicromolar para o mP2X7R. Já o composto A804598, 2-ciano1-[(1S)-1-feniletil]-3-quinolina-5-guanidina, apresentou perfis de inibição sobre os receptores rP2X7R, hP2X7R e mP2X7R, em baixas concentrações, na faixa de 9-11 nM (Coddou et al. 2001).

Recentemente foi publicado um estudo que demonstrou a eficácia e a tolerância, em humanos com artrite reumatoide, do composto CE-244,535 (um antagonista seletivo em teste *in vitro* e *in vivo*). Porém, esse composto não apresentou significativo aumento da eficácia em relação ao tratamento atual (Stock et al. 2012). Pesquisas utilizando moléculas seletivas e com potencial inibitório sobre o P2X7R têm oferecido novos "insights" sobre a farmacologia deste receptor, e seu potencial terapêutico . Cabe ressaltar que existem poucos estudos sobre a ação de produtos naturais sobre esse receptor, sinalizando, dessa forma, a necessidade de explorar mais o campo acerca do potencial farmacológico de moléculas com atividade antagonista obtidas da natureza, para o tratamento de doenças relacionadas a este receptor.

1.8. PERFIL FARMACOLÓGICO DO P2X7R

O perfil farmacológico do P2X7R encontra-se bem descrito na literatura (tTabela 3). Tal perfil caracteriza-se por ter o BzATP (EC_{50} = 25 µM), seguido do ATP (EC_{50} > 100 µM) seu agonista fisiológico. Cabe ressaltar que a potência do ATP aumenta quando retiram-se os íons cálcio e magnésio da solução (Coddou et al. 2011). Além desses, existem os agonistas parciais, como o 2-meSATP e o ATPγS e também os que possuem baixo efeito de ativação sobre o receptor, como o αβ-meATP e o βγ-meATP (Coddou et al. 2011).

Em relação aos antagonistas, o rP2X7R, assim como o rP2X4R, possuem um perfil de resistência ao antagonista de ampla ação sobre os P2Rs, a suramina (Surprenant et al. 2006). Porém, um análogo da suramina, o composto NF279,

possui um [IC_{50} = 3 µM] para ohP2X7R, entretanto é necessária uma concentração maior para o rP2X7R, [IC_{50} = 100 µM] (Klapperstuck et al. 2000). Existem outros antagonistas já conhecidos, como o PPADS, que possuem a função de antagonizar os sinais e correntes de cálcio nas células de mamíferos, com IC_{50} que varia de 1 a 60 µM (Gunosewoyo et al. 2007), merece destaque o MRS2159, um potente antagonista para rP2X1R, que respondeu efetivamente em inibir os rP2X7R, hP2X7R e o mP2X7R. Além desses, podemos mencionar o o ATP oxidado, um antagonista irreversível, que requer um período de mais de uma hora para o início do seu efeito em concentrações em torno de 100 µM a 300 µM (Gunosewoyo et al. 2010). O BBG é caracterizado como sendo um antagonista reversível para o rP2X7R [IC_{50} = 15µM] e para o hP2X7R [IC_{50} = 250µM] (Coddou et al. 2011). Até o momento o IC_{50} do BBG para o P2X7R de camundongo não foi determinado, porém, no nosso primeiro manuscrito determinamos este valor [IC_{50} = 1.2 µM].

Além dos compostos caracterizados farmacologicamente com atividade inibitória sobre o P2X7R, existem outros tipos de compostos que agem em outras vias e/ou receptores, mas também demonstram atividade antagonista sobre esse receptor. Dentre os quais podemos citar os bloqueadores de calmodulina dependente de proteína quinase II, como por exemplo, o 1[N,O-bis(5-isoquinolinosulfonil)-N-metil-L-tirosil]-4-fenilpiperazina (KN62) e o calmidazólio, ambos inibem a corrente de influxo de cálcio induzida pelo BzATP no hP2X7R (Brown et al. 2002).

1.8. PRODUTOS NATURAIS

Os produtos naturais oriundos de plantas, animais e microrganismos vêm sendo utilizados há milhares de anos no tratamento de doenças humanas, constituindo assim, a base da medicina tradicional conhecida em todo o mundo. A Medicina Tradicional Chinesa e a Medicina Nipo-Chinesa, na Ásia, bem como a Medicina Alternativa na América constituem alguns exemplos desse uso (Koehn. 2005; Balunas. 2005; Itokawa et al. 2008).

As informações sobre o do uso medicinal dessas plantas foram transmitidas de geração a geração ao longo do tempo. Dessa forma, essas propriedades instigaram a curiosidade científica e já no início do século XIX, no ano de 1805, ocorreu o descobrimento da morfina, seu isolamento e purificação. A partir desse acontecimento, diversas moléculas ativas têm sido descobertas para o tratamento de diferentes patologias diretamente ou na forma de análogos semissintéticos (Njuguna et al. 2012). Temos como exemplos disso, a descoberta e o uso clínico da quinina e da artemisina, as quais representam apenas dois dos inúmeros compostos naturais que possuem atividade antiplasmodial, e que são utilizadas como fármacos de primeira linha até hoje. Além disso, elas serviram de base para pesquisa e síntese de diversos compostos antimaláricos semissintéticos (Njuguna et al. 2012).

No contexto da flora brasileira, o Brasil detém cerca de 20 % da biodiversidade mundial, a qual se concentra principalmente na floresta Amazônica, a maior floresta do Planeta e fonte de matérias-primas (Pereira 2004). Embora exista uma imensa diversidade biológica na Amazônia, o conhecimento sobre as

espécies que a constituem e suas relações filogenéticas é muito restrito. Assim como sobre os microrganismos e as interações entre os seres que a compõem (Pereira 2004). Por isso, estudos acerca da exploração do potencial da nossa flora contribuem para o enriquecimento do conhecimento acadêmico sobre a biodiversidade brasileira.

Na busca por novos medicamentos eficazes, e potencialmente menos tóxicos, para enfermidades que afligem a população, a pesquisa com produtos naturais vem aumentando e recebendo investimentos. Atualmente, estima-se que cerca de 30 % dos medicamentos utilizados na medicina moderna provêm de produtos naturais. Exemplos interessantes dessa aplicabilidade são a morfina e os salicilatos (Calixto 2005). Também podemos citar os agentes antineoplásicos, visto que cerca de 75 % deles provêm de produtos naturais, como por exemplo, o taxol e a vincristina (Mann 2002).

No entanto, na literatura existem trabalhos que caracterizam o emprego clínico de extratos e frações vegetais no tratamento de doenças e consequentemente para descoberta de suas ações sobre alvos específicos como enzimas, transportadores ou canais iônicos envolvidos em processos fisiológicos e patológicos importantes (Barbosa-Filho et al. 2006). No trabalho de Barbosa-Filho et al. 2006; foi feita uma revisão quanto às plantas e às substâncias encontradas que possuem atividade inibitória sobre a acetilcolinesterase. Foram relatadas 309 plantas e 260 substâncias isoladas, evidenciando assim a relevância do estudo de produtos naturais para obtenção de uma atividade específica sobre alvos celulares ou enzimáticos.

1.8.1 MICRORGANISMOS E METABOLISMO SECUNDÁRIO

Desde a descoberta da penicilina em 1928, por Fleming, a partir do fungo *Penicillium chrysogenum,* têm ocorrido avanços e descobertas no campo da atividade biológica de microrganismos e seus metabólitos secundários. Posteriormente, outra descoberta importante, a da ciclosporina em 1979, a partir do fungo *Tolypocladium inflatum,* levou a um avanço fundamental na medicina no campo do transplante de órgãos. Antes da descoberta desse fármaco, a durabilidade de um enxerto era curta, muitos não passavam das primeiras horas pós-operatórias, pois o sistema imune do receptor atacava o enxerto intensamente, promovendo a rejeição. Devido à ação imunossupressora sobre os linfócitos T, a ciclosporina age impedindo que o sistema imune do receptor ataque o órgão enxertado, prevenindo assim, a rejeição, um fator indesejável para o sucesso do transplante (Starzl et al. 1981).

Na atualidade, os estudos acerca de microrganismos e de seus metabólitos secundários têm sido considerados uma fonte rica para o isolamento de moléculas com atividade microbicida. Um recente trabalho foi publicado por Mohandas et al. (2012), que caracteriza a atividade inibitória de metabólitos secundários produzidos por um grupo de bacilos (*Bacillus thuringiesis, Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis, Bacillus megaterium* e *Bacillus cereus*) sobre a β-Lactmase de bactérias resistentes a meticilina. Além desse dado, o mesmo trabalho também relata a atividade microbicida de produtos obtidos do metabolismo secundário de fungos (Actinomicetos), os quais são responsáveis por cerca de 80 % da produção de moléculas com potencial atividade antibiótica

(Mohandas et al. 2012). Outro artigo publicado por Al-Sohaibani et al. (2012), também relata a atividade inibitória de compostos isolados de *Streptococcus mutans* sobre a formação do biofilme oral e no controle da colonização e acumulação da cárie. A atividade antiviral de extratos fúngicos e seus compostos sobre o vírus da Influenza A, também foram relatados recentemente (Teplyakova et al. 2012).

Um estudo *in vitro* também demonstrou a atividade antioxidante de 49 extratos de fungos endolíticos isolados de uma planta (*Scapania verrucosa* Heeg) (Zeng et al. 2011). Anteriormente, Guo et al. (2008), já havia relatado as atividades microbicida e antitumoral, *in vitro,* de compostos isolados do extrato etéreo de *Scapania verrucosa* Heeg e do fungo endofítico isolado (*Chaetomium fusiforme*) (Guo et al. 2008).

Nesse contexto, podemos citar o recente trabalho de Santiago et al. 2012, no qual foi evidenciada a atividade antitumoral e leishmanicida de fungos endolíticos originários da Antártica, onde há a prevalência de basidiomicetos no solo e no ambiente subglacial das geleiras, como por exemplo, *Cryptococcus liquefaciens*, uma levedura constituinte do gênero *Cryptococcus sp*. Ascomicetos são raramente encontrados em ambientes extremamente gelados, embora eles sejam reconhecidos como contaminantes de comidas congeladas (Butinar et al. 2011). Entretanto, Butinar et al. (2011), descreveram a presença de ascomicetos em regiões árticas, como por exemplo, da espécie *Metschnikowia bicuspidata* do gênero *Metschnikowia sp.* Além desses, existem outros fungos que colonizam

regiões com temperatura baixa, como por exemplo, a *Acarospora algicola,* que habita ambientes gelados, como o sul do Brasil, em especial o Paraná.

1.8.2 PRODUTOS NATURAIS E RECEPTORES P2X7R

Em relação aos receptores P2, há poucos trabalhos que relatam a atividade antagonista de produtos naturais sobre esses receptores (Quadro 1); Shemon et al. (2004), relataram o bloqueio do fluxo de cátions mediado pelo P2X7R por quelaritrina, um alcalóide benzofenantridinico. Essa ação, porém, não foi seletiva, visto que os compostos apresentavam efeito inibitório sobre enzimas, como a PKC e a alanina aminotransferase. Buchanan et al. (2006) evidenciaram o efeito antagonista possivelmente específico para P2X7R por uma nova classe de alcalóides pirrolo-imidazóis tetraméricos obtidos por estudos de relação estrutura atividade (SAR) (Buchanan et al. 2007). Em 2007, esse mesmo grupo isolou a nifatoxina C, um alcaloide, a partir de uma espécie de esponja do mar (*Callyspongia sp*). Também podemos relatar o estudo de Said et al. (2007), o qual estudou a ação de óleos vegetais de *Olea europaea L; Capsicum sp; Zea mays; camelina sativa* e *Aleurites moluccana,* sobre a ativação de morte celular relacionada ao P2X7R. Somente o óleo de *Capsicum sp* demonstrou atividade agonista e levou a intensa ativação da morte celular via esse receptor (Said et al. 2007). Ainda nesse contexto, Marques-da-Silva et al. 2011 relataram a inibição do poro associado à ativação do P2X7R pela colchicina, com inibição na produção de citocinas (IL1-β e INF-γ), assim como de mediadores inflamatórios (ROS e NO).

Recentemente, foi relatado o significativo efeito antagonista sobre o P2X7R das frações do extrato metanólico de *Rheedia logifolia,* promovendo uma ação analgésica do extrato sobre a dor de origem inflamatória (Santos et al. 2011). Liu et al. 2010 descreveram a ação antagonista da emodina, um derivado antraquinônico da *Rheum officinale Baill* sobre esse receptor. Gunosewoyo et al. 2009 sugeriram o estudo da química de compostos sintéticos e naturais e o screening biológico como ferramentas para a descoberta de antagonistas do P2X7R (Gunosewoyo et al. 2009). Uma descrição mais detalhada sobre produtos naturais com ação antagonista sobre o P2X7R pode ser vista nos manuscritos em anexo (vide anexo 1 e 2).

No contexto do "screening", *in vitro,* de novas moléculas com possíveis atividades antagonistas sobre o P2X7R, as metodologias mais utilizadas atualmente não permitem a triagem de vários compostos ao mesmo tempo. Não são accessíveis e demandam tempo na obtenção e análise dos resultados. Podemos citar como exemplos: a citometria de fluxo, a microfluorimetria para detecção da corrente de cálcio intracelular, a microscopia de fluorescência e a eletrofisiologia. No presente trabalho, desenvolvemos um ensaio adequado para a avaliação seletiva e viável da atividade do P2X7R, utilizando uma metodologia espectrofotométrica (primeiro manuscrito).

Quadro 1. Compostos naturais com ação antagonista sobre P2X7R

Artigo	**Espécie Biológica**	**Composto Estudado/IC_{50}**
Shemon et al. 2004	*Chelidonium majus* (*Papaveraceae*)	Quelaritrina (Alcalóide benzofenantridinico) IC_{50} = 10 µM
Buchanan et al. 2006	*Stylissa flabellata* (Esponja do mar)	Estilissadinas A e B IC_{50} = 0.7 µM e 1.8 µM
Buchanan et al*. 2007*	*Callyspongia sp* (Esponja do mar)	Nifatoxina C (Alcalóide) IC_{50} = N.D
Said et al. 2007	*Olea europaea L; Capsicum sp; Zea mays; camelina sativa*; *Aleurites moluccana*	Óleos Vegetais IC_{50} = N.D
Gunosewoyo et al.2009	Alcaloides obtidos de estudos sobreestrutura - atividade (SAR)	Massadina (IC_{50} = N.D) Protoberberina (IC_{50} = 0.3 µM)
Santos et al. 2011	*Rheedia longifolia* (Guttiferae)	Amentoflavona IC_{50} = 2 µg/mL
Liu et al. 2010	*Rheum officinale Baill*	Emodina IC_{50} = 500 nM
Marques-da-Silva et. al. 2011	*Colchicum.sp*	Colchicine EC_{50} = 540 µM Macrófago peritoneal murino EC_{50} = 290 µM Oócitos de *Xenopus laevis*

2. OBJETIVOS

2. OBJETIVO GERAL

Identificar extratos e/ou frações bioativas que possam ser candidatas à obtenção de um potencial antagonista do receptor P2X7.

2.1. OBJETIVOS ESPECÍFICOS

1. Padronizar a metodologia para o "screening" dos extratos e suas frações a serem testados;

2. Identificar extratos dos diferentes biomas com atividade antagonista para o receptor P2X7 *in vitro*;

3. Determinar o IC_{50} dos extratos e suas frações com atividade antagonista para o receptor P2X7;

4. Avaliar a ação dos extratos e suas frações sobre a atividade do ATP extracelular, liberação de citocinas e outras vias moduladas pelo receptor P2X7, utilizando a IC_{50} dos mesmos, na tentativa de encontrar um antagonista;

5. Avaliar a atividade dos extratos e suas frações sobre o receptor P2X7 através do ensaio de eletrofisiologia;

6. Avaliar a ação dos extratos e suas frações em modelos *in vivo* de dor neuropática e inflamatória e de úlcera gástrica.

3. METODOLOGIA

1 - Cultura de Células: Utilizamos placa opaca de 96 poços (Corning), onde foram plaqueadas em triplicata células J774.G8 ou U-937 na concentração de $4X10^5$ células/poço, e foram cultivadas em meio RPMI com 10% de SFB em estufa de 37ºC com atmosfera de 5% de CO2 por 24 h. Após este período, o meio de cultivo foi trocado por uma salina extracelular (em mM: 150 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2 e 10 HEPES; pH 7.4) e então foram feitos os tratamentos.

2 - Tratamento das células: Nos testes utilizamos dois antagonistas conhecidos para o receptor P2X7. As células foram tratadas ou com BBG (Brilhant Blue G), que é um antagonista competitivo reversível, numa concentração de [100 µM]. No caso do antagonista irreversível ATPox. (ATP oxidado), utilizamos a dose descrita na literatura [300 µM]. As células foram tratadas com BBG por 15 minutos, ou por 60 minutos com o ATP ox. Em estufa B.O.D a temperatura de 37 º C, com atmosfera de 5% de CO2 antes da estimulação com o agonista (ATP). No tratamento com os extratos, as células foram plaqueadas em placas de 96 poços opaca e receberam o tratamento de 100 µg/ml de cada extrato por 15 min, antes da estimulação com o agonista (ATP). O tratamento com ATP consistiu na incubação das células com 5mM desse agonista por 10 min.

3 - Obtenção dos extratos fúngicos: As frações que foram analisadas são oriundas da colaboração científica com a Dra. Tânia Maria de Almeida Alves, chefe do Laboratório de Química de Produtos Naturais do CPqRR. As amostras foram coletadas dos biomas brasileiro e antártico. As frações foram enviadas

na forma de filme seco, em placas de polipropileno, com 96 poços de 250-300 microlitros de capacidade, fundo em “V”, não estéreis. Cada poço continha dois miligramas de cada amostra na forma de filme seco; As placas foram submetidas à irradiação por microondas visando minimizar contaminação microbiológica.

4 - Triagem da atividade dos extratos sobre o P2X7R: O experimento se baseou na quantificação da incorporação do iodeto de propídeo (IP) [50nM] pelas células utilizadas (J774.G8 e U-937), através da abertura do poro membranar pelo receptor ativado, a partir da leitura em espectrofotômetro (Spectramas M5) com λ emissão = 488nm e λ absorbância = 590nm. Foram utilizadas células tratadas com os extratos [100 μg/ml]. Alguns poços foram tratados com extratos e 5 mM de ATP, para averiguar a ação antagonista dos extratos isoladamente e outros poços foram tratados somente com as frações para averiguar a citotoxicidade das mesmas. O controle do experimento foi tratado com brilhant blue G (BBG) [100 nM] e o controle positivo foi tratado com Triton X-100 [0.01%]. Os extratos que demonstraram atividade foram submetidos à avaliação dos seus respectivos IC´s$_{50}$ através do tratamento das células com cada extrato [100 μg/mL - 2 ng/mL], onde a dose que promoveu uma inibição de 50% sobre a ativação do receptor foi eleita como sendo seu respectivo IC$_{50}$. Esta análise foi feita através do teste estatístico descrito no item 15.

5 – Avaliação da liberação de IL-1β via P2X$_7$R: As células J774.G8 e U-937 foram tratadas com os extratos nos seus respectivos IC´s$_{50}$ na presença ou não

do tratamento com ATP [5 mM], também foram utilizados como controles, poços tratados com BBG [100 nM] na presença ou não do ATP. Após uma hora de tratamento os sobrenadantes das culturas foram coletados e foi realizado um ELISA para detecção de IL-1β de acordo com as especificações do kit da RD Systems. O resultado foi obtido a partir da leitura das amostras no comprimento de onda de 450 nm em espectrofotômetro (Spectramax M5);

6 – Ensaio de citotoxicidade: As células J774.G8 e U-937 foram tratadas com os extratos em concentrações até quatro vezes maiores que os seus respectivos IC´s_{50} por 24 h. Após este período essas células receberam ou não o tratamento com ATP [5 mM] por 1 h., também foram utilizados como controles, poços tratados com BBG [100 nM] por 24 h. na presença ou não do ATP por 1 h. e poços tratados com Triton 0. 1 % por 1 h. Após todos os tratamentos e períodos de incubações, os sobrenadantes das culturas foram coletados e foi realizada a detecção da LDH, uma enzima presente no citoplasma da célula, uma vez rompida à membrana plasmática, essa enzima é detectável no sobrenadante da cultura, indicando dessa forma que houve dano celular. Foi feita a detecção da LDH de acordo com as especificações do kit da Doles, Goiânia, GO – Brasil. O resultado foi obtido a partir da leitura no comprimento de onda de 510 nm em espectrofotômetro (Spectramax M5).

7 - Ensaio para detecção de Nitrito: Utilizando o mesmo protocolo citado nos itens 4 e 5, o Nitrito, um produto estável da oxidação de NO, foi medido através de espectrofotômetro após adição do reagente Griess (Sigma) no sobrenadante das células previamente tratadas. Após 10-15 min em

temperatura ambiente, a absorbância do cromóforo foi medida no comprimento de onda de 540 nm (Spectramax M5).

8 - Ensaio para detecção de ROS: Células J774.G8 e U937 foram submetidas ao tratamento com os extratos e os controles segundo a metodologia descrita no item 2, por uma hora. Após este período o estresse oxidativo foi avaliado utilizando-se o corante dihidroetídeo (DHE). Este protocolo se baseou no tratamento das células com DHE [10 nM] por 20 minutos e a fluorescência foi determinada através da análise pelo espectrofotômetro (Spectramax M5) nos comprimentos de onda para excitação (490 nM) e emissão (570 nM).

9 - Medida da atividade dos P2XRs por eletrofisiologia: A técnica de patch-clamp permite a avaliação dos P2XRs que são ionotrópicos. As correntes iônicas foram registradas em condições de alto selamento elétrico entre a membrana da célula e o microeletródio utilizado para realizar os registros. De forma simplificada, uma micropipeta, contendo o eletródio, preenchida com solução salina toca a superfície celular e, posteriormente, foi aplicada uma sucção. Em condições apropriadas surgiu um selamento Giga Ôhmico (GΩ) a partir do qual foi possível registrar correntes de canais unitários ou correntes macroscópicas de baixa condutância. Essa configuração é denominada de cell-attached. As pipetas utilizadas para o experimento na configuração cell-attached foram preparadas de capilares de borossilicato com filamento 1,2 mm (World Precision Instruments, Inc; New Haven, E.U.A.), utilizando um puxador de pipetas (Microelectrode Puller, Narishe Group, NY, E.U.A.). As avaliações foram feitas utilizando um amplificador eletrônico (Axopatch-1D Axon

Instruments, Inc; San Mateo; E.U.A.) que permitiu fixar o potencial e medir as correntes dos canais iônicos. Este amplificador foi controlado por um computador padrão IBM, através do programa Axoscope 8.0 (Axon Instruments, Inc; San Mateo, E.U.A.), que realizou os protocolos de pulso e armazenou as medidas do amplificador. O amplificador foi ligado ao computador, através de uma interface AD/DA Digidata 1320 (Axon Instruments, Palo Alto, CA, E.U.A.), que digitalizou os registros numa freqüência de 50 KHz. A resistência em série da pipeta foi corrigida até (75-85%) em todos os experimentos e a capacitância da célula foi anulada (20-30%) pelo amplificador. O valor de capacitância utilizado para essa anulação correspondeu ao valor da capacitância da membrana celular que foi utilizado para normalização das correntes, já que apresentou uma relação direta com a área da membrana celular.

10 – Avaliação da incorporação de corante aniônico via P2X7R: O experimento se baseou na quantificação da incorporação do Lucifer Yellow (LY) [3mM] pelas células utilizadas neste trabalho, através da abertura do poro membranar pelo receptor ativado, a partir da leitura em espectrofotômetro (Spectramas M5) com λ emissão = 485nm e λ absorbância = 528nm. Foram utilizadas células tratadas com a dose correspondente ao IC_{50} dos extratos. 50% foram tratadas com extratos e 5 mM, de ATP para averiguar a ação antagonista dos extratos, e 50% foram tratadas somente com os extratos como controle. O controle do experimento foi tratado com brilhant blue G (BBG) [100 nM] e o controle positivo foi tratado com Triton X-100 [0.01 %].

11 - Teste da Formalina: camundongos receberam injeção subplantar de 20 µL de formalina 2,5% para a medida da reatividade em segundo(s) dos animais em lamber ou morder a pata de 0-5 min (1 fase) e de 15-30 min (2 fase) após injeção de formalina. Os tratamentos foram realizados oralmente, através da gavagem dos extratos ativos *in vitro* [200 µg/kg], ou com BBG [100 mg/kg]. Tivemos também como controles, um grupo tratado com morfina [10 mg/kg] via intraperitoneal (para dor neuropática) e outro grupo tratado com diclofenaco de sódio [50 mg/kg] via oral (gavagem) para dor inflamatória.

12 – Avaliação da atividade gastro-protetora: Foram utilizados camundongos Swiss (20-30 g), de ambos os sexos para os estudos da atividade gastro-protetora, os animais foram mantidos em ciclo claro e escuro de 12/12 horas, mantidos em jejum por 24 h com livre acesso à água. Os extratos que demonstraram ação antagonista via P2X7 *in vitro,* foram administrados via oral por gavage (200 µg/k), 30 minutos antes de receberem o estímulo ulcerogênico (200 µL de Etanol 70% v/v). BBG [100 mg/kg] (antagonista), ou Lansoprazol [25 mg/kg] (agente gastro-protetor comercializado) foram utilizados como controles. Após três horas, os animais foram eutanasiados e seus estômagos foram extraídos e abertos, para contagem e registro fotográfico do número de lesões geradas.

13 - Animais – Foram utilizados camundongos Swiss (20-30 g), de ambos os sexos para os estudos de dor e inflamação Os animais foram mantidos em ciclo claro e escuro de 12/12 horas, com livre acesso à ração e água. Os animais foram fornecidos pelo Biotério Central da Fundação Oswaldo Cruz. Os procedimentos experimentais estão devidamente registrados e autorizados

pela Comissão de Ética no Uso de Animais – CEUA/FIOCRUZ sob o Certificado de Licença número 0118/02.

14 - Análise Histológica: Foram realizados cortes histológicos dos estômagos dos animais submetidos ao protocol de úlcera por etanol. Os cortes foram fixados em formol tamponado a 10 % e foram processadas por técnica rotineira de inclusão em parafina e enviadas para processamento histológico e confecção de lâminas com cortes de 4 µm. As secções obtidas foram coradas com Hematoxilina e Eosina.

15 - Análise Bioquímica: Com o soro obtido dos animais submetidos ao protocolo de indução de úlcera por etanol foram feitas as análises bioquímicas. Estas análises permitiram a avaliação de uma possível toxicidade aguda, já que para determinação de uma toxicidade crônica, seria necessário o tratamento destes animais por um tempo maior e em doses diferentes. Foram analisados os níveis séricos das transaminases hepáticas (AST e ALT) e da creatinina, pois são parâmetros utilizados para avaliação de toxicidade hepática e renal. Utilizamos para estas análises os Kits da Labtest, Lagoa Santa, Minas Gerais, Brasil.

16 - Análise Estatística: Primeiramente analisamos se os dados obtidos seguiam uma curva gaussiana, para que de acordo com a análise fosse utilizado um programa paramétrico ou não paramétrico adequado. Os dados obtidos foram analisados estatisticamente através de testes *t*-Student e análise de variância ANOVA, utilizando-se como parâmetro de significância $p < 0,05$ (Instat Graph Pad).

4.RESULTADOS

PlosOne (Aceito)

An Improved Method for P2X7R Antagonist Screening

Rômulo José Soares-Bezerra1*, Natiele Carla da Silva Ferrira1, Anael Viana Pinto Alberto1, André Gustavo Calvano Bonavita1, Antônio Augusto Fidalgo-Neto1, Valber da Silva Frutuoso2 & Luiz Anastacio Alves1

1. Laboratory of Cellular Communication, Oswaldo Cruz Institute – FIOCRUZ, Brazil;

2. Laboratory of Immunopharmacology, Oswaldo Cruz Institute – FIOCRUZ, Brazil.

An Improved Method for P2X7R Antagonist Screening

Rômulo José Soares-Bezerra[1], Natiele Carla da Silva Ferreira[1], Anael Viana Pinto Alberto[1], André Gustavo Calvano Bonavita[1], Antônio Augusto Fidalgo-Neto[1], Valber da Silva Frutuoso[2] & Luiz Anastacio Alves[1]*

1. Laboratory of Cellular Communication, Oswaldo Cruz Institute – FIOCRUZ, Brazil;
2. Laboratory of Immunopharmacology, Oswaldo Cruz Institute – FIOCRUZ, Brazil.

*** Corresponding Author**
E-mail: alveslaa@gmail.com
Address: Laboratório de Comunicação Celular – sala 39 Pav 108 IOC – Fiocruz
Avenida Brasil 4365, Manguinhos, RJ – Brazil - CEP-21045-900.
Tel: +55-21-2562-1809
Fax: +55-21-2562-1772

Abstract

The P2X7 receptor (P2X7R), a member of the P2X ionotropic receptor family, is physiologically activated by ATP. When activated by high concentrations of ATP (i.e., at inflammation sites), this receptor is capable of forming a pore that allows molecules of up to 900 Da to pass through. This receptor is upregulated in several diseases, particularly leukemia, rheumatoid arthritis and Alzheimer's disease. A selective antagonist of this receptor could be useful in the treatment of P2X7R activation-related diseases. The major difficulty in evaluating drug screening is measuring P2X7R activity in cell cultures and biological systems, and some methods use flow cytometry and/or fluorescence microscopy to measure the uptake of fluorescent dyes with molecular weights of up to 900 Da, such as propidium iodide (PI) and Lucifer Yellow (LY). Several fluorometric assays have been described, but the applicability, reproducibility and accuracy of these methods has not been properly evaluated. In the present study, we evaluated several parameters using *in vitro* protocols to validate a high-throughput screening (HTS) method to identify P2X7R antagonists. We generated dose-response curves to determine the EC_{50} value of the known agonist ATP and the IC_{50} values for the known antagonists Brilliant Blue G (BBG) and oxidized ATP (OATP). The values obtained were consistent with those found in the literature (0.6-0.9 mM, 0.9-1.2 µM and 100 µM for ATP, BBG and OATP, respectively). The Z-factor, an important statistical tool that can be used to validate the robustness and suitability of an HTS assay, was 0.635 for PI uptake and 0.867 for LY uptake. No inter-

operator variation was observed, and the results obtained using our improved method were reproducible. Our data indicate that our assay is suitable for the selective and reliable evaluation of P2X7 activity in multiwell plates using spectrophotometry-based methodology. This assay method might improve the high-throughput screening of conventional chemical or natural product libraries for possible candidate P2X7R antagonist or agonist compounds.

Keywords: P2X7R, antagonist, screening, drug discovery, spectrophotometry

Introduction

Drug Discovery

Herbal medicines have been used worldwide for millennia to treat many diseases. Just over 200 years ago, the first pharmacologically active compound, morphine, was isolated from a plant; since then, it has been used as a potent analgesic. It has been estimated that more than 400,000 plant species exist. Plants have formed the basis of therapeutic medical interventions throughout human history. Despite the availability of modern technology, plants are commonly used to treat various ailments and continue to serve as the basis for many pharmaceutical products [1]. The development of drugs to treat human diseases has been based mainly on a variety of plant-derived compounds. Well-known examples include aspirin, morphine, reserpine, digitalis and quinine. We observed an era during which drugs could be isolated, purified, studied and administered in precise doses. Pharmaceutical research expanded after the 1940s, and approximately 80% of drugs developed since then have been derived from natural sources and their analogs or identified through micro-organism screening [2]. In contrast, the focus of drug discovery efforts has shifted from natural products to synthetic compounds in recent decades [3]. Although the expansion of synthetic medicinal chemistry in the 1990s resulted in a decrease in the proportion of new drugs obtained directly from natural products, some synthetic drugs continue to be based on molecules obtained from natural compounds.

Structure-Activity Relationship (SAR) studies, combinatorial chemistry and molecular modeling based on receptor structure are other means by which molecules with

selective agonistic or antagonistic activity toward cellular targets can be rationally obtained and synthesized [4].

A wide variety of screening methods can be used for drug discovery; however, only some of these methods are inexpensive, rapid and efficient. Therefore, the approval of new molecular entities has decreased over the past 15 years [5]. An increase in successful drug discovery, especially for drugs targeting difficult chronic targets, is clearly desirable. Thus, it is important to improve drug discovery screening methods.

P2X7R antagonists as promising therapeutic tools

The roles of ATP and its receptors in physiological and pathophysiological processes have been recognized [6].

Purines and pyrimidines are widely distributed in organisms, where they can act as signaling mediators, promoting various biological effects through purinergic receptors (P1 and P2 receptors). P2 receptors are divided into two families: P2Y and P2X. P2Y receptors (P2YR) are G protein-coupled receptors, whereas P2X receptors (P2XR) are ionotropic receptors [7].

Seven mammalian P2XR subtypes (P2X1-7) have been cloned [8]. P2X7R is expressed in many physiological systems, including the endocrine, cardiovascular, immune, nervous, respiratory, reproductive and digestive systems, as well as in muscular tissues. In the immune system, P2X7R is expressed in several cell types, including macrophages, monocytes, dendritic cells, lymphocytes and mast cells [8].

When activated, P2XR allows for the passage of ions based on an electrochemical gradient. P2X7R is unique in its ability to act as either a low conductance channel (approximately 10 pS) or as a nonselective pore with high conductance (approximately 400 pS). The open pore conformation allows the passage of molecules of up to 900 Da [8]. Based on this property, fluorescent dyes are used in functional assays that evaluate the activation of this receptor; such dyes include ethidium bromide (EB) (mw = 394.31 Da), Lucifer Yellow (LY) (mw = 457.24 Da), YO-PRO-1 (mw = 629 Da) and propidium iodide (PI) (mw = 668.39 Da). Several authors have reported that the formation of a large conductance channel following P2X7R activation is related to the interaction of

this receptor with the Pannexin 1 channel; to date, this finding remains controversial [9]. A recently published study by our group found no correlation between P2X7R and the Pannexin 1 large conductance channel in macrophages [10].

P2X7R plays a crucial role in many physiological processes, including activation of the inflammatory response during pain transduction [11,12] as well as apoptotic and necrotic processes. Moreover, P2X7R participation has been suggested to play a role in several pathologies, including acute leukemia in children [13], neurodegenerative [14] and parasitic diseases [15] and renal diseases, such as glomerulonephritis [16]. Additionally, upregulation of this receptor has been observed in tumor cells [17,18].

Due to the findings outlined above, these nucleotide receptors are attracting attention for use in the development of drugs for the treatment of a number of disorders [19-23]. In addition, purinergic receptors have received increasing attention as potential therapeutic targets [24]. The pharmaceutical industry has made a significant investment in researching novel, selective and potent antagonists for these receptors that might be useful as therapeutic agents. Existing antagonists of these receptors are not fully specific, acting on different cellular targets or on both P2R subtypes [8].

Several fluorometric methodologies that can be used to screen for molecules with P2X7R antagonistic activity have been described in the literature [25-28]. For example, Baxter *et al.* (2003) described the use of a 96-well plate and ethidium bromide in the context of an HTS assay to identify antagonistic compounds, but no essential information regarding the evaluation of the methodology used was published [25]. In the same year, Alcaraz *et al*. described a method using a white 96-well plate and ethidium bromide, in which the analyses were performed using a plate fluorimeter [26]. However, the protocol required a 90-minute incubation at room temperature, and cell viability, possible fluorimetric interference between the white wells and ethidium bromide, and the standardization of this method were not discussed. Another study published in 2009 by Michel *et al.* described an HTS method using a 96-well plate fluorescence reader (Flex Station, Molecular Devices, Sunnyvale, California, United States); the protocol involved treating cells with antagonists for 40 minutes. Prior to the agonist or EB applications, all treatments were performed at room temperature; however, the median incubation time after

treatment, the cell viability, the doses of the agonists or antagonists used and the accuracy and reproducibility of this method were not described [28]. Recently, Namovic *et al.* (2012) described a functional HTS assay evaluating P2X7R-induced pore formation using a fluorescence imaging plate reader (FLIPR) and an expensive automated platform, which allowed YO-PRO-1 uptake to be detected in the presence of P2X7R-specific agonists and antagonists in different species. However, the uptake of other dyes (e.g., anionic dyes) and agonist molecules was not tested, nor was possible interference of the Pannexin 1 channel on dye permeation through a P2X7R-induced pore [27].

The purpose of the present study was to develop a fast and low-cost protocol for the identification of natural compounds with potential P2X7R antagonistic or agonistic effects. To that end, we used a spectrophotometric microplate reader, which allowed us to simultaneously test between 96 and 386 compounds. We took advantage of the ability of this receptor to open a non-selective pore that promotes the uptake of fluorescent dyes under stimulation with high doses of ATP (> 100 µM). We used J774.G8 cells that natively express P2X7R [10] to evaluate the ATP-dependent dose-response (EC_{50}) and the inhibitory concentration (IC_{50}) of Brilliant Blue G (a reversible blocker). To verify that dye uptake was occurring through the Pannexin 1 channel, we assessed an experimental protocol using a Pannexin 1 antagonist (mefloquine) and found no correlation between the pore activity of P2X7R and Pannexin 1 channel activity, as previously reported [10]. Thus, we developed a method that has high sensitivity and reproducibility and low cost and may be useful for testing many compounds simultaneously.

Experimental Section

Reagents

ATP (adenosine 5'-triphosphate), Brilliant Blue G (BBG), PI, Triton X-100, HEPES, NaCl, KCl, $MgCl_2$, $CaCl_2$ and RPMI 1640 were purchased from Sigma Chemical Co., St. Louis, MO (USA). Fetal bovine serum (FBS) was obtained from Gibco BRL (USA).

Cells

The J774.G8 macrophage cell line, which was previously demonstrated by us to natively express P2X7R and the Pannexin 1 channel [10], as well as other P2 subtypes, including P2X4R and P2Y1, P2Y2 and P2Y4 receptors [29], was routinely maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C under an atmosphere containing 5% CO_2. Every three days, the medium was changed, and the cells were adjusted to a density of $4x10^6$ cells per 150 cm^2 cell culture flask (Corning). Prior to evaluating the method that was improved upon in this study, cell viability was assessed using Trypan Blue exclusion. Once the range of viability exceeded 90%, the spectrophotometric assay was conducted.

Spectrophotometric measurement of P2X7R pore activity

Our experimental approach consisted of the measurement of PI or LY uptake by J774.G8 cells through the P2X7R-associated pore. J774.G8 cells ($4x10^5$/well) were plated in opaque 96-well plates (Corning) and maintained in culture with RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C under an atmosphere containing 5% CO2 for 24 h. After this time, the medium was exchanged for an extracellular saline solution (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 HEPES, pH 7.4. The following protocol was then performed: The cells were stimulated with ATP [5 mM] for 15 minutes at 37ºC under an atmosphere containing 5% CO_2. PI [50 nM] or LY [3 mM] was then added to the cell cultures for 5 minutes (to avoid the uptake of these dyes via other mechanisms, such as pinocytosis) in the treatments using LY. After this incubation, the saline solution was exchanged to eliminate interference from non-permeated dye. The plates were then assessed using a spectrophotometer (SpectraMax M5, Molecular Devices, Sunnyvale, California, United States); PI uptake was measured by excitation at 488 nm -λ and emission at 590 nm λ, whereas LY uptake was measured by excitation at 485 nm -λ and emission at 528 nm λ. Three wells were used for each control group: The positive control group was treated with Triton X-100 [0.1%] (which was directly added to the control wells), the negative control group was treated only with extracellular saline

solution, and the remaining groups were treated with ATP [5 mM] following exposure to dye (PI or LY) for longer than 5 minutes.

Drug Treatments

The protocols including treatment with the P2X7 antagonist (the competitive antagonist, BBG [0.048-100 μM]) or the irreversible antagonist (oxidized ATP (OATP), [0.048-400 μm]) were the same as those described above. Three wells were used to test each concentration. BBG-treated wells were incubated for 15 minutes at 37ºC under an atmosphere containing 5% CO_2, whereas OATP-treated wells were incubated for 60 minutes at 37ºC under an atmosphere containing 5% CO_2. After both treatments, ATP stimulation was applied for 15 minutes; then, PI or LY was added for an additional 5 minutes before spectrophotometric analysis.

Imaging analysis

In addition to the spectrophotometric analyses, we also performed fluorescence microscopy analyses (Nikon Eclipse TE2000S) as previously described to verify dye uptake in ATP-activated cells. Images were captured using a Nikon digital camera system.

Lactate Dehydrogenase (LDH) detection assay

The supernatants of J774.G8 cells that had been previously treated for 15 minutes with five different concentrations of ATP were performed according to the instructions included in the LDH detection kit, which was purchased from Doles (Goiania, GO, Brazil).

Cytotoxicity assay using the MTT technique

J774.G8 cells ($4x10^5$ cells/well) were treated for 15 minutes with five different concentrations of ATP, after which point, the medium was changed and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) was added. After incubation for 3 h at 37ºC, the reactions were interrupted with DMSO, and the absorbance was measured using a spectrophotometer (SpectraMax M5, Molecular Devices, Sunnyvale, California, United States) at 450 nm.

Data analysis

Each sample was measured in triplicate, and all experiments were repeated a minimum of three times by each operator. In the present study, two operators participated in all of the experimental steps. The fit was evaluated using the R^2 coefficient [33]. The data are expressed as the means ± standard deviation. To determine whether the samples followed a Gaussian distribution, we used two normality tests: D'Agostino's K-squared test and Pearson's chi-squared test. When the data followed a Gaussian distribution, they were compared using one-way ANOVA, followed by Tukey's post-hoc analysis. Data that did not follow a Gaussian distribution were compared using the Mann-Whitney U test. *P* values of 0.05 or less were considered significant. The tests used are specified in the figure legends and were two-tailed and paired. We also assessed the quality of our improved HTS assay by calculating the Z-factor, which is a valuable tool that can be used to evaluate the robustness and suitability of HTS assays and that can be useful for assay optimization and validation [34]. This parameter is calculated using the following equation:

$$Z = 1 - \frac{3SD \text{ of sample} + 3SD \text{ of control}}{|\text{mean of sample} - \text{mean of control}|}$$

The SD values represent the standard deviation values (of the control or sample). We calculated the Z-factor using ATP as a control, and the means of the samples were calculated using the dye uptake assay values for PI or LY, accordingly.

Results and Discussion

Effects of ATP on PI uptake in J774.G8 cells

To determine whether our screening method is reliable and reproducible, we established a dose-response curve using ATP. We plated cells in opaque 96-well plates and added various concentrations of ATP from 0.321 to 25 mM. After 15 minutes, we added PI and measured the absorbance using a spectrophotometer. The calculated EC_{50} of ATP using our method was 0.7389 ± 0.02 mM. The dose-response curve for ATP using two different operators is shown in Figure 1. This value of EC_{50} was similar to that described in the literature for the activation of selective dye permeation pathways by P2X7R [35]. Our method resulted in a low EC_{50} value compared to values obtained using other methodologies described in the literature (Table 1), confirming the high accuracy of this method. Inter- and intra-operator mensurements did not differ significantly, and the median difference between the daily measurements made by the two operators was 0.877 ($p<0.05$) (Figure 1). We evaluated the cytotoxicity of the ATP treatments at the concentrations described above for 15 minutes using the LDH and MTT assays. No toxicity was observed until 1 hour had elapsed (as shown in Figures 6 and 7).

Antagonist-mediated inhibition of the P2X7 receptor

We next determined whether the main P2X7R competitive antagonist, BBG, could block the pore associated with the receptor in a dose-dependent manner and with a similar IC_{50} value. Brilliant Blue G (BBG) is an analog of FD&C blue dye, a widely used food additive that is commonly used as a selective P2X7R antagonist [36]. Some authors have suggested that the low toxicity and high selectivity of BBG make this compound useful for blocking the potential adverse effects of P2X7R activation in several pathological conditions [36].

As expected, BBG blocked PI transport, and the IC_{50} value ranged from 0.9 to 1.2 µM (Figure 2). As mentioned previously, the inter- and intra-operator variations in the IC_{50} value were not significant, and the dose-response curves did not differ significantly (Figure 2). An IC_{50} value of 6.71± 0.05 µM for mP2X7R is reported in the literature [27], and we compared this value to our results because our model was based on a mouse macrophage cell line (J774.G8). Using our improved methodology, we obtained IC_{50} values for BBG and mP2X7R that ranged from 0.9 to 1.2 uM. These values are lower than those reported previously, indicating a more sensitive and specific antagonistic action on this receptor.

After examining the antagonistic activity of BBG on P2X7R using our methodology, we evaluated the activity of other antagonist molecules on this receptor. For this purpose, we used a specific irreversible antagonist, oxidized ATP (OATP). As shown in Figure 3, we established an IC_{50} value [100 µM] and an inhibitory curve for oxidized ATP [0.048-400 µM]; these values are less than those obtained using other methods (Table 1). To confirm the data that we obtained using other methodologies that are currently used to screen molecules with activity at this receptor, we used a dye uptake protocol based on J774.G8 cells treated with known P2X7R agonists and antagonists at specific concentrations that are known to activate only P2X7R [9] prior to conducting fluorescence microscopy analyses (Figure 4). The images confirmed that the P2X7 pore was activated by ATP and that it was blocked by both BBG and OATP. When we compared the IC_{50} and EC_{50} values obtained using this improved methodology with values obtained using other methodologies, our results were more sensitive and accurate than those obtained using methods that are usually used to screen compounds with P2X7R activity (Table 1).

Some authors have described the existence of two activated pores that are related to P2X7R activation, one of which allows for the passage of cationic molecules and the other of which allows for the uptake of anionic molecules [35]. We conducted an experiment to assess our method of evaluating the inhibition of anionic dye uptake by the P2X7R pore. As shown in Figure 5, we treated J774.G8 cells with ATP [5 mM], the antagonists BBG [100 nM] and OATP [300 µM] and an anionic dye, LY [3 mM]. The results show the inhibitory effects of these antagonists on dye uptake, thereby validating our method of investigating compounds with P2X7R antagonistic activity by inhibiting the uptake of anionic molecules

by the activated pore. To confirm the robustness of our HTS assay, we used a statistical parameter termed the Z-factor, which is used to evaluate the suitability of a high-throughput screening assay based on the range of the signal and the internal variation of the equipment. A score of $1>Z\geq0.5$ indicates a good assay, and a score of $Z=1$ indicates a perfect assay. Our calculated Z-factor for the HTS assay was 0.635 using PI and 0.876 using LY [34].

Pannexin 1 has been implicated as the protein associated with the P2X7R pore [30]. For this reason, we examined whether our methodology was specific for P2X7R pore activity. As seen in Figure 6, treatment with the Pannexin 1 antagonist, mefloquine, and the P2X7R agonist ATP did not block dye uptake, indicating that this uptake may have occurred via the open P2X7R pore rather than via the Pannexin 1 channel. This result reinforces the applicability of our standardized methodology to verify the blockage or non-blockage of dye uptake through P2X7R activation only. When taken together, our results validated the improved methodology developed in this study and demonstrated its applicability to P2X7R antagonist screening. P2X7R is primarily expressed in immune cells and cells of hematopoietic origin, such as mast cells and microglia, and has a longer carboxy-terminal tail than the six other members of the P2X receptor family. As mentioned previously, this receptor plays a crucial role in a significant number of pathophysiological conditions.

Pharmaceutical companies have expressed great interest in the development of new medicines, and the rate of patent protection loss is significantly greater than the rate of new drug discovery. Given this consideration, the market for new pharmaceutical compounds is relatively fragile because a small number of products represent a significant portion of the market. For example, eight products accounted for 58% of Pfizer's annual worldwide sales of $44 billion in 2007 [2]. When such drugs lose their patent protection, the associated sales revenue can decrease by 80%. In addition, the competition from generic drug manufacturers, who do not participate in the drug discovery process, is intense; generic manufacturers produce 67% of all drugs prescribed in the United States [2].

Many methodologies are used to screen currently available drugs. However, some features, such as the speed of execution and reliability, are critical. Furthermore, it is important that these screening methods are able to test a large number of candidates.

Currently, high-throughput screening (HTS) programs in drug discovery rely mainly on combinatorial chemistry libraries. When compared to the very large number of natural products, combinatorial chemistry libraries are relatively limited with respect to molecular diversity. Our improved methodology might possess great advantages over currently available methodologies because it can easily be used to screen a wide spectrum of compounds of synthetic or natural origin.

Conclusions

Synthetic compounds are currently being tested and patented by the pharmaceutical industry as potential antagonists for clinical use [37]. There is a strong clinical and industrial interest in specific P2X7R antagonists for use in the treatment of many diseases, such as those related to inflammation or pain, as well as cancer. Advances in the selection and development of new P2 receptor antagonists will certainly be relevant and is of significant technological interest. To facilitate and speed the discovery of potential antagonists, the development of a simple, rapid and relatively inexpensive method for testing compounds on a large scale is necessary. In the present study, an improved method was standardized and tested using known P2X7R antagonists, generating results that validated this protocol; this method is applicable to the selection of new compounds with P2X7Y inhibitory activity.

Acknowledgments

This work was supported by grants from the CNPq, FAPERJ, PAPES V/Fiocruz-CNPq and Oswaldo Cruz Institute.

Figure legends:

Figure 1: ATP dose-response curve. J774.G8 cells were plated in opaque 96-well plates and treated with various concentrations of ATP from 0.321 to 25 mM in combination with PI [50 nM]. The data represent the means ± S.D. of three independent experiments. The results were analyzed using the Mann-Whitney U test; * $p<0.05$.

Figure 2: BBG dose-response curve. J774.G8 cells were plated in opaque 96-well plates and treated with various concentrations of BBG from 0.048 to 100 μM in combination with PI [50 nM] and ATP [5 mM]. The data represent the means ± S.D. of three independent experiments. The results were analyzed using the Mann-Whitney U test; * $p<0.05$..

Figure 3: OATP dose-response curve. J774.G8 cells were plated in opaque 96-well plates and treated with various concentrations of OATP from 0.048 to 400 μM. The data represent the means ± S.D. of three independent experiments. The results were analyzed using the Mann-Whitney U test; * $p<0.05$.

Figure 4: Evaluation of the inhibitory activity of BBG on P2X7 using fluorescence microscopy. J774.G8 cells were treated as follows: (a) Control (J774 plus PI [50 nM]), (b) control (0.1% Triton-X 100), (c) control cells plus ATP [5 mM], (d) cells treated with BBG [100 nM] plus ATP [5 mM] and (e) cells treated with OATP [300 μM] plus ATP [5 mM]. The profiles are representative of three to seven independent experiments that were performed in triplicate. The images were captured using a Nikon digital camera system with a total magnification of 43.8X.

Figure 5: Anionic dye uptake. J774.G8 cells were plated in opaque 96-well plates after the following treatments: a) Cells + LY [3 mM], b) cells + ATP [5 mM] + LY [3 mM], c) cells + 0.1% Triton X-100 [3 mM], d) cells + BBG [100 nM] + ATP [5 mM] + LY [3 mM] and e) cells + OATP [300 μM] + ATP [5 mM] + LY [3 mM]. The data represent the means ± S.D. of three independent experiments. The results were analyzed using ANOVA, followed by Tukey's post-hoc test; * $p<0.05$.

Figure 6: MTT cytotoxicity assay. J774.G8 cells that were treated for 15 minutes with five concentrations of ATP were analyzed. (a) Untreated cells, (b) cells + Triton X-100 [0.1%], (c) cells + ATP [5 mM], (d) cells + ATP [10 mM], (e) cells + ATP [15 mM], (f) cells + ATP [20 mM] and (g) cells + ATP [25 mM]. The data represent the means ± S.D. of three independent experiments. The results were analyzed using ANOVA, followed by Tukey's post-hoc test; * $p<0.05$.

Figure 7: LDH assay in J774.G8 Cells. The supernatants of cells that had been treated for 15 minutes with five concentrations of ATP were analyzed. (a) Untreated cells, (b) cells + Triton X-100 [0.1%], (c) cells + ATP [5 mM], (d) cells + ATP [10 mM], (e) cells + ATP [15 mM], (f) cells + ATP [20 mM] and (g) cells + ATP [25 mM]. The data represent the means ± S.D. of three independent experiments. The results were analyzed using ANOVA, followed by Tukey's post-hoc test; * $p<0.05$.

FIGURE 1

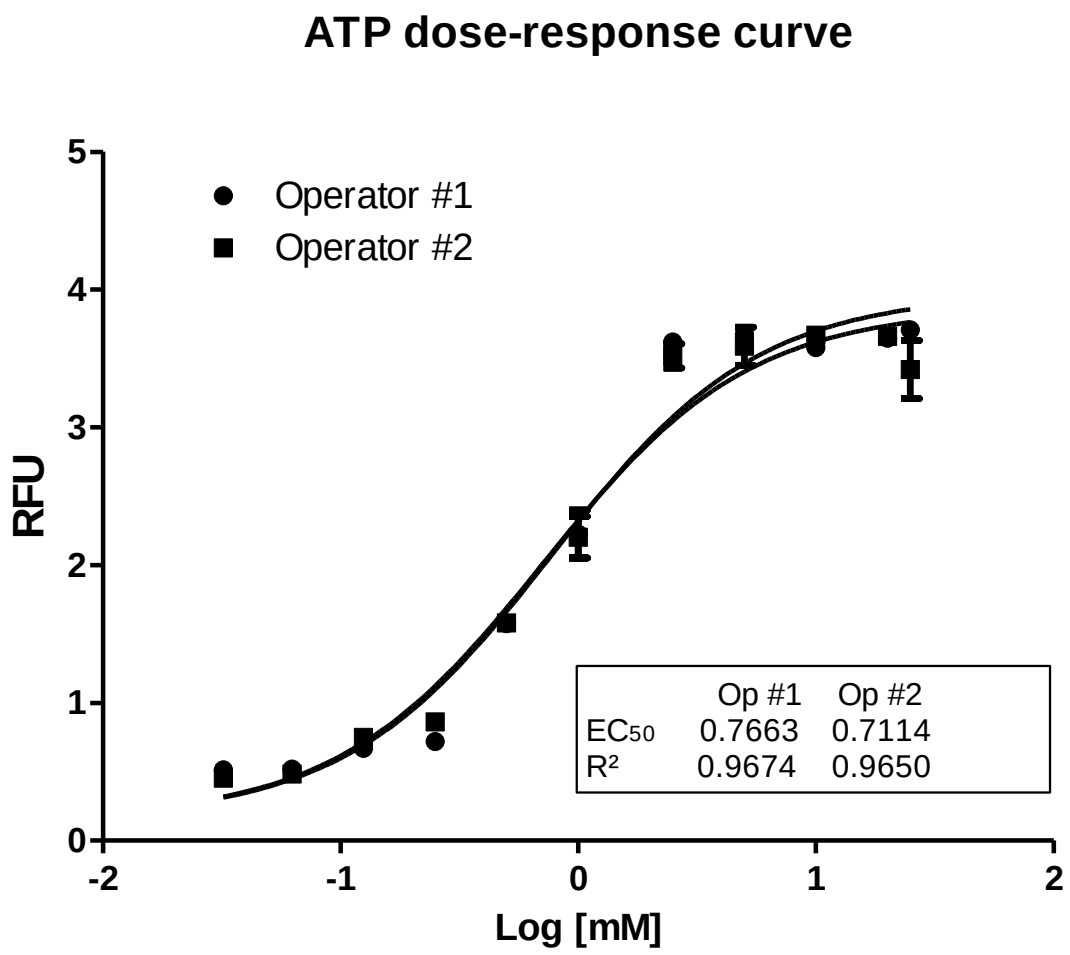

ATP dose-response curve
Operator #1
Operator #2
RFU
Log [mM]
Op #1 Op #2
EC50 0.7663 0.7114
R² 0.9674 0.9650

FIGURE 2

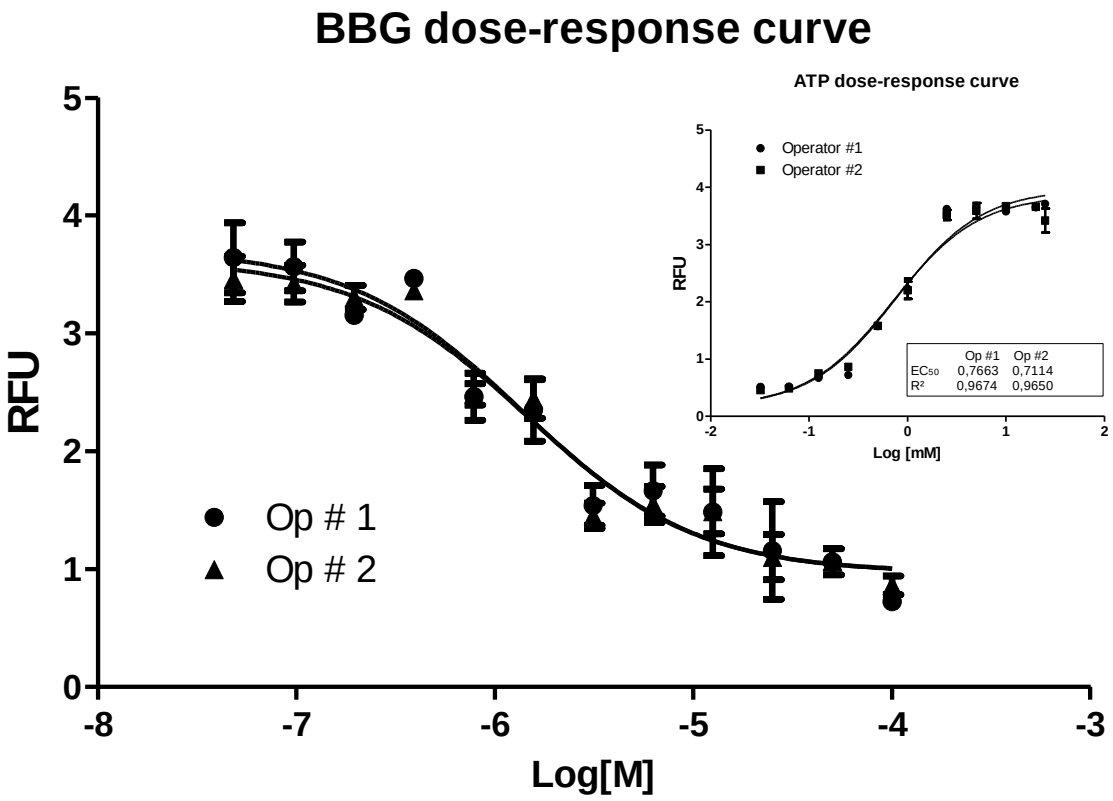

BBG dose-response curve
RFU
Log[M]
Op # 1
Op # 2
ATP dose-response curve
Operator #1
Operator #2
RFU
Log [mM]
Op #1 Op #2
EC50 0,7663 0,7114
R² 0,9674 0,9650

FIGURE 3

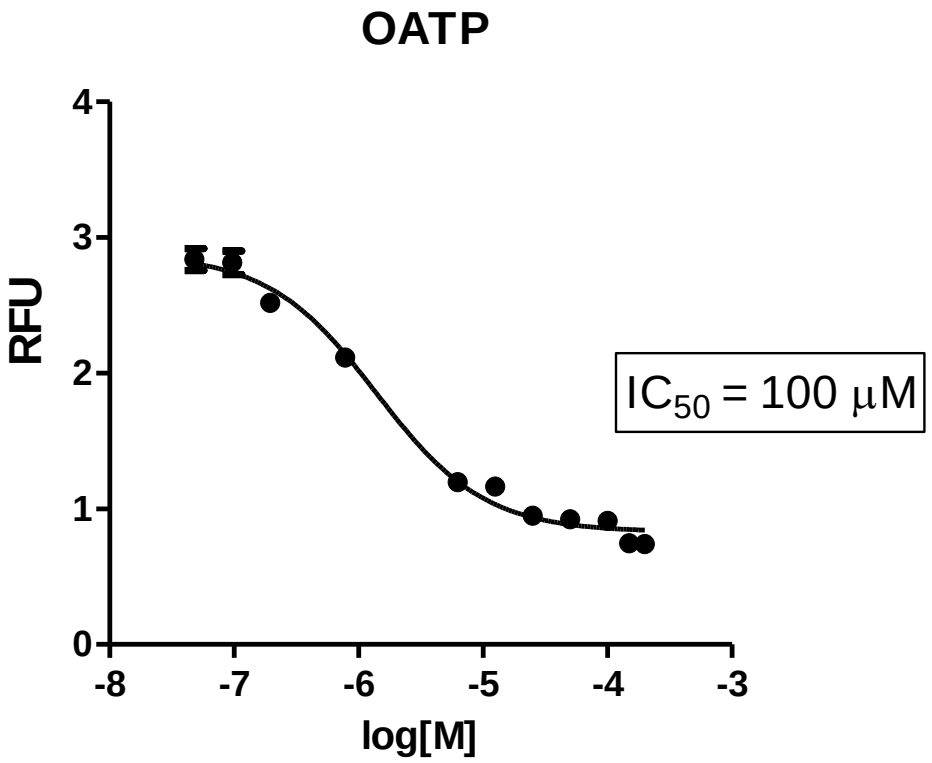


FIGURE 4

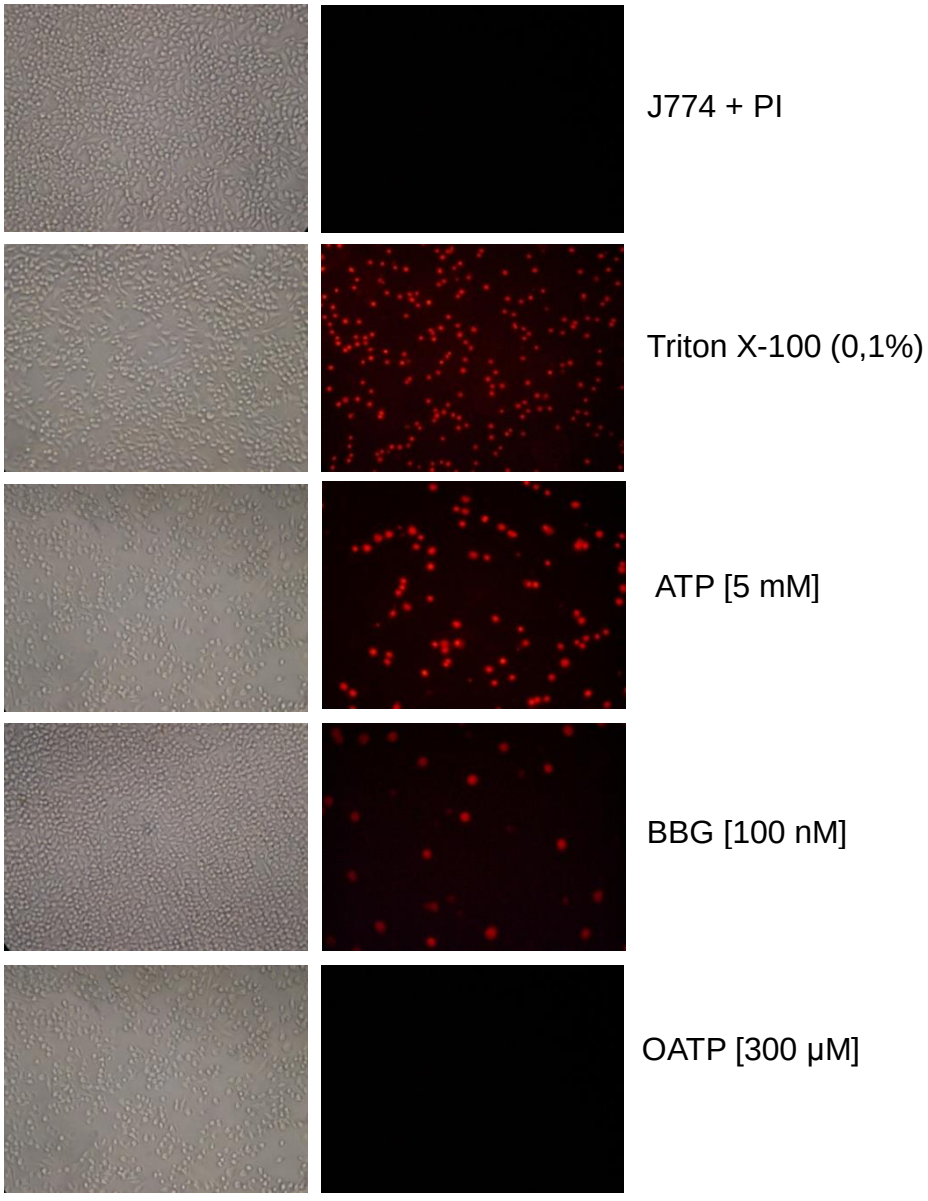


FIGURE 5

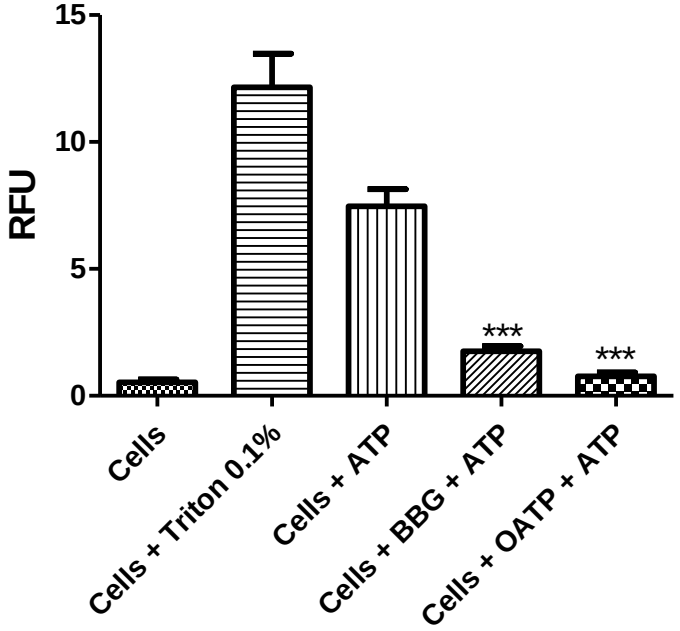


FIGURE 6

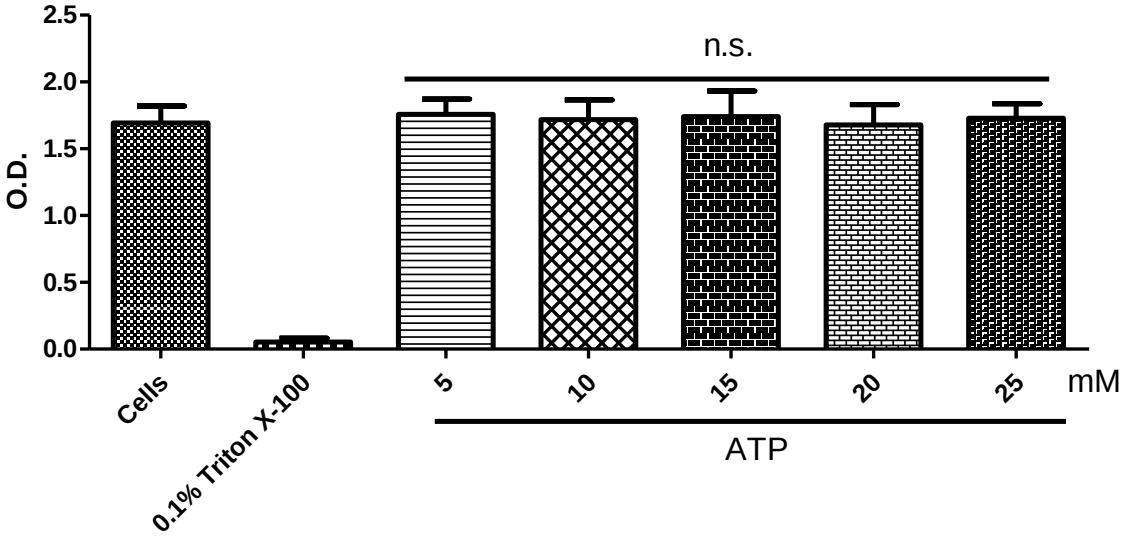


FIGURE 7

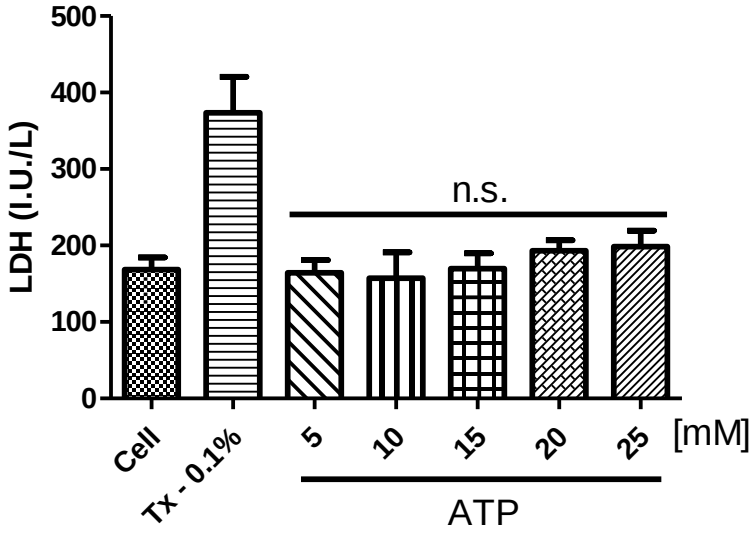


Table 1. IC_{50} or EC_{50} values for P2X7R agonists or antagonists using different methods.

	ATP (EC_{50})	BBG (IC_{50})*	OATP (IC_{50})	Reference
Cytometry	2-4 mM	N.D.**	300 uM	Coddou *et al*. 2011
Electrophysiology	2-4 mM	N.D.**	300 uM	Coddou *et al.* 2011
FLIPR	N.D.**	6.71± 0.05 uM	N.D.**	Namovic *et al*. 2012
Spectrophotometry	0.6-0.9 mM	0.9-1.2 uM	100 uM	Present study

*IC_{50} for natively expressed mP2X7R in J774.G8 cells.

**N.D. = Not determined

References:

1. Pan SY, Pan S, Yu ZL, Ma DL, Chen SB, et al. (2010) New perspectives on innovative drug discovery: an overview. J Pharm Pharm Sci 13: 450-471.

2. Li JW, Vederas JC (2009) Drug discovery and natural products: end of an era or an endless frontier? Science 325: 161-165.

3. Zhu F, Qin C, Tao L, Liu X, Shi Z, et al. (2011) Clustered patterns of species origins of nature-derived drugs and clues for future bioprospecting. Proc Natl Acad Sci U S A 108: 12943-12948.

4. Williams M (1996) Challenges in developing P2 purinoceptor-based therapeutics. Ciba Found Symp 198: 309-321.

5. Mitchell W, Matsumoto S (2011) Large-scale integrated super-computing platform for next generation virtual drug discovery. Curr Opin Chem Biol 15: 553-559.

6. Lister MF, Sharkey J, Sawatzky DA, Hodgkiss JP, Davidson DJ, et al. (2007) The role of the purinergic P2X7 receptor in inflammation. J Inflamm(Lond) 4: 5.

7. Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50: 413-492.

8. Burnstock G (2009) Purinergic signalling: past, present and future. Braz J Med Biol Res 42: 3-8.

9. Coddou C, Yan Z, Obsil T, Huidobro-Toro JP, Stojilkovic SS (2011) Activation and regulation of purinergic P2X receptor channels. Pharmacol Rev 63: 641-683.

10. Alberto AV, Faria RX, Couto CG, Ferreira LG, Souza CA, et al. (2013) Is pannexin the pore associated with the P2X7 receptor? Naunyn Schmiedebergs Arch Pharmacol 386: 775-787.

11. Buchanan MS, Carroll AR, Addepalli R, Avery VM, Hooper JNA, et al. (2007) Natural Products, Stylissadines A and B, Specific Antagonists of the P2X7 Receptor, an Important Inflammatory Target1. The Journal of Organic Chemistry 72: 2309-2317.

12. Santos JA, Fidalgo-Neto AA, Faria RX, Simoes A, Calheiros AS, et al. (2011) Effect of Rheedia longifolia leaf extract and fractions on the P2X receptor in vitro: novel antagonists? J Med Food 14: 920-929.

13. Chong JH, Zheng GG, Zhu XF, Guo Y, Wang L, et al. (2010) Abnormal expression of P2X family receptors in Chinese pediatric acute leukemias. Biochem Biophys Res Commun 391: 498-504.

14. Takenouchi T, Sekiyama K, Sekigawa A, Fujita M, Waragai M, et al. (2010) P2X7 receptor signaling pathway as a therapeutic target for neurodegenerative diseases. Arch Immunol Ther Exp (Warsz) 58: 91-96.

15. Cascabulho CM, Menna-Barreto RF, Coutinho-Silva R, Persechini PM, Henriques-Pons A (2008) P2X7 modulatory web in Trypanosoma cruzi infection. Parasitol Res 103: 829-838.

16. Taylor SR, Turner CM, Elliott JI, McDaid J, Hewitt R, et al. (2009) P2X7 deficiency attenuates renal injury in experimental glomerulonephritis. J Am Soc Nephrol 20: 1275-1281.

17. Gorodeski GI (2009) P2X7-mediated chemoprevention of epithelial cancers. Expert Opin Ther Targets 13: 1313-1332.

18. Mann J (2002) Natural products in cancer chemotherapy: past, present and future. Nat Rev Cancer 2: 143-148.

19. Di Virgilio F, Baricordi OR, Romagnoli R, Baraldi PG (2005) Leukocyte P2 receptors: a novel target for anti-inflammatory and anti-tumor therapy. CurrDrug TargetsCardiovascHaematolDisord 5: 85-99.

20. Burnstock G (2001) Purinergic and Pyrimidinergic Signaling I - Molecular, Nervous and Urinogenitary System Function. In: Abbracchio MP, Williams M, editors. Hnadbook of Experimental Pharmacology. Berlin: Springer-Verlag. pp. 89-127.

21. Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev 87: 659-797.

22. Burnstock G (2008) Purinergic signalling and disorders of the central nervous system. NatRev Drug Discov 7: 575-590.

23. Burnstock G (2009) Purinergic regulation of vascular tone and remodelling. AutonAutacoidPharmacol 29: 63-72.

24. Williams M, Jarvis MF (2000) Purinergic and pyrimidinergic receptors as potential drug targets. Biochem Pharmacol 59: 1173-1185.

25. Baxter A, Bent J, Bowers K, Braddock M, Brough S, et al. (2003) Hit-to-Lead studies: the discovery of potent adamantane amide P2X7 receptor antagonists. Bioorg Med Chem Lett 13: 4047-4050.

26. Alcaraz L, Baxter A, Bent J, Bowers K, Braddock M, et al. (2003) Novel P2X7 receptor antagonists. Bioorg Med Chem Lett 13: 4043-4046.

27. Namovic MT, Jarvis MF, Donnelly-Roberts D (2012) High throughput functional assays for P2X receptors. Curr Protoc Pharmacol Chapter 9: Unit 9 15.

28. Michel AD, Ng SW, Roman S, Clay WC, Dean DK, et al. (2009) Mechanism of action of species-selective P2X(7) receptor antagonists. Br J Pharmacol 156: 1312-1325.

29. Coutinho-Silva R, Persechini PM (1997) P2Z purinoceptor-associated pores induced by extracellular ATP in macrophages and J774 cells. Am J Physiol 273: C1793-C1800.

30. Iglesias R, Locovei S, Roque A, Alberto AP, Dahl G, et al. (2008) P2X7 receptor-Pannexin1 complex: pharmacology and signaling. Am J Physiol Cell Physiol 295: C752-760.

31. Grundken C, Hanske J, Wengel S, Reuter W, Abdulazim A, et al. (2011) Unified patch clamp protocol for the characterization of Pannexin 1 channels in isolated cells and acute brain slices. J Neurosci Methods 199: 15-25.

32. Dahl G, Qiu F, Wang J (2013) The bizarre pharmacology of the ATP release channel pannexin1. Neuropharmacology.

33. Ludbrook J (1997) Comparing methods of measurements. Clin Exp Pharmacol Physiol 24: 193-203.

34. Zhang JH, Chung TD, Oldenburg KR (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen 4: 67-73.

35. Cankurtaran-Sayar S, Sayar K, Ugur M (2009) P2X7 receptor activates multiple selective dye-permeation pathways in RAW 264.7 and human embryonic kidney 293 cells. Mol Pharmacol 76: 1323-1332.

36. Peng W, Cotrina ML, Han X, Yu H, Bekar L, et al. (2009) Systemic administration of an antagonist of the ATP-sensitive receptor P2X7 improves recovery after spinal cord injury. Proc Natl Acad Sci U S A 106: 12489-12493.

37. Gunosewoyo H, Kassiou M (2010) P2X purinergic receptor ligands: recently patented compounds. Expert Opin Ther Pat 20: 625-646.

A Ser Submetido Após Registro de Patente

Potential antagonistic activity of Three Fungi Extracts on P2X7R with Inhibition of Its Physiologic Pathways

Soares-Bezerra R. J[1], Ferreira N.C.S[1], Alves M.T [3], Zani L.C[3], Francisco M.F[3], Faria R.X[1], Frutuoso V.S[2], Alves L.A[1]*.

1. Laboratório de Comunicação Celular, Instituto Oswaldo Cruz, Fiocruz; 2. Laboratório de Imunofarmacologia, Instituto Oswaldo Cruz, Fiocruz, Brazil; 3. Laboratório de Química de Produtos Naturais, Centro de Pesquisas Químicas René Rachou, Fiocruz – Minas Gerais, Brazil.

Potential Antagonistic Activity of Three Fungi Extracts on P2X7R with Inhibition of Physiologic Pathways

Soares-Bezerra R. J[1], Ferreira N.C.S[1], Alves M.T [3], Zani L.C[3], Francisco M.F[3], Faria R.X[1], Frutuoso V.S[2], Alves L.A[1*]

1. Laboratório de Comunicação Celular, Instituto Oswaldo Cruz, Fiocruz; 2. Laboratório de Imunofarmacologia, Instituto Oswaldo Cruz, Fiocruz, Brazil; 3. Laboratório de Química de Produtos Naturais, Centro de Pesquisas Químicas René Rachou, Fiocruz – Minas Gerais, Brazil.

*E-mail: alveslaa@gmail.com

Address: Laboratório de Comunicação Celular -
IOC - Fiocruz - Pav. 108 - Sala 36 - AV. Brasil 4365, Manguinhos
CEP-21045-900, Rio de Janeiro, RJ. , Brasil.

Tel: +55-21-2562-1809

Abstract

P2X7R is a purinergic receptor that is involved in important physiological and metabolic functions and also in pathological processes, principally those related to the inflammatory process. Despite its relevance, until recently there has not been a selective agonist or antagonist compound useful in the treatment of diseases linked to P2X7R. In this context, the research for a selective, safe, and potent compound that can be used in clinical trials has received special attention from the pharmaceutical's companies. In this sense, many drugs that are used today in the treatment of any pathology have chemical structures based on natural products of some botanical species that have been used in popular medicine for years. The majority of medicines are extracted from natural products; approximately 120 pharmaceutical products are derived from plants, and 75% were discovered from popular medicine. This work was based on a vast investigation of plant and fungi extracts from different biomes, searching for antagonistic activity on P2X7R. As a result, after testing 1800 different extracts, we found three from fungi: 8067, 8549, and 8568. Two are from the Antarctic continent: 8549 and 8568, and another: 8067, is from the Paraná coast of- Brazil. First, the IC50 of these extracts was identified, and with these values tests were performed on some physiological and metabolic functions of P2X7R, such as the capacity of anionic or cationic pore formations, release of IL-1β, and production of NO and ROS. The in vivo activity was also performed in pain and ulcer models. The results showed a promising antagonistic activity of these extracts on P2X7R, which can be future candidates for a Bioproduct with potential clinical applicability for treatment of diseases related to P2X7R.

Keywords: P2X7R, antagonist, Natural Products, drug discovery.

Introduction

P2XR were discovered in the decade of the 90's with the advance of molecular biology. There are seven subtypes (P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, and P2X7) cloned in mammalian cells [1]. P2X7R is expressed in many systems, including the endocrine, cardiovascular, immune, nervous, respiratory, reproductive, and digestive systems, in addition to muscular tissues. In the immune system many cell types were identified, including macrophages, monocytes, dendritic cells, lymphocytes, and mast cells [1].

When activated, the P2XR allows the passage of ions according to the electrochemical gradient. The P2X7 receptor (P2X7R) is unique for its ability to convert from a low conductance channel (~15 ps) to a nonselective pore with large conductance (~400 PS), which allows the passage of molecules up to 900Da, such as fluorodyes (Yo-Pro 1, Propidium iodide, Lucifer yellow, and ephedrine bromide) [2].

Until nowadays, the known antagonists of the P2X7 receptor were not fully understood as they act with different targets in the cell [3]. Research for new antagonists with specificity for this receptor is necessary due to its crucial role in many physiological processes, such as the activation of the immune and inflammatory response; [3], and in the apoptosis and necrosis processes. Moreover, the participation of P2X7R was suggested in several pathologies, including Alzheimer's, Inflammatory diseases, Neuropathic pain, Rheumatoid Arthritis, Myeloid Acute leukemia, and Diabetes [5]. Additionally, in tumor cells there is an up-regulation of this receptor [6]; [7];[8].

Since the discovery of Penicillin by Fleming in 1928 from the *Penicillium chrysogenum* fungus, the research for biological activity of microorganisms and

their secondary metabolites has advanced. Years later, another important discovery, that of Cyclosporin in 1979, arose from the *Tolypocladium inflatum* fungus. This new drug brought a large advance in the medicine of organ transplantation, as it has an immunosuppressive action on T lymphocytes, preventing graft disease [9].

Recently, activity against Leishmania and tumor cells was found in an endophitic Antartic fungus [10]. There is a prevalence of basidiomicetes in the soil and subglacial enviromment of glaciers, where *Cryptococcus liquefaciens* p.e, a yeast of *Cryptococcus sp* is found. Ascomicetes are rarely found in cold environments, although they are encountered on frozen contaminated food [11]. On the other hand, a study published by Butinar *et al*., 2011, described the presence of Ascomicetes in Arctic environments, such as the *Metschnikowia bicuspidata* yeast from the *Metschnikowia sp* genus. Other types of fungus colonize cold enviromments, like *Acarospora algicola,* which is present in the south of Brazil, spefically in Paraná [11].

In this study we found three fungus extracts that had promising *in vitro* and *in vivo* activities on the inhibition of P2X7R. This can be applied in the scenario of the research for a selective compound that can be useful in the treatment of inflammatory diseases related to P2X7R.

Materials and Methods

Reagents

ATP (adenosine 5'-triphosphate), oxidized ATP (OATP), Brilliant Blue G (BBG), Propidium Iodide (PI), Lucifer yellow (LY), Triton X-100, HEPES, NaCl, KCl, $MgCl_2$, $CaCl_2$, Griess reagent modified, LPS from *Escherichia coli*, Dihidroetidium (DHE), and RPMI 1640 were purchased from Sigma Chemical Co., St. Louis, MO (USA). Fetal bovine serum (FBS) was obtained from Gibco BRL (USA), ELISA Kit for detection of IL-1β was purchased from R&D Systems, Minneapolis, MN (USA). kit for detection of LDH was purchased from Doles (Goiania, GO, Brazil).

Cells

J774G8 macrophage murine cell line and U937 human monocyte cell line were routinely maintained in culture with RPMI 1640 medium supplemented with 10% fetal bovine serum, at 37℃ in a 5% CO_2 atmosphere.

Animals

In this study utilized male Swiss Mice (20-30 g), obtained from the central institutional bioterium, and all experimental procedures utilized were approved by the Ethics Committee for Animal Use of FIOCRUZ (N. 0118/02).

Extracts Treatment

The experimental approach consisted of the measurement of PI or LY uptake by J774G8 or U937 cells through P2X7R associated pore. Based on

this, $4x10^6$ cells/well were plated in a 96 well opaque plate (Falcon), and the cells were treated with the extracts [100 µg/mL] or the P2X7R competitive antagonist: BBG [100 µM]. After all treatments, the plate was incubated at 37ºC with atmosphere of 5% of CO_2 for 15 min. The next step was the treatment with or without the P2X7R agonist, ATP [5mM], for 10 min at 37ºC with an atmosphere of 5% CO_2. Later, PI [50 nM] or LY [3mM] was added to cell culture for more 5 minutes, and the plate was read in a spectrophotometer (SpectraMax M5 -λ of excitement 488 nm and λ of reading 590 nm for PI read, and-λ of excitement 485 nm and λ of reading 528 nm for LY read). Before each experiment the medium was changed using an extracellular saline solution (in mM): 150 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, and 10 HEPES, pH7.4. The maximum permebilization control was made using Triton X-100 [0,1%] directly over the cells. The viability control was made with a cell in the presence of saline solution only. The extracts that showed a positive activity were tested again to determinate the IC_{50} value by a dose-response curve.

Extracts

The extracts tested in this work were obtained by a scientific collaboration with Dr. Tânia Maria de Almeida of the Natural Products Chemistry Laboratory of Centro de Pesquisas Químicas René Rachou/FIOCRUZ, Minas Gerais, Brazil.

ELISA assay for detection of IL-1β

The detection of IL-1β in the supernatants of cells previously incubated by 1 h in the presence or absence of the test extract´s IC_{50} and was performed by the instructions from the ELISA Kit for detection of IL-1β from R&D Systems, Minneapolis, MN (USA).

Assay for detection of Lactate Dehydrogenase (LDH)

The detection of LDH activity in the supernatants of cells previously incubated by 24 h in the presence or absence of four times the extract's IC_{50} and/or more 1h in the presence of ATP was performed by the instructions from the kit for detection of LDH purchased from Doles (Goiania, GO, Brazil).

Assay for detection of Nitrite

Nitrite, a stable oxidation product of NO, was measured spectrophotometricaly by adding Griess reagent (Sigma) in the same volume of cell culture supernatants previously incubated in the presence or absence of the extract's IC_{50}. After 10-15 minutes at room temperature, the absorbance of the chromophore was measured at 540 nm (Spectramax M5).

Assay for detection of ROS

The oxidative stress in the J774.G8 and U937 cells after treatments with the extracts or antagonist (BBG) or agonist (ATP), was measured using the dihidroetidium (DHE) dye. The protocol was based on the treatment with DHE [10nM] for 20 minutes, after all treatments previously described. The

fluorescence was determined using a spectrophotometer (Spectramax M5) at excitation and emission wavelengths of 490 and 570 nm, respectively.

Electrophysiologic measurements

Whole-cell patch clamp experiments were performed at 37ºC by using an Axopatch-1D amplifier (Axon Instruments). Cells were transferred to a chamber mounted into a microscope stage. Patch pipettes (with 1.2-mm outer di-ameter) were pulled from IBBL borosilicate glass capillaries (World Precision Instruments). Whole-cell configuration was performed as previously described [14].

Series resistance was 5-11 MΩ for all experiments in standard saline (solution A), and no compensation was applied for currents less than 1500 pA. Above this level, currents were compensated by 88%. Measurements were discarded when the series resistance increased substantially. MPM (mean–standard deviation, 34.98–11.03 pF; n=31) cell capacitance was measured by applying a 20-mV hy-perpolarizing pulse from a holding potential of 20 mV; capacitive transient was then integrated and divided by the amplitude of the voltage step (20 mV).

Currents were filtered with a corner frequency of 5 kHz (8-pole Bessel filter), digitized at 20-50 kHz using a BNC-2110 interface (National Instruments), and acquired on a personal computer by using Axoscope software and pCLAMP 9.0 (Axon Instruments). Recordings were then filtered by using a low-pass digital filter with a cut-off fre-quency of 500 Hz in the software (WinEDR V2.6.6.; Uni-versity of Strathclyde).

Saline solutions for electrophysiology

Different saline solutions were used in the pipette or in the bath, depending on the protocol. The bath solution (in mM) consisted of the following: 150 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, and 10 HEPES (pH, 7.4); the pipette solution (in mM) consisted of the following: 150 KCl, 5 NaCl, 1 MgCl2 10 HEPES, and 0.1 EGTA (pH, 7.4).

Drug application

Patch clamp experiments were carried out under perfu-sion (RC-24 chamber, Warner Instrument Corp.) to confirm the data obtained by micropipette application. All drugs were dissolved in saline solution immediately before use. Ion currents were studied by pulse application of ATP (from 1 to 30 s).

Data analysis

All experiments were performed at least three times. The data presented are mean ± S.E.M. Statistical analysis was performed by 1-way ANOVA. *P* values of 0.05 or less were considered significant.

Results

Extracts Treatment

In order to verify whether if the extracts had an inhibition of pore formation of P2X7R, we tested the extracts [100µg/mL] **Figure 1**, and the extracts that showed inhibitory activity. Afterward the IC_{50} in J774.G8 Cells:

Figure 2, 3 and 4, and in U937 Cells (**Figure 5, 6 and** 7) was determined. As a result, observed values obtained from U937 cell treatments were less than obtained from J774.G8 cells. This demonstrates a possible sensitivity of human cells to these fungus extracts, which suggests that future treatments in humans will require only low dosages of these compounds. We also tested the capacity of the compounds to antagonize the anionic pore, **Figure 8.** The three studied fungical extracts showed a pattern of antagonism of this receptor that is significantly the same as the BBG for both cationic or anionic pore formation, the known antagonist.

ELISA assay for detection IL-1β

As a result, our data suggest a significant inhibition of the release of IL-1β by the treatment on the IC_{50} of each fungus extract in relation to control treated with ATP. These patterns were observed on both J774.G8 and U937, **Figures 9 and 10**.

Assay for detection of Lactate Dehydrogenase (LDH)

Our next step was to verify if these fungus extracts could be cytotoxic for the cells in the IC_{50} concentrations. Our data show no significant cytotoxicity of the three extracts on the J774.G8 or U937 cells, and neither do treatments with ATP **Figures 11 and 12**.

Assay for detection of Nitrite

The next step was to evaluate the activity of these three fungical extracts on a physiologic function related to P2X7R, the production of Nitric Oxide, when

the macrophages are activated. As expected, the treatment with our extracts inhibited the NO production significantly. As such we can conclude that these compounds antagonize this receptor and its physiologic function, **Figure 13 and 14**.

Assay for detection of ROS

In **Figure 15**, as observed in the previous results of assays for detection of the release of other inflammatory mediators analyzed in this paper, the fungi extracts tested also demonstrated significant inhibitory activity on the release of ROS, confirming their anti-inflammatory activity by antagonism of P2X7R.

Electrophysiologic measurements

As shown in **Figure 16**, the three fungi extracts inhibited the ionic current formation by the P2X7R activated with ATP [1mM], in a dose dependent manner, but it is shown that higher or less doses than the IC_{50} values of each one also had an inhibitory effect. This demonstrates the antagonistic activity of the extracts in the pore formation of their activated receptor.

Discussion

Nowadays the research for new antagonistic compounds for the P2X7 receptor is more powerful and selective, and has low side effects. The research has been growing and has gained high financial support from the pharmaceutical industry [4]. The receptor is implicated with many pathological processes, and a new antagonist with high security and efficacy could be useful in the treatment of a diverse set of diseases, including cancer [6].

Currently there are many compounds synthesized and patented by large industry groups, such as the anticonceptional masculine pill (NF449), that acts on P2X1 receptor that is present in the vas deferens, acting on the contractile function, preventing the flow of spermatozoa (study in rats). The Clopidogrel: An antiplatelet currently on the market with action on P2Y2R and the Minodronic Acid (Astellas Pharma): used for pain treatment, acting on P2X3R. There are also three compounds that are on clinical trial for the rheumatoid arthritis treatment, acting on P2X7R (Pfizer: CE-224535, AstraZeneca: AZD-9056, and Evotec: EVT-401 [4]; [3]. These compounds must be submitted to clinical trials to elucidate the side effects and toxicicity. Thereby, any advance on the research for new antagonists to P2X7R, will certainly gain the clinical and pharmaceutical interests.

In this context, our study has high relevance and is supported by the antagonistic potential activity to P2X7R related to the fungus extracts studied (8067, 8549, and 8568). We confirm the previous statement based on the results in this work, which showed the significant inhibitory activity of the extracts on the cationic or anionic pore formations, as the inhibition of the IL-1 release, which pathway is also modulated by the P2X7R receptor [12]. Another pathway related to the activation of P2X7R, that of Nitric Oxide production [13], was also inhibited by these fungical compounds; the same result was observed in the ROS detection assay. And to evaluate a putative pharmacological inhibitory effect of these extracts on the P2X7R, we performed an eletrophysiological measurement, which reveals a significant inhibition of its extracts on the ionic current formation by the P2X7R associated pore, which

reveals an inhibitory effect in the opening of this pore. Together, our results demonstrated an inhibitory effect of these compounds with low cytotoxicity until four times the IC_{50} of each extract, as it was shown by the LDH release test.

These results certainly will take the attention of pharmaceutical companies to elaborate a bioproduct in the future, which could be useful in the clinic for the treatment of diseases modulated by its receptor.

References:

1. Burnstock, G., *Purinergic signalling: past, present and future.* Braz J Med Biol Res, 2009. **42** (1): p. 3-8.
2. Coddou, C., S.S. Stojilkovic, and J.P. Huidobro-Toro, *Allosteric modulation of ATP-gated P2X receptor channels.* Rev Neurosci, 2011. **22** (3): p. 335-54.
3. Coddou, C., et al., *Activation and regulation of purinergic P2X receptor channels.* Pharmacol Rev, 2011. **63** (3): p. 641-83.
4. Gunosewoyo, H. And M. Kassiou, *P2X purinergic receptor ligands: recently patented compounds.* Expert opinion on therapeutic patents, 2010. **20** (5): p. 625-46.
5. Li, Z., D. Liang, and L. Chen, *Potential therapeutic targets for ATP-gated P2X receptor ion channels.* Assay Drug Dev Technol, 2008. **6** (2): p. 277-84.
6. Mann, J., *Natural products in cancer chemotherapy: past, present and future.* Nat Rev Cancer, 2002. **2**: p. 143-148.
7. White, N. And G. Burnstock, *P2 receptors and cancer.* Trends Pharmacol Sci, 2006. **27** (4): p. 211-7.
8. Li, X., et al., *Decreased expression of P2X7 in endometrial epithelial pre-cancerous and cancer cells.* Gynecologic oncology, 2007. **106** (1): p. 233-43.
9. Starzl, T.E., et al., *Liver transplantation, 1980, with particular reference to cyclosporin-A.* Transplant Proc, 1981. **13** (1 PT 1): p. 281-5.
10. Santiago, I.F., et al., *Leishmanicidal and antitumoral activities of endophytic fungi associated with the Antarctic angiosperms Deschampsia antarctica Desv. And Colobanthus quitensis (Kunth) Bartl.* Extremophiles, 2012. **16** (1): p. 95-103.
11. Butinar, L., T. Strmole, and N. Gunde-Cimerman, *Relative incidence of ascomycetous yeasts in arctic coastal environments.* Microb Ecol, 2011. **61** (4): p. 832-43.
12. Clark, A.K., et al., *P2X7-dependent release of interleukin-1beta and nociception in the spinal cord following lipopolysaccharide.* J Neurosci, 2010. **30** (2): p. 573-82.
13. Sperlagh, B., et al., *ATP released by LPS increases nitric oxide production in raw 264.7 macrophage cell line via P2Z/P2X7 receptors.* Neurochem. Int., 1998. **33** (3): p. 209-215.
14. Faria RX, Defarias FP, Alves LA: Are second messengers crucial for opening the pore associated with P2X7 receptor? Am J Physiol Cell Physiol., 2005. **288**: p. 260–271.

Legends to figures:

Figure 1: Extracts Treatment. J774.G8 cells were subjected to the following treatment scheme: (a) Cell + PI., (b) cell + Triton 0.1% + PI., (c) Cell + ATP [5 mM] + PI., (d) Cell + BBG [100 nM] + ATP [5 mM] + PI., (e) Cell + BBG [100 nM] + PI., (f) Cell + DMSO 0.05% + PI., (g) Cell + extract 8067 [100 µg/mL] + PI., (h) Cell + extract 8067 [100 µg/mL] + ATP [5 mM] + PI., (i) Cell + extract 8549 [100 µg/mL] + PI., (j) Cell + extract 8549 [100 µg/mL] + ATP [5 mM] + PI., (l) Cell + extract 8568 [100 µg/mL] + PI., (m) Cell + extract 8568 [100 µg/mL] + ATP [5 mM] + PI. Date present as mean ± S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$

Figure 2: Curve dose-response of 8067 in J774.G8 Cells. Cells were treated with the extract in a range of [100 µg/mL – 2 ng/mL] + ATP [5 mM] + PI. Date represents mean ± S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 3: Curve dose-response of 8549 in J774.G8 Cells. Cells were treated with the extract in a range of [100 µg/mL – 2 ng/mL] + ATP [5 mM] + PI Date represents mean ± S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 4: Curve dose-response of 8568 in J774.G8 Cells. Cells were treated with the extract in a range of [100 µg/mL – 2 ng/mL] + ATP [5 mM] + PI Date represents mean ± S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 5: Curve dose-response of 8067 in U937 Cells. Cells were treated with the extract in a range of [100 µg/mL – 2 ng/mL] + ATP [5 mM] + PI Date represents mean ± S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 6: Curve dose-response of 8549 in U937 Cells. Cells were treated with the extract in a range of [100 µg/mL – 2 ng/mL] + ATP [5 mM] + PI Date represents mean ± S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 7: Curve dose-response of 8568 in U937 Cells. Cells were treated with the extract in a range of [100 µg/mL – 2 ng/mL] + ATP [5 mM] + PI Date represents mean

± S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * p<0,05.

Figure 8: Lucifer Yellow uptake assay of J774.G8 Cells. J774.G8 cells were subjected to the following treatment scheme: (a) Cell + LY., (b) Cell + DMSO 0.05% + LY., (c) cell + Triton 0.1% + LY., (d) Cell + ATP [5 mM] + LY., (e) Cell + BBG [100 nM] + LY., (f) Cell + BBG [100 nM] + ATP [5 mM] + LY., (g) Cell + extract 8067 [100 µg/mL] + LY., (h) Cell + extract 8549 [100 µg/mL] + LY., (i) Cell + extract 8568 [100 µg/mL] + LY., (j) Cell + extract 8067 [100 µg/mL] + ATP [5 mM] + LY., (l) Cell + extract 8549 [100 µg/mL] + ATP [5 mM] + LY, (m) Cell + extract 8568 [100 µg/mL] + ATP [5 mM] + LY. Date represents mean ± S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * p<0,05.

Figure 9: ELISA for IL-1β detection of J774.G8 Cells. The supernatant of Cells previously treated with the following scheme were analyzed: (a) Cells without treatments., (b) Cells + DMSO 0.05%., (c) Cells + ATP [5 mM]., (d) Cells + BBG [100 nM]., (e) Cells + BBG [100 nM] + ATP [5 mM]., (f) Cells + extract 8067 [2.1 µg/mL]., (g) Cells + extract 8549 [2.6 µg/mL]., (h) Cells + extract 8568 [3.8 µg/mL]., (i) Cells + extract 8067 [2.1 µg/mL] + ATP [5 mM]., (j) Cells + extract 8549 [2.6 µg/mL] + ATP [5 mM]., (l) Cells + extract 8568 [3.8 µg/mL]. Date represents mean ± S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * p<0,05

Figure 10: ELISA for IL-1β detection of U937 Cells. The supernatant of Cells previously treated with the following scheme were analyzed: (a) Cells without treatments., (b) Cells + DMSO 0.05%., (c) Cells + ATP [5 mM]., (d) Cells + BBG [100 nM]., (e) Cells + BBG [100 nM] + ATP [5 mM]., (f) Cells + extract 8067 [0.69 µg/mL]., (g) Cells + extract 8549 [0.92 µg/mL]., (h) Cells + extract 8568 [1.5 µg/mL]., (i) Cells + extract 8067 [0.69 µg/mL] + ATP [5 mM]., (j) Cells + extract 8549 [0.92 µg/mL] + ATP [5 mM]., (l) Cells + extract 8568 [1.5 µg/mL]. Date represents mean + S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * p<0,05.

Figure 11: Assay for LDH of J774.G8 Cells. The supernatant of Cells previously treated by 24 h with four times the IC_{50} of each extract were analyzed: (a) Cells without treatments., (b) Cells + DMSO 0.05%., (c) Cells + Triton 0.1%., (d) Cell + ATP [5 mM]., (e) Cell + BBG [100 nM]., (f) Cell + BBG [100 nM] + ATP [5 mM]., (g) Cell + extract 8067 [8.4 µg/mL]., (h) Cell + extract 8067 [8.4 µg/mL] + ATP [5 mM]., (i) Cell + extract 8549 [10.4 µg/mL]., (j) Cell + extract 8549 [10.4 µg/mL] + ATP [5 mM]., (l) Cell + extract 8568 [15.2 µg/mL]., (m) Cell + extract 8568 [15.2 µg/mL] + ATP [5 mM]. Date

represents mean ± S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * p<0,05.

Figure 12: Assay for LDH of U937 Cells. The supernatant of Cells previously treated by 24 h with four times the IC_{50} of each extract were analyzed: (a) Cells without treatments., (b) Cells + DMSO 0.05%., (c) Cells + Triton 0.1%., (d) Cell + ATP [5 mM]., (e) Cell + BBG [100 nM]., (f) Cell + BBG [100 nM] + ATP [5 mM]., (g) Cell + extract 8067 [2.76 µg/mL]., (h) Cell + extract 8067 [2.76 µg/mL] + ATP [5 mM]., (i) Cell + extract 8549 [3.68 µg/mL]., (j) Cell + extract 8549 [3.68 µg/mL] + ATP [5 mM]., (l) Cell + extract 8568 [6.0 µg/mL]., (m) Cell + extract 8568 [6.0 µg/mL] + ATP [5 mM]. Date represents mean ± S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * p<0,05.

Figure 13: Nitrite Detection of J774.G8 Cells. The supernatant of Cells previously treated with the following scheme were analyzed: (a) Cells without treatments., (b) Cells + DMSO 0.05%., (c) Cells + LPS [1 µg/mL]., (d) Cell + ATP [5 mM]., (e) Cell + BBG [100 nM]., (f) Cell + BBG [100 nM] + ATP [5 mM]., (g) Cell + extract 8067 [2.1 µg/mL]., (h) Cell + extract 8067 [2.1 µg/mL] + ATP [5 mM]., (i) Cell + extract 8549 [2.6 µg/mL]., (j) Cell + extract 8549 [2.6 µg/mL] + ATP [5 mM]., (l) Cell + extract 8568 [3.8 µg/mL]., (m) Cell + extract 8568 [3.8 µg/mL] + ATP [5 mM]. Date represents mean ± S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * p<0,05.

Figure 14: Nitrite Detection of U937 Cells. The supernatant of Cells previously treated with the following scheme were analyzed: (a) Cells without treatments., (b) Cells + DMSO 0.05%., (c) Cells + LPS [1 µg/mL]., (d) Cell + ATP [5 mM]., (e) Cell + BBG [100 nM]., (f) Cell + BBG [100 nM] + ATP [5 mM]., (g) Cell + extract 8067 [0.69 µg/mL]., (h) Cell + extract 8067 [0.69 µg/mL] + ATP [5 mM]., (i) Cell + extract 8549 [0.92 µg/mL]., (j) Cell + extract 8549 [0.92 µg/mL] + ATP [5 mM]., (l) Cell + extract 8568 [1.5 µg/mL]., (m) Cell + extract 8568 [1.5 µg/mL] + ATP [5 mM]. Date represents mean ± S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * p<0,05.

Figure 15: ROS Detection of J774.G8 Cells. After the following treatments, the cells were labeled with DHE and the ROS detection was assayed. (a) Cells without treatments., (b) Cells + H_2O_2 20%., (c) Cell + ATP [5 mM]., (d) Cell + BBG [100 nM]., (e) Cell + BBG [100 nM] + ATP [5 mM]., (f) Cell + extract 8067 [2.1 µg/mL]., (g) Cell + extract 8549 [2.6 µg/mL]., (h) Cell + extract 8568 [3.8 µg/mL]., (i) Cell + extract 8067 [2.1 µg/mL] + ATP [5 mM]., (j) Cell + extract 8549 [2.6 µg/mL] + ATP [5 mM]., (l) Cell +

extract 8568 [3.8 µg/mL] + ATP [5 mM]. Date represents mean + S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 16: Electrophysiologic measurements. The J774.G8 cells were treated with diferent concentrations of each fungi extracts to evaluate te inhibitory effect of them on the ionic current formation by the opened pore related to the activation of the P2X7R. Date represents mean + S.D. Of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Fig.1

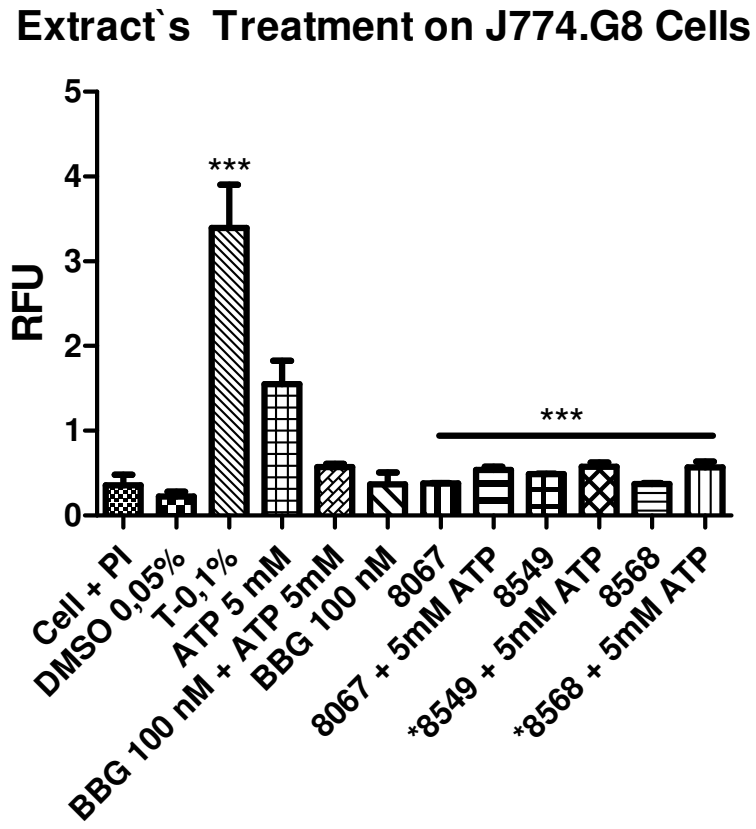
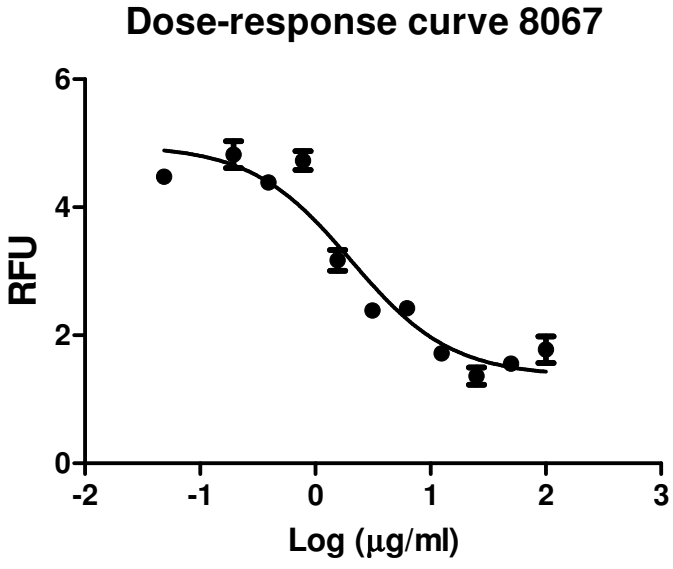
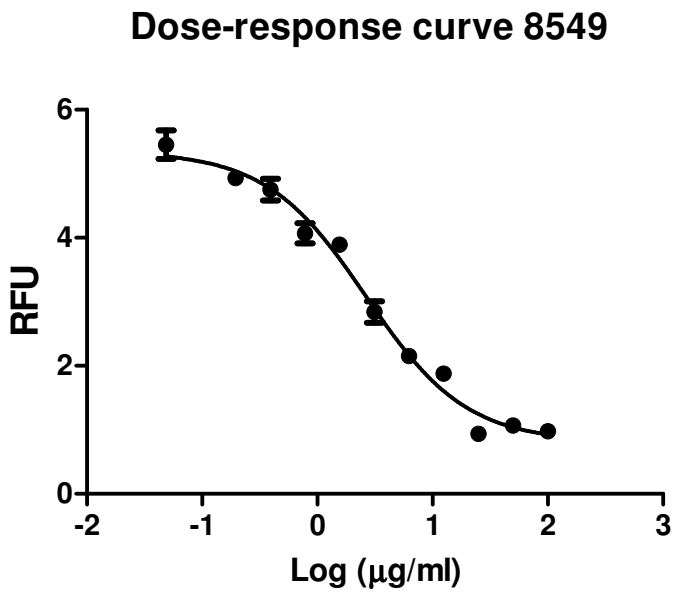


Fig.2



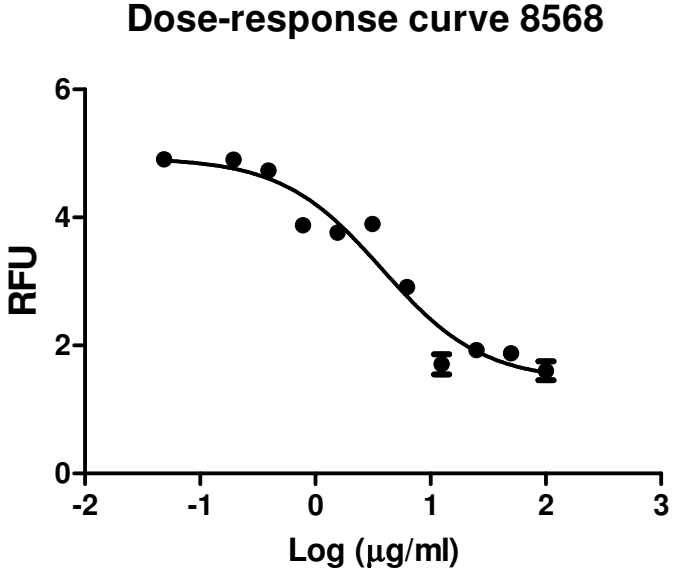
IC_{50}= 2.1 µg/ml

Fig.3



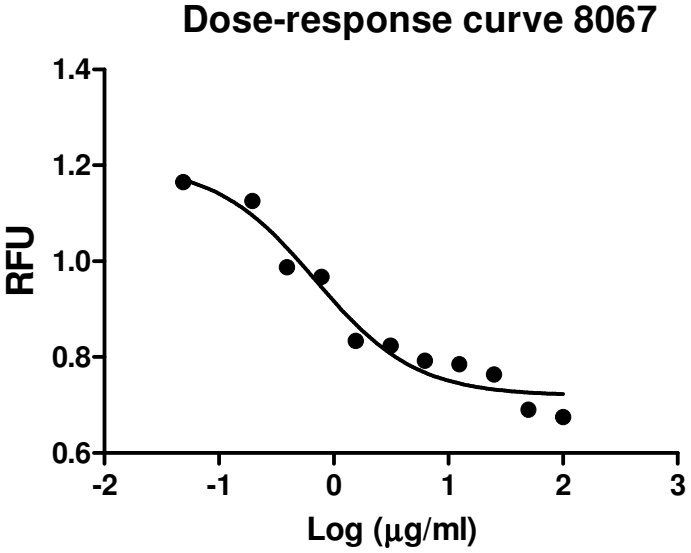
IC_{50}= 2.6 µg/ml

Fig.4



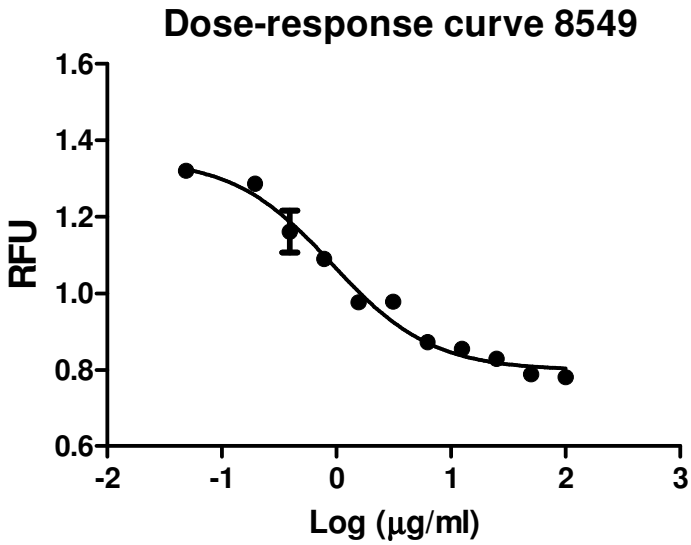
IC_{50}= 3.8 µg/ml

Fig.5



IC_{50}= 0.69 µg/ml

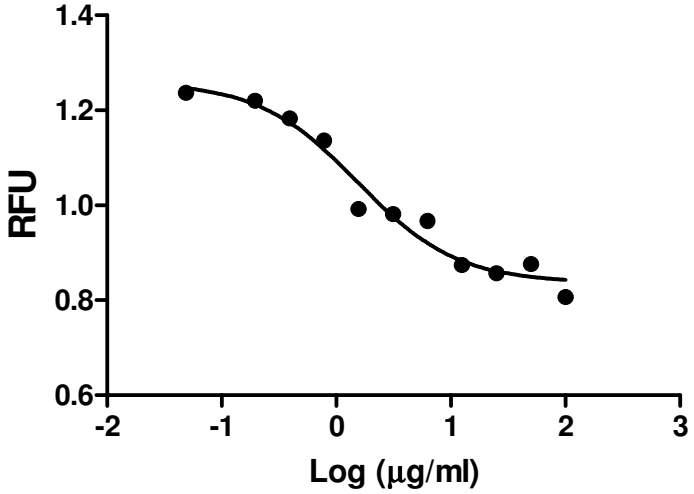
Fig.6



IC_{50}= 0.92 µg/ml

Fig.7

Dose-response curve 8568



IC_{50}= 1.5 µg/ml

Fig.8

LUCIFER YELLOW UPTAKE ASSAY

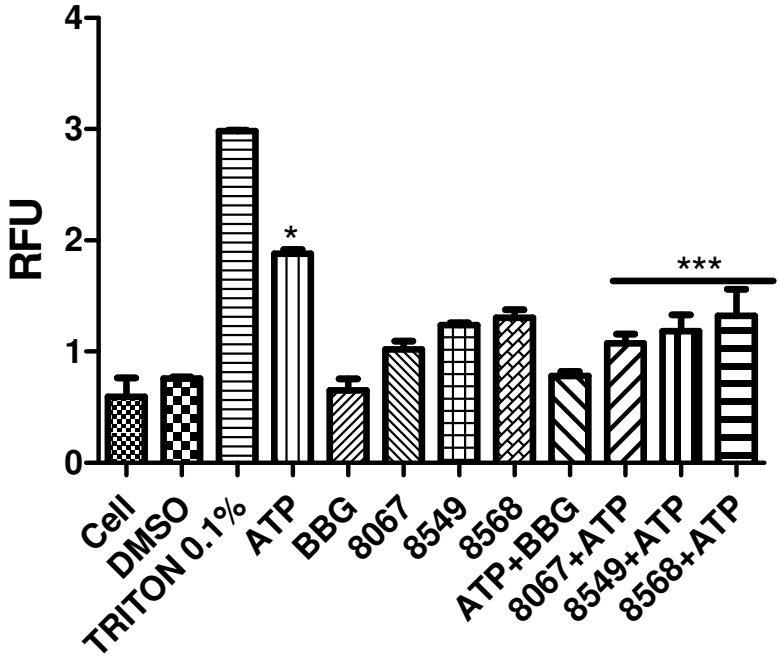


Fig.9

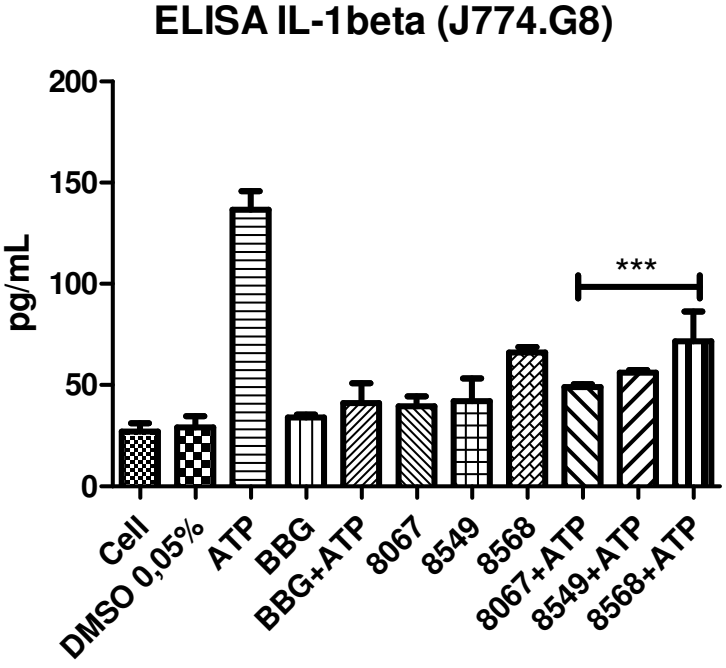

ELISA IL-1beta (J774.G8)
pg/mL
200
150
100
50
0
***
Cell
DMSO 0,05%
ATP
BBG
BBG+ATP
8067
8549
8568
8067+ATP
8549+ATP
8568+ATP

Fig.10

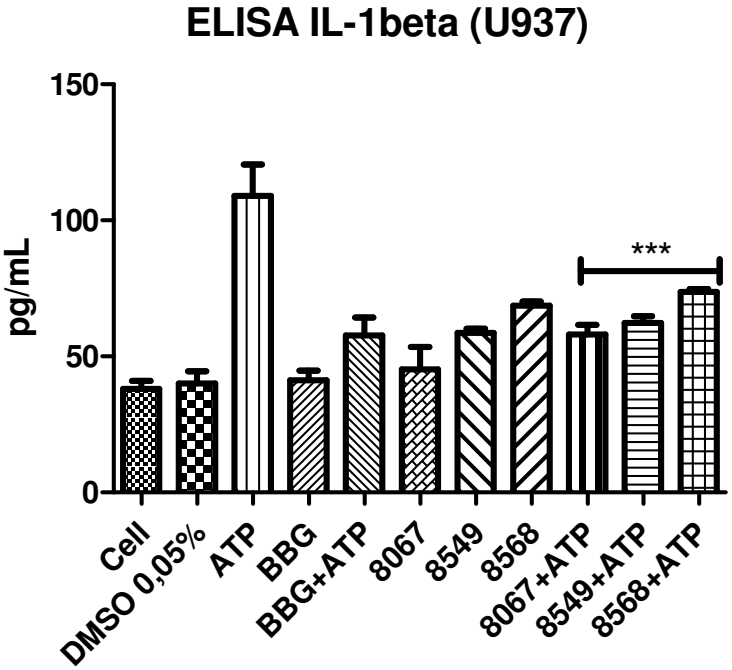

ELISA IL-1beta (U937)
pg/mL
150
100
50
0
***
Cell
DMSO 0,05%
ATP
BBG
BBG+ATP
8067
8549
8568
8067+ATP
8549+ATP
8568+ATP

Fig.11

LDH ASSAY (J774.G8 Cells)

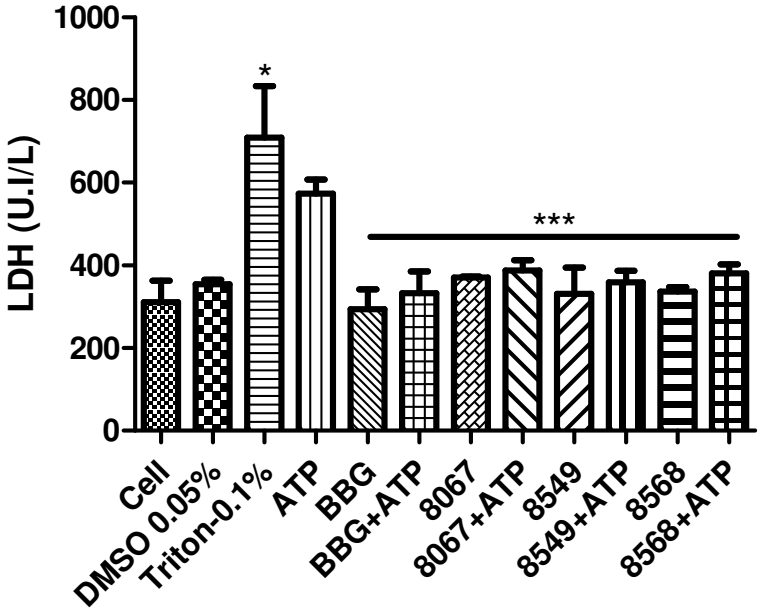


Fig.12

LDH ASSAY (U937 Cells)

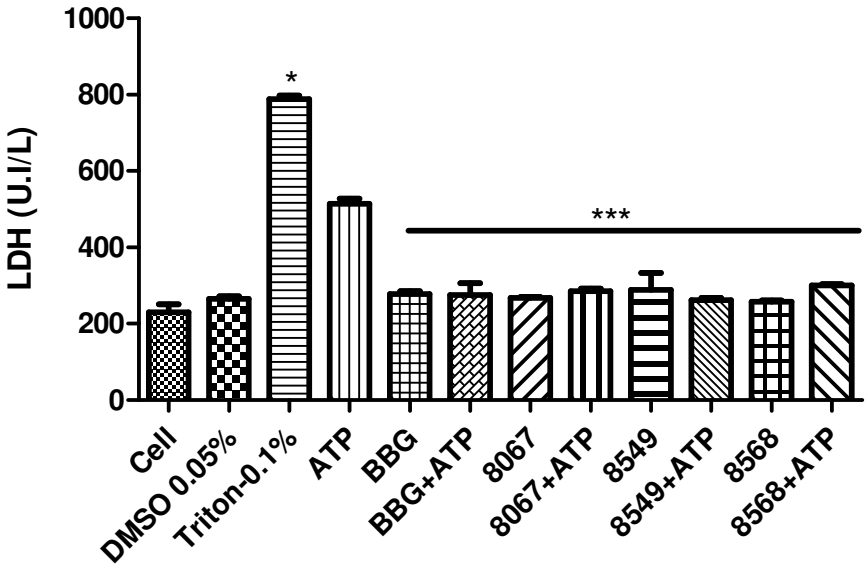


Fig.13

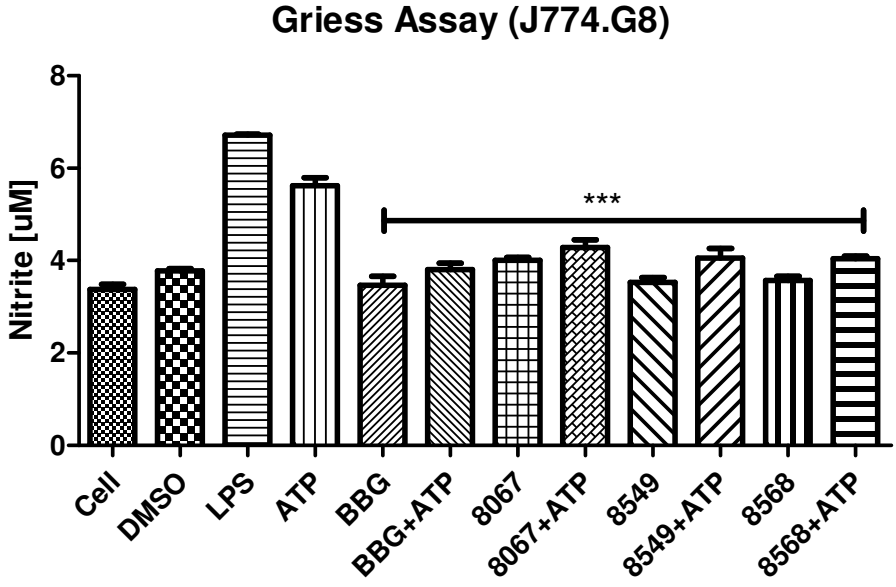

Griess Assay (J774.G8)
Nitrite [uM]
8
6
4
2
0
***
Cell
DMSO
LPS
ATP
BBG
BBG+ATP
8067
8067+ATP
8549
8549+ATP
8568
8568+ATP

Fig.14

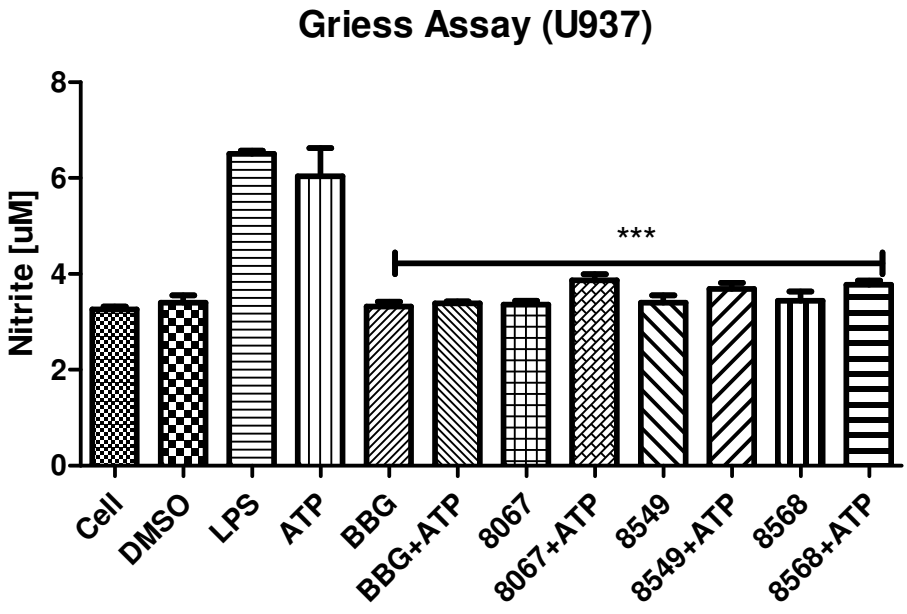

Griess Assay (U937)
Nitrite [uM]
8
6
4
2
0
***
Cell
DMSO
LPS
ATP
BBG
BBG+ATP
8067
8067+ATP
8549
8549+ATP
8568
8568+ATP

Fig.15

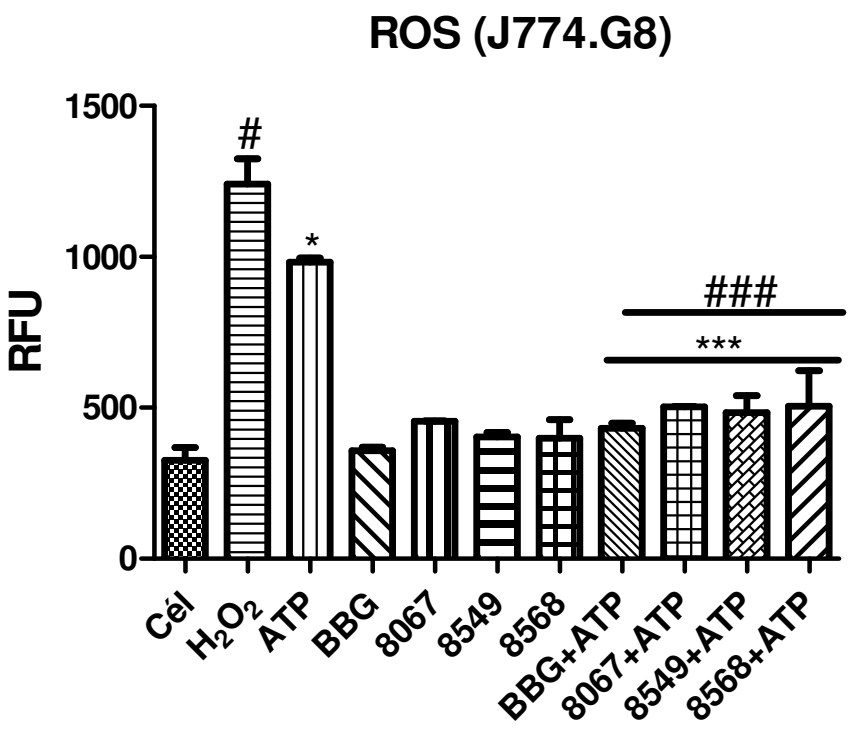
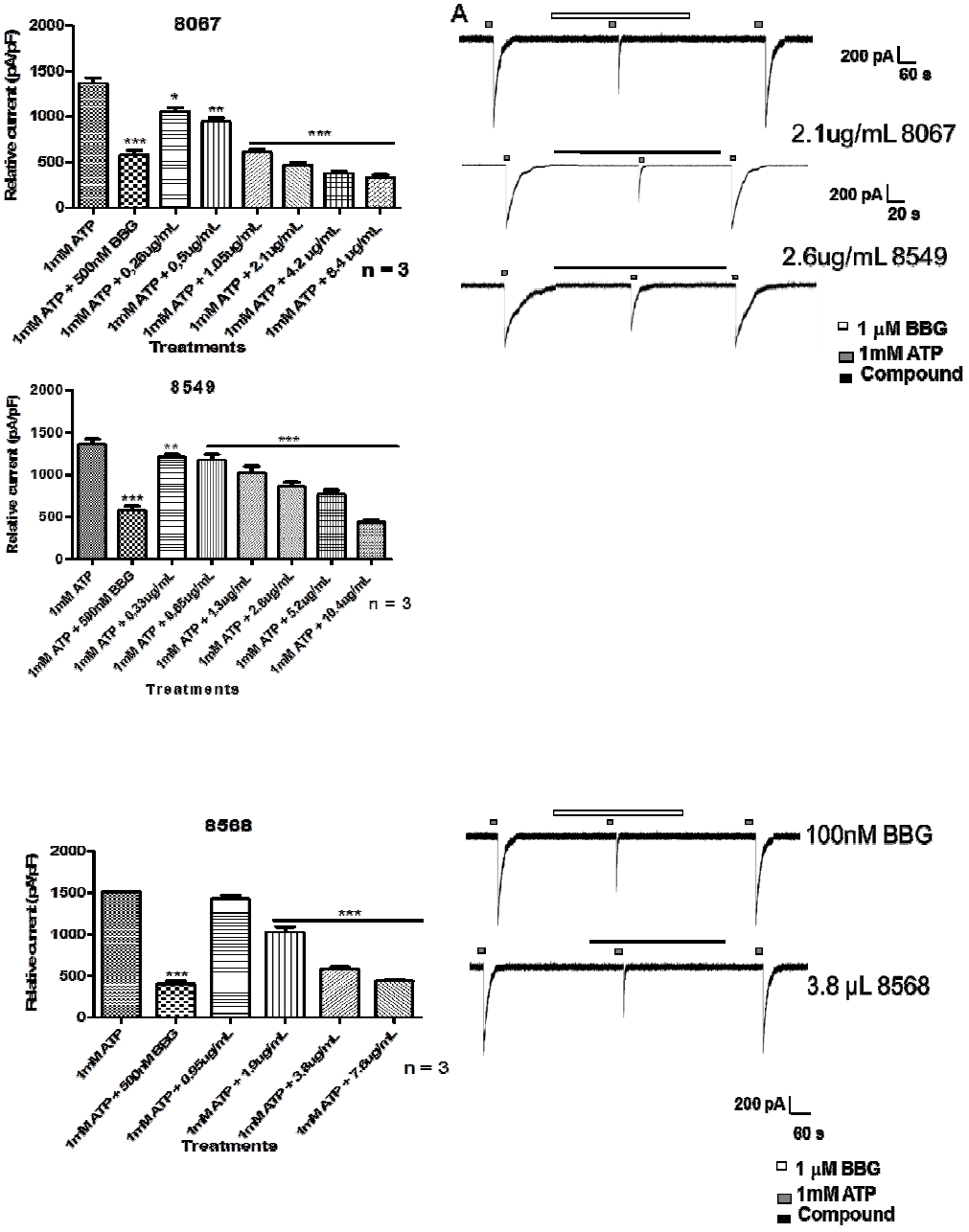

ROS (J774.G8)
RFU
1500
1000
500
0
#
*
###
***
Cél
H2O2
ATP
BBG
8067
8549
8568
BBG+ATP
8067+ATP
8549+ATP
8568+ATP

Fig.16


8067
Relative current (pA/pF)
2000
1500
1000
500
0
***
*
**
***
1mM ATP
1mM ATP + 500nM BBG
1mM ATP + 0.26ug/mL
1mM ATP + 0.5ug/mL
1mM ATP + 1.05ug/mL
1mM ATP + 2.1ug/mL
1mM ATP + 4.2 ug/mL
1mM ATP + 8.4 ug/mL
n = 3
Treatments
A
200 pA
60 s
2.1ug/mL 8067
200 pA
20 s
2.6ug/mL 8549
1 μM BBG
1mM ATP
Compound
8549
Relative current (pA/pF)
2000
1500
1000
500
0
***
**
***
1mM ATP
1mM ATP + 500nM BBG
1mM ATP + 0.33ug/mL
1mM ATP + 0.65ug/mL
1mM ATP + 1.3ug/mL
1mM ATP + 2.6ug/mL
1mM ATP + 5.2ug/mL
1mM ATP + 10.4ug/mL
n = 3
Treatments
8568
Relative current (pA/pF)
2000
1500
1000
500
0
***
***
1mM ATP
1mM ATP + 500nM BBG
1mM ATP + 0.95ug/mL
1mM ATP + 1.9ug/mL
1mM ATP + 3.8ug/mL
1mM ATP + 7.6ug/mL
n = 3
Treatments
100nM BBG
3.8 μL 8568
200 pA
60 s
1 μM BBG
1mM ATP
Compound

A Ser Submetido Após o Registro de Patente

The Analgesic, Anti-Inflammatory and Gastroprotective Activities of Three Fungi Extracts and its possible correlation with the P2X7R

Soares-Bezerra R. J[1], Ferreira N.C.S[1], Alves M.T [3], Zani L.C[3], Francisco M.F[3], Calheiros A.S[2], Souza C.Z[2], Azeredo J.A[2], Lima-Quaresma K.[1], Alves L.A[1] , Frutuoso V.S[2]*.

1. Laboratório de Comunicação Celular, Instituto Oswaldo Cruz, Fiocruz; 2. Laboratório de Imunofarmacologia, Instituto Oswaldo Cruz, Fiocruz, Brazil; 3. Laboratório de Química de Produtos Naturais, Centro de Pesquisas Químicas René Rachou, Fiocruz – Minas Gerais, Brazil.

The Analgesic, Anti-Inflammatory, and Gastroprotective Activities of Three Fungi Extracts and its possible correlation with the P2X7R

Soares-Bezerra R. J[1], Ferreira N.C.S[1], Alves M.T [3], Zani L.C[3], Francisco M.F[3], Calheiros A.S[2], Souza C.Z[2], Azeredo J.A[2], Lima-Quaresma K.R.F[1], Alves L.A[1] , Frutuoso V.S[2]*.

1. Laboratório de Comunicação Celular, Instituto Oswaldo Cruz, Fiocruz; 2. Laboratório de Imunofarmacologia, Instituto Oswaldo Cruz, Fiocruz, Brazil; 3. Laboratório de Química de Produtos Naturais, Centro de Pesquisas Químicas René Rachou, Fiocruz – Minas Gerais, Brazil.

*E-mail: Frutuoso@ioc.fiocruz.br

Address: Laboratório de Imunofarmacologia -

IOC - Fiocruz - Pav. 108 - Sala 01 - AV. Brasil 4365, Manguinhos

CEP-21045-900, Rio de Janeiro, RJ. , Brasil.

Tel: +55-21-2562-1752

Abstract

The search for new drugs with less side effects and more efficacy to treat inflammatory diseases continues to be a challenge difficult to solve. Among the drugs used today to treat these diseases, nonsteroidal anti-inflammatory drugs are widely used as the first line treatment for these diseases, although their major side effect is ulcer formation in the gastric system, which is another disease that is related to an increase in the patient's morbidity and mortality rates, and its treatment is based on the prescription of less safe drugs. In this context, we analyzed the analgesic, anti-inflammatory, and gastroprotective activities of three fungi extracts that showed an antagonistic activity *In vitro* on the P2X7R, which is a purinergic receptor that is physiologically activated by ATP released from apoptotic or necrotic cells during an inflammatory process. The activation of its receptor is related to the release of pro-inflammatory citokines, i.e. IL-1β and other inflammatory mediators as NO and ROS. We tested these extracts using *in vivo* mice models to the neuropathic and inflammatory pain and ulcer by 70% ethanol. Our results demonstrated both analgesic and anti-inflammatory activities, in addition to a protective effect on the gastric epithelium of the treated animals. Our results could take the attention of pharmaceutical laboratories that are searching for a compound with these activities to synthesize a byproduct that could be useful in the clinical treatments.

Keywords: P2X7R, analgesic, anti-inflammatory, ulcer, antagonist.

Introduction

The Purinergic receptors are activated by extracellular purines and pyrimidines, are subdivided into two classes, the P2Y and P2X receptors. Among the P2X receptors is included the P2X7 receptor, which is widely expressed in hematopoietic lineage cells and in some Central Nervous System cell types. Among these cell types, we can cite: mast cells, erythrocytes, monocytes, macrophages peripheral dendritic cells, B and T lymphocytes, Langerhans cells, microglia, Schwann cells, and astrocytes [1].

There are many agonists featured to P2XR [2], but ATP is the only one that activates the P2X7 receptor under physiological conditions, and with no pathological conditions its concentration in the extracellular environment is low. The higher concentrations of ATP in the extracellular space are related to pathological conditions, when cells that store ATP in the cytoplasm at higher concentrations than the extracellular space tend to release it, leading to an activation of the P2X7 receptor. This release represents a danger signal, since its activation occurs in the process where cellular death is occurring by apoptosis, or necrosis that are typically related to the inflammatory process. Beyond that, the P2X7R is activated in the range of millimolar concentration, differently from the other P2X receptors, which are activated in the range of micromolar concentrations [3];[1];[2].

The activation of its receptor is related to activation of many intracellular signal pathways, some of which result in the release of inflammatory mediators such as cytokines IL-1β, IL-6, IL-18, and tumor necrosis factor-α (TNF-α). This also mediates the apoptosis in polymorph nuclear cells and induces the formation of giant multinucleated cells. The exposure of the P2X7R to high concentrations of ATP [>100Mm] or for a long period of time results in the opening of a membrane pore that allows the uptake of molecules up to 900 Da [4].

The release of these inflammatory mediators by the P2X7R activation contributes to the intensification of certain destructive processes, leading to the

destruction of tissue and structures such as cartilage, as it occurs in the context of rheumatoid arthritis [5]. In this case, that are many studies reporting the use of synthetic compounds with antagonistic effects on the P2X7R in the treatment of rheumatoid arthritis [2] [10].

The role of ATP as a neurotransmitter is already known, as is its participation in the acute and chronic nociception context [2] [10]. Among the P2X receptors involved in this process, the P2X7R is seen as a promising target since its activation leads to the superoxide products released, which act on the peripheral nerve cells, thereby contributing to the sensorial transduction of neuropathic pain [6]. Previous studies have shown the participation of the P2X7R in the context of pain and inflammation [4];[7], and our group also studied the participation of its receptor in the context of inflammatory pain and ulcers [8].

Gastric ulcers are intrinsically related to an imbalance between the production of chloridric acid and pepsin and the secretion of protective factors that protect the gastric mucosa, i.e. bicarbonate, mucus, prostaglandins, polyamines, nitric oxide, dopamine, and sulphydryl compounds. However, the major factors that lead to the development of gastric ulcer are associated with the *H. pylori* infections and the use of nonsteroidal anti-inflammatory drugs [9]. The search for safer drugs to treat this disease has taken special attention, and natural products have been identified as a source to find new safer gastroprotective compounds [10]. In the context of purinergic signaling, the activation of P2 receptors, their relationship with gastric ulcer pathology, and their contributions on the nociceptive signaling were also described [10].

The search for an antagonistic molecule has been the focus of many research groups and pharmaceutical laboratories around the world [10]. Thus, the natural products emerge as a source for discovery of novel effective molecules with inhibitory effects on the P2X receptors. In this study, we described the anti-inflammatory, analgesic and the gastroprotective activities of three fungi extracts, two obtained from the Antartic environment (8549 and 8568) and one from the south coast of Brazil (8067). The search for a molecule

with these characteristics is the current goal of many scientific groups and pharmaceutical companies to elaborate an effective compound that could be useful in the clinical treatments of inflammatory diseases, pain disorders, and gastroprotection [11].

Materials and Methods

Extracts

The extracts (8067, 8549 and 8568) tested in this work were from the Natural Products Chemistry Laboratory of Centro de Pesquisas Químicas René Rachou/FIOCRUZ - Minas Gerais – Brazil.

Biochemical Analysis

We collected the blood from the animals submitted to the ulcer experimental protocol and after centrifugation. The serum was acquired and utilized for the biochemical analysis to evaluate the subclinical toxicity through the investigation of the hepatic and renal damage on their functions. We analyzed the blood levels of AST (Aspartate Aminotransferase), ALT (Alanine Aminotransferase), which are two markers to evaluate the hepatic function. Also investigated were creatinine (an ammonia byproduct, which is excreted by the functional kidneys) blood levels. These assays were performed using biochemical assay kits pursached from Labtest, Lagoa Santa, Minas Gerais, Brasil.

Animals

Adult male Swiss Webster mice weighing 20–30 g were obtained from the Animal Breeding Unit, Oswaldo Cruz Foundation and housed in the animal facilities of Pavilhão Hélio Peggy, Oswaldo Cruz Institute, Oswaldo Cruz Foundation with free access to food and water. All experimental protocols were approved (protocols L-002-08 and L-0260-05) by the Institutional Animal Welfare Committee

Ulcer Model

The animals (male Swiss mice with 20-30g) were maintained by deprivation of food and free access to water for 24 h. After which these animals were separated into groups of eight animals each. One group was orally treated with 8067's extract [200 µg/mL]; another with 8549's extract [200 µg/mL] and another with 8568's extract [200 µg/mL]. The control groups were treated with 25 mg/Kg of Lansoprazole or saline or the P2X7R antagonist: BBG [100 mg/Kg]. After 30 minutes of treatment, they were orally treated with 200 µL of Ethanol [70% v/v]. Then, three hours later, all animals were euthanatized and the stomachs were opened and examined to count the number of ulcerations.

Pain Model

The animals (male Swiss mice with 20-30g), were separated by groups with eight animals each. The treatments with the extracts were orally as described in the ulcer protocol. We utilized as controls: one group orally treated with Sodium Diclofenac [50 mg/Kg], an anti-inflammatory drug, and another group intraperitoneally treated with Morphin [10 mg/Kg], an analgesic drug. After one hour of treatment, all animals received a formalin injection into the hind paw (the pain inductor agent) and the time that each animal spent licking the hind paw was counted at 0 to 5 minutes to analyze the activity of these extracts on the neuropathic pain, and again after 15 to 30 minutes to evaluate the activity of the treatments on inflammatory pain.

Histological assessment

Stomachs from ulcer model were fixed at 10% neutral-buffered formalin, then sectioned and embedded in paraffin. Sections (4µm) were deparaffinized, stained with haematoxylin and eosin, and then examined under a light microscope to evaluate the occurrence of ulcer areas in the tissue slices.

Results

With the aim to investigate the action of these three fungi extracts in the context of neuropathic pain, inflammatory pain and gastric ulcer formation

through the inhibition of the P2X7R. We applied the methodologies previously described and obtained the following results: as shown in the **Figure** 1, the fungus extracts: 8067 and 8549 demonstrated a significant inhibitory effect on the neuropathic pain, and this effect was more potent than that promoted by the commercial drug (Sodium Diclofenac) or the competitive antagonist of P2X7R (BBG). But in the context of the inflammatory pain, the three extracts: 8067, 8549, and 8668, were significantly efficient on the inhibition of this pain. This shows an analgesic effect related to these extracts, probably by inhibition of the release of inflammatory mediators, as shown by previous studies [4]; [5]. Further, all extracts tested in this work, as shown in **Figure** 2, demonstrate a significantly gastroprotective activity, and it was more effective than that of the commercial drug (Lanzoprazole) or that of the competitive antagonist (BBG) in the presence of 70% Ethanol. These results were confirmed with the pictures of stomach lesions **Figure** 6-7 and also through the histological assessment **Figure** 8-9, which demonstrated that the previous treatment with the three fungus extracts, significantly decreased the ulcer formation evoked by 70% Ethanol. The BBG had a clear gastroprotective activity, which was the same as that of Lanzoprazole, indicate a possible mechanism which links the P2X7R and the mechanisms that lead to the development of ulcers. Additionally we performed some tests of subclinical toxicity with the blood collected from the animals in the experimental day, and we utilized them to evaluate the biochemistry dosage of creatinine, AST, and ALT. As illustrated in **Figures** 3 and 4, these extracts did not damage the liver, since the blood levels of these hepatic enzymes shown no difference in relation to the saline control group. The same was observed with the blood levels of creatinine **Figure** 5. Thus, the tested extracts here were not toxic to the animals, and these natural compounds demostrated efficacy and security *in vivo.*

Discussion

The search for a safe new molecule to effectively treat inflammatory diseases, pain disorders, and gastric ulcers, without increased side effects has been the main interest of many research groups a pharmaceutical laboratories

worldwide [6]. In this context the natural products emerge as a source of molecules that can be applied in these treatments [6].

Based on the reasons above, our main interest of these studies was to evaluate the activity of three fungi extracts by inhibition of a purinergic receptor (P2X7), whose activation is known to correlate with the development process of diseases that afflict a high percentage of the worldwide population: the inflammatory diseases, pain disorders, and gastric ulcers [6]; [8];[9].

The activity and half inhibitory concentration of these extracts were accessed in one of our previous studies (unpublished data). In the mentioned study we evaluated the activity of these extracts in animal models that mimic the context of inflammation, pain, and gastric ulcers. Regarding the neuropathic pain, only two extracts demonstrated inhibition (8067 and 8549), but in the inflammatory pain context, all three extracts (8067,8549, and 8568) showed a significant inhibition of this pain. The data confirm our previous findings that showed the inhibitory activity of these extracts on physiologic pathways related to the activation of P2X7R, i.e. the release of IL-1beta and other inflammatory mediators (NO and ROS). Based on these data, we can confirm the participation of the receptor in the inflammatory context and the anti-inflammatory activity of these extracts, in addition to the antagonistic effect on P2X7R. The extract 8568 was not able to inhibit the neuropathic pain because the mechanisms and the context between the neuropathic and inflammatory pain are different, them this extract can be selective to act in the inflammatory context, including the pain.

Neuropathic pain can be evoked by the action of superoxide radicals in peripheral nervous terminations, as we noted in our previous study, and the antagonistic activity of these extracts on P2X7R are capable of inhibiting the release of superoxide radicals by inhibition of the receptor. This suggests the inhibitory mechanism of the extracts 8067 and 8568 in neuropathic pain.

One of the factors that leads to gastric ulcer formation is the use of nonsteroidal anti-inflammatory, which causes an inhibition on the secretion of prostaglandins that act on the protection of the gastric epithelium. The extracts

tested in this study show a significant inhibition of gastric ulcer formation, showing a possible mechanism through the inhibition on the acid gastric secretion or other mechanism, including the overt release of production´s factors, like mucus, as we observed in the gastric epithelium photographs (fig.7).

Together, our findings indicate the discovery of three natural compounds with potential activities, which could be applicable in the scenario of drug discovery and treatment of inflammatory diseases, with analgesic and gastroprotective actions.

Legends

Figure 1: Pain Model. In this model both neuropathic and inflammatory pain were analyzed, with the count of the paw licking. The animal groups were subdivided as: a) Saline; b) Morphin [10mg/kg]; c) diclofenac [50mg/kg]; d) BBG [100mM]; e) Extract 8067 [200µL/mL]; f) Extract 8549 [200µL/mL]; and G) Extract 8568 [200µL/mL]. Date represents mean + S.D. of five independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 2: Ulcer Model. The protective effect of the three fungi extracts was tested by challenge with 70% Ethanol. The animal groups were subdivided as a) Saline; b) Ethanol 70%; c) Lansoprazole + 70% Ethanol; d) BBG [100nM]; e) BBG [100nM] + 70% Ethanol; f) Extract 8067 [200µL/mL]; g) Extract 8549 [200µL/mL]; h) Extract 8568 [200µL/mL]; i) Extract 8067 [200µL/mL] + 70% Ethanol; j) Extract 8549 [200µL/mL] + 70% Ethanol ; l) Extract 8568 [200µL/mL] + 70% Ethanol . Date represents mean + S.D. of five independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 3: AST quantification was made with the blood collected in the experimental day of the ulcer assay. As shown, the extracts 8067,8549 and 8568 were not toxic to the animals, even with co-treatment with 70% Ethanol. Date represents mean + S.D. of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 4: ALT quantification was analyzed in the blood collected in the experimental day of the ulcer assay. The same pattern showed in the AST quantification result was present here, confirming the non-hepatic toxicity of these extracts. Date represents mean + S.D. of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 5: Creatinine quantification assay was performed to evaluate a possible renal toxicity. Interestingly, it was not found, and it confirmed the non toxicity and the security, in vivo, of the future use of these compounds in other animal models. Date represents mean + S.D. of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 6: Image of Ulcer Lesions in the Stomach Epthelium of control groups: a) Saline; b) Lansoprazole [25mg/Kg] + 70% Ethanol; c) 70% Ethanol; d) BBG [100mg/Kg]; e) BBG [100mg/Kg] + 70% Ethanol. We recorded by photography of the stomach of animals after 30 min. Of treatment with the extracts and the challenge with 70% Ethanol. Date represents mean + S.D. of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 7: Image of Ulcer Lesions in the Stomach Epthelium of extract treated groups: a) 8067 [200 µg/mL]; b) 8067 [200 µg/mL]; + 70% Ethanol; c) 8549 [200 µg/mL]; d) 8549 [200 µg/mL] + 70% Ethanol; e) 8568 [200 µg/mL]; f) 8568 [200 µg/mL] + 70% Ethanol;. We recorded by photography of the stomach of animals after 30 min. of treatment with the extracts and the challenge with 70% Ethanol. Date represents mean + S.D. of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 8: Histological assessment of Ulcer Lesions in the Stomach Epthelium of control groups: a) Saline; b) Lansoprazole [25mg/Kg] + 70% Ethanol; c) 70% Ethanol; d) BBG [100mg/Kg]; e) BBG [100mg/Kg] + 70% Ethanol. Of treatment with the extracts and the challenge with 70% Ethanol. Date represents mean + S.D. of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

Figure 9: Histological assessment of Ulcer Lesions in the Stomach Epthelium of extratct treated groups: a) 8067 [200 µg/mL]; b) 8067 [200 µg/mL]; + 70% Ethanol; c) 8549 [200 µg/mL]; d) 8549 [200 µg/mL] + 70% Ethanol; e) 8568 [200 µg/mL]; f) 8568 [200 µg/mL] + 70% Ethanol. Of treatment with the extracts and the challenge with 70% Ethanol. Date represents mean + S.D. of three independent experiments. The result was analyzed by ANOVA and Turkey * $p<0,05$.

References

1. Skaper, S.D., P. Debetto, and P. Giusti, The P2X7 purinergic receptor: from physiology to neurological disorders. FASEB J, 2010. 24(2): p. 337-45.

2. Coddou, C., et al., Activation and regulation of purinergic P2X receptor channels. Pharmacol Rev, 2011. 63(3): p. 641-83.

3. Lister, M.F., et al., The role of the purinergic P2X7 receptor in inflammation. Journal of inflammation (London, England), 2007. 4: p. 5-5.

4. Lister, M.F., et al., The role of the purinergic P2X7 receptor in inflammation. J Inflamm (Lond), 2007. 4: p. 5.

5. Caporali, F., et al., Human rheumatoid synoviocytes express functional P2X7 receptors. J Mol Med (Berl), 2008. 86(8): p. 937-49.

6. Liang, S., et al., P2X receptors and modulation of pain transmission: focus on effects of drugs and compounds used in traditional Chinese medicine. Neurochem Int, 2010. 57(7): p. 705-12.

7. Kobayashi, K., et al., Induction of the P2X7 receptor in spinal microglia in a neuropathic pain model. Neurosci Lett, 2011. 504(1): p. 57-61.

8. Santos J.A.A., F.-N.A.A., Faria R.X., Simões A.P.S., Calheiros A.S., Bérenger A.L., Castro-Faria-Neto H.C., Frutuoso V.S., Alves L.A. , Effect of Rheedia longifolia aqueous leaves extract and fractions from methanol extract

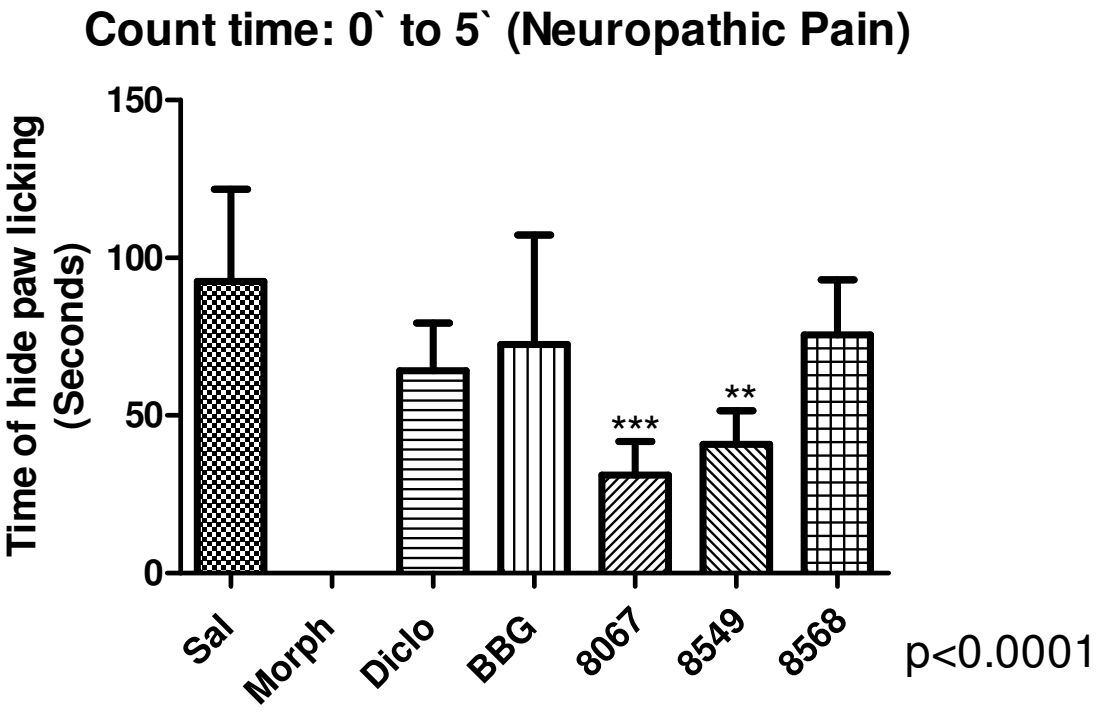
on P2X7 receptor in vitro: novel antagonists? . Journal of Medicinal food, 2011. submetido.

9. Gilberto Santos Cerqueira, G.d.S.e.S., Emiliano Rios Vasconcelos, Ana Paula Fragoso de Freitas, Brinell Arcanjo Moura, Danielle Silveira Macedo, Augusto Lopes Souto, José Maria Barbosa Filho, Luzia Kalyne de Almeida Leal, Gerly Anne de Castro Brito, Caden Souccar, Glauce Socorro de Barros Viana, Effects of hecogenin and its possible mechanism of action on experimental models of gastric ulcer in mice. European Journal of Pharmacology, 2012. 683: p. 260–269.

10. K. Dang, K.B., 2 K. Lamb,1 and G. F. Gebhart1, Gastric Ulcers Evoke Hyperexcitability and Enhance P2X Receptor Function in Rat Gastric Sensory Neurons. J Neurophysiol., 2005. 93: p. 3112–3119.

11. Gunosewoyo, H. and M. Kassiou, P2X purinergic receptor ligands: recently patented compounds. Expert opinion on therapeutic patents, 2010. 20(5): p. 625-46.

Fig.1


Count time: 0` to 5` (Neuropathic Pain)
Time of hide paw licking (Seconds)
150
100
50
0
***
**
Sal
Morph
Diclo
BBG
8067
8549
8568
p<0.0001

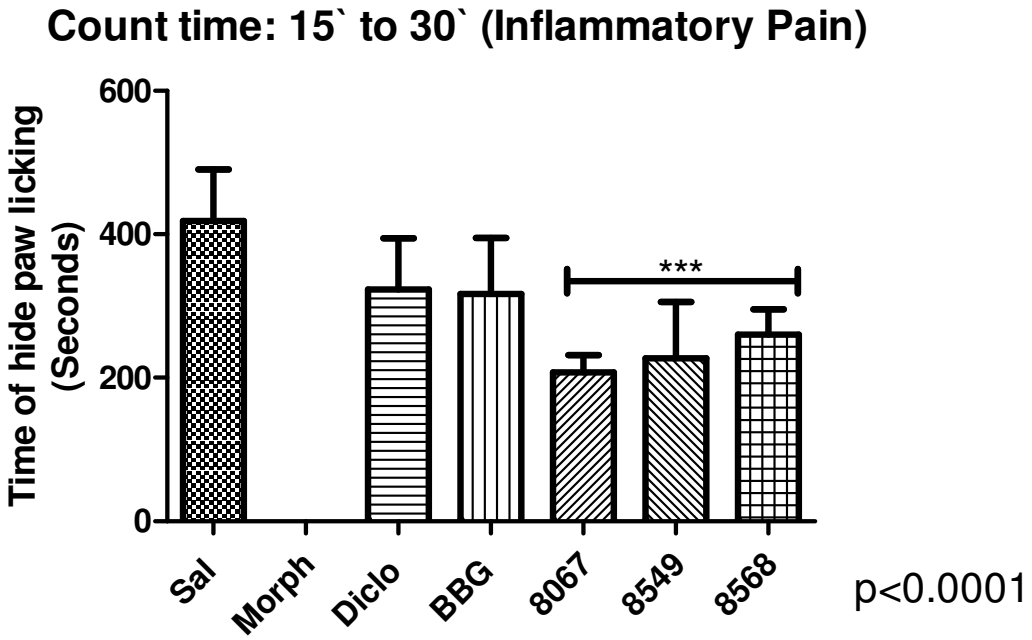

Count time: 15` to 30` (Inflammatory Pain)
Time of hide paw licking (Seconds)
600
400
200
0
***
Sal
Morph
Diclo
BBG
8067
8549
8568
p<0.0001

Fig.2

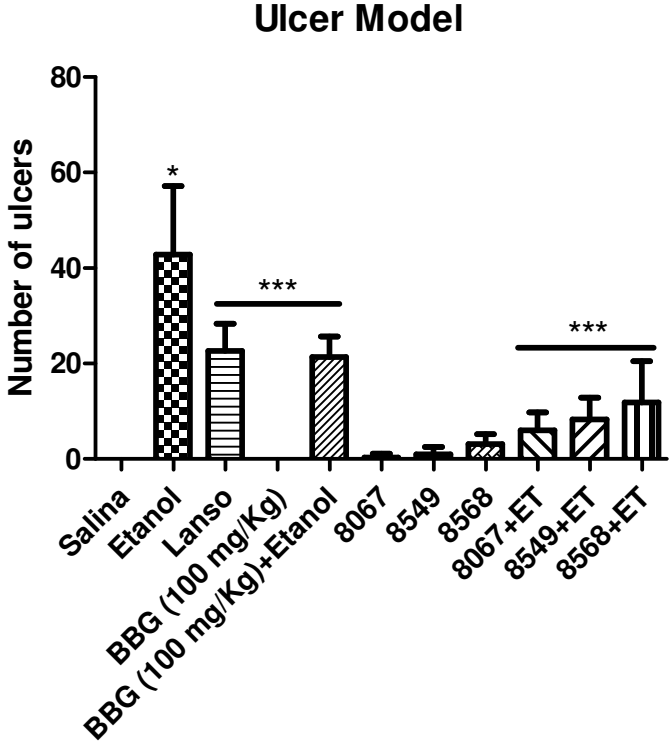
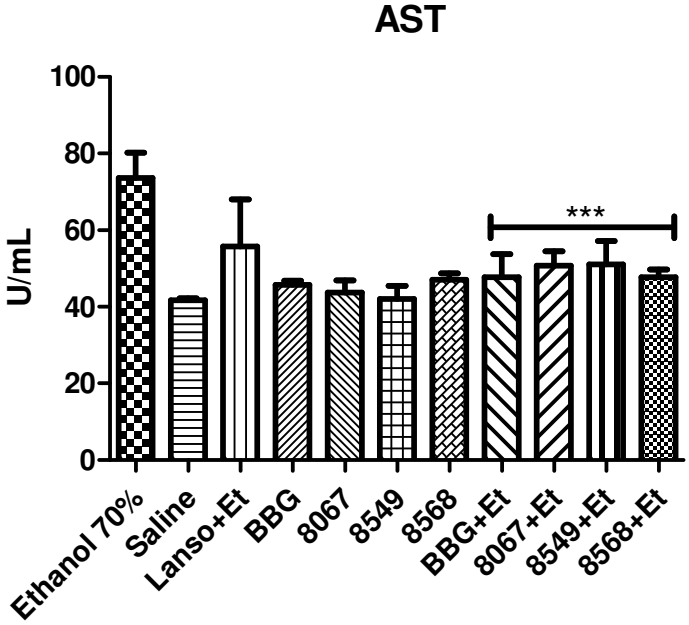


Fig.3



p<0.0001

Fig.4

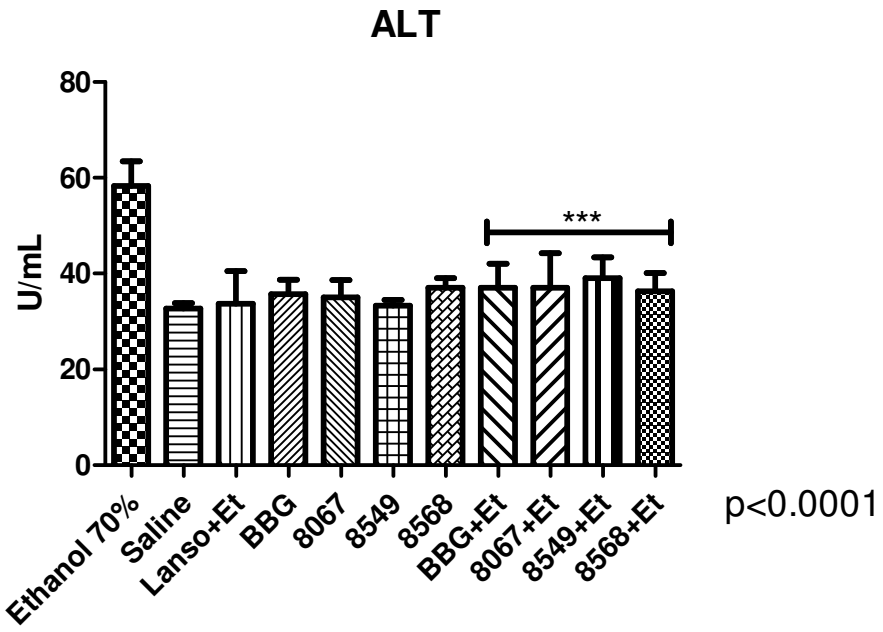

ALT
U/mL
80
60
40
20
0
***
Ethanol 70%
Saline
Lanso+Et
BBG
8067
8549
8568
BBG+Et
8067+Et
8549+Et
8568+Et
p<0.0001

Fig.5


Creatinine
mg/dL
2.0
1.5
1.0
0.5
0.0
***
Ethanol 70%
Saline
Lanso+Et
BBG
8067
8549
8568
BBG+Et
8067+Et
8549+Et
8568+Et
p<0.0001

Fig.6

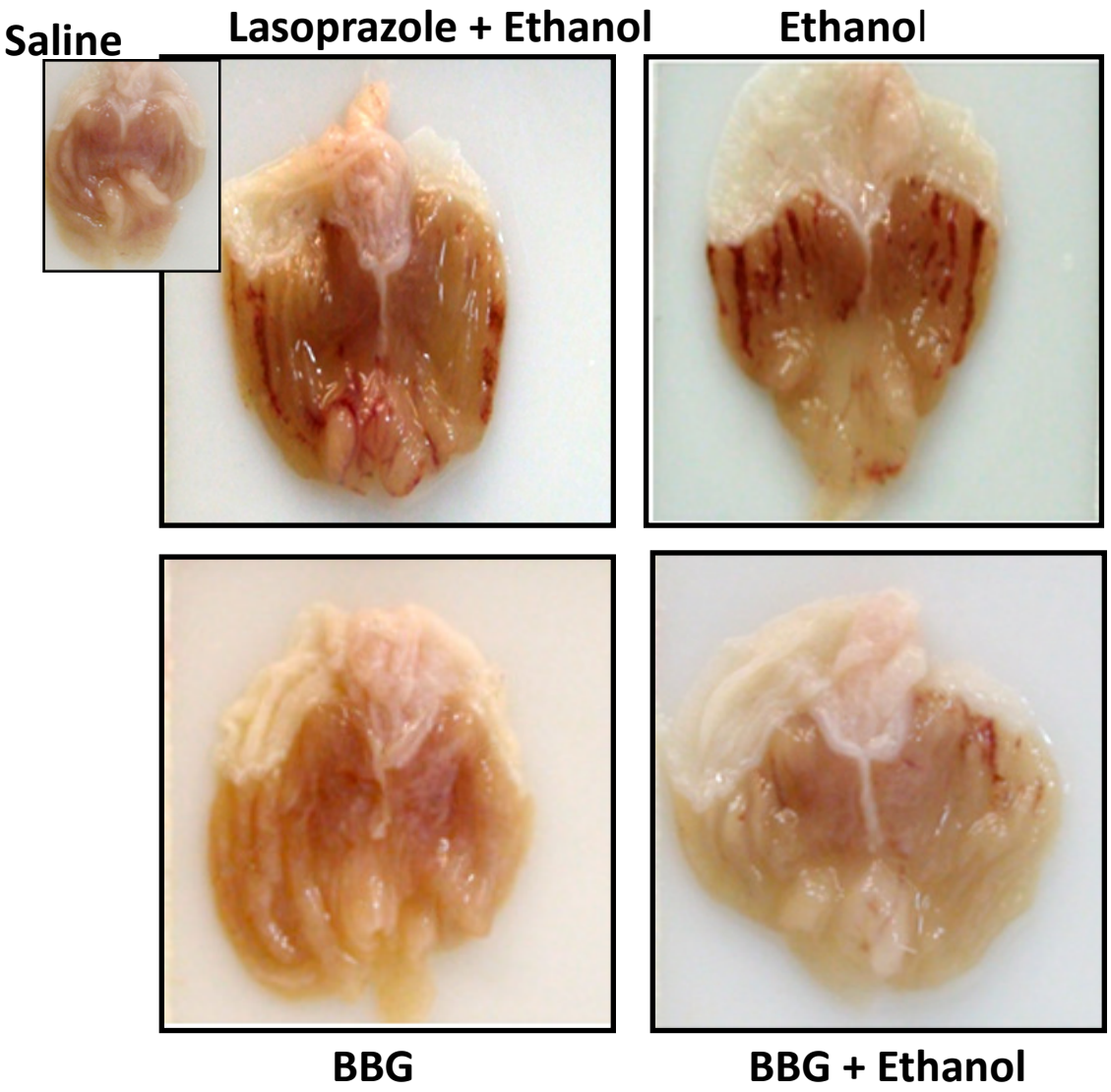


Fig.7

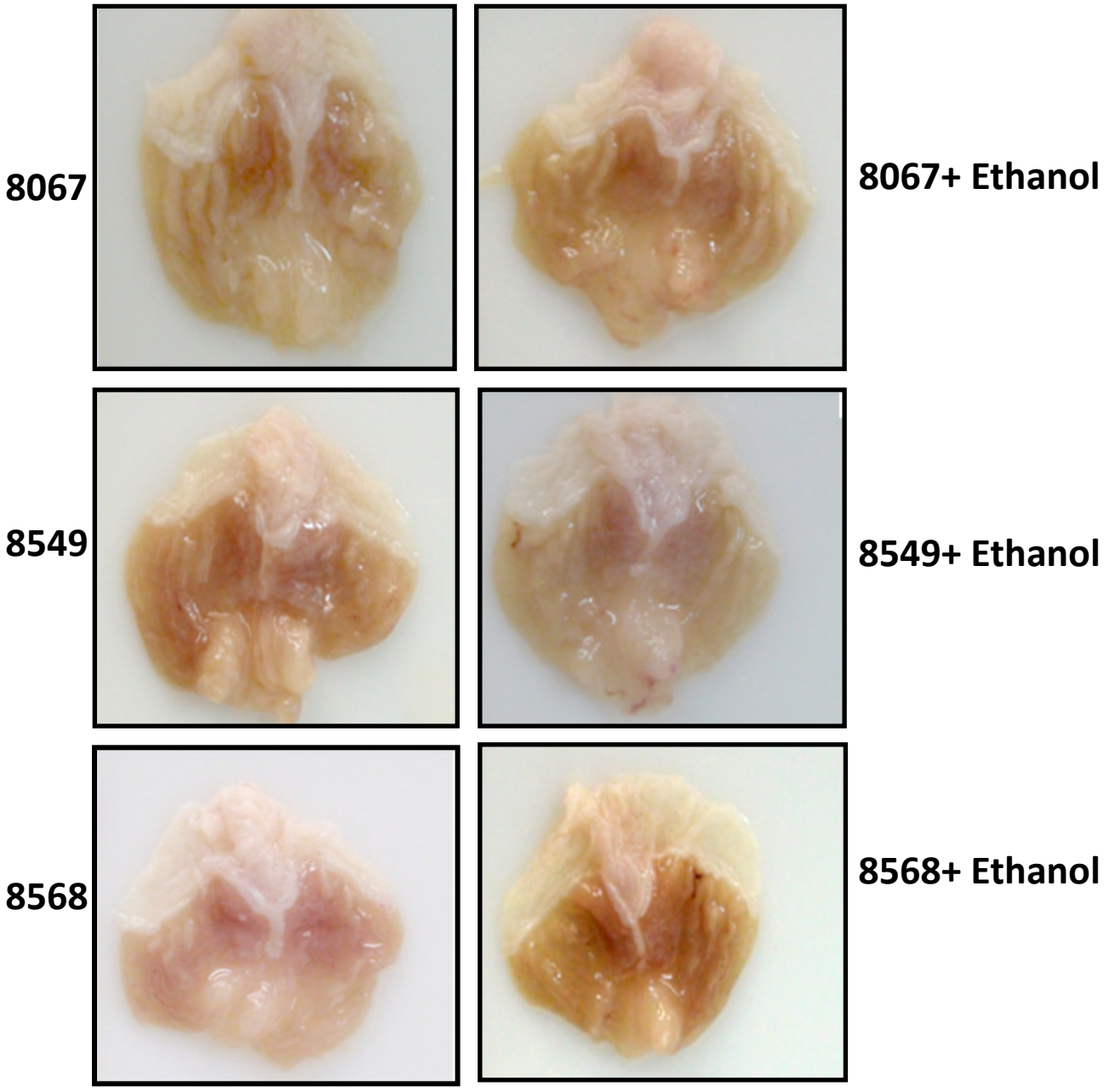


Fig.8

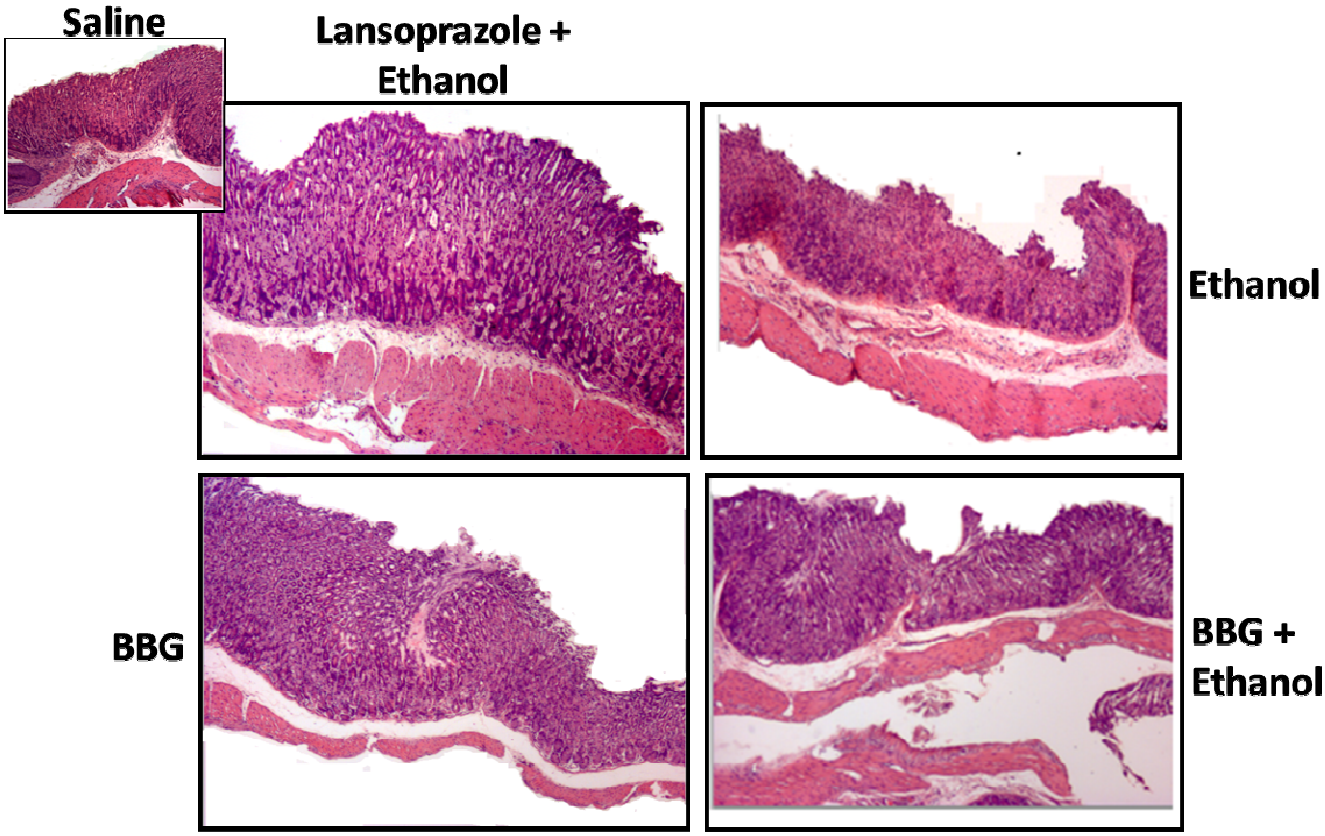
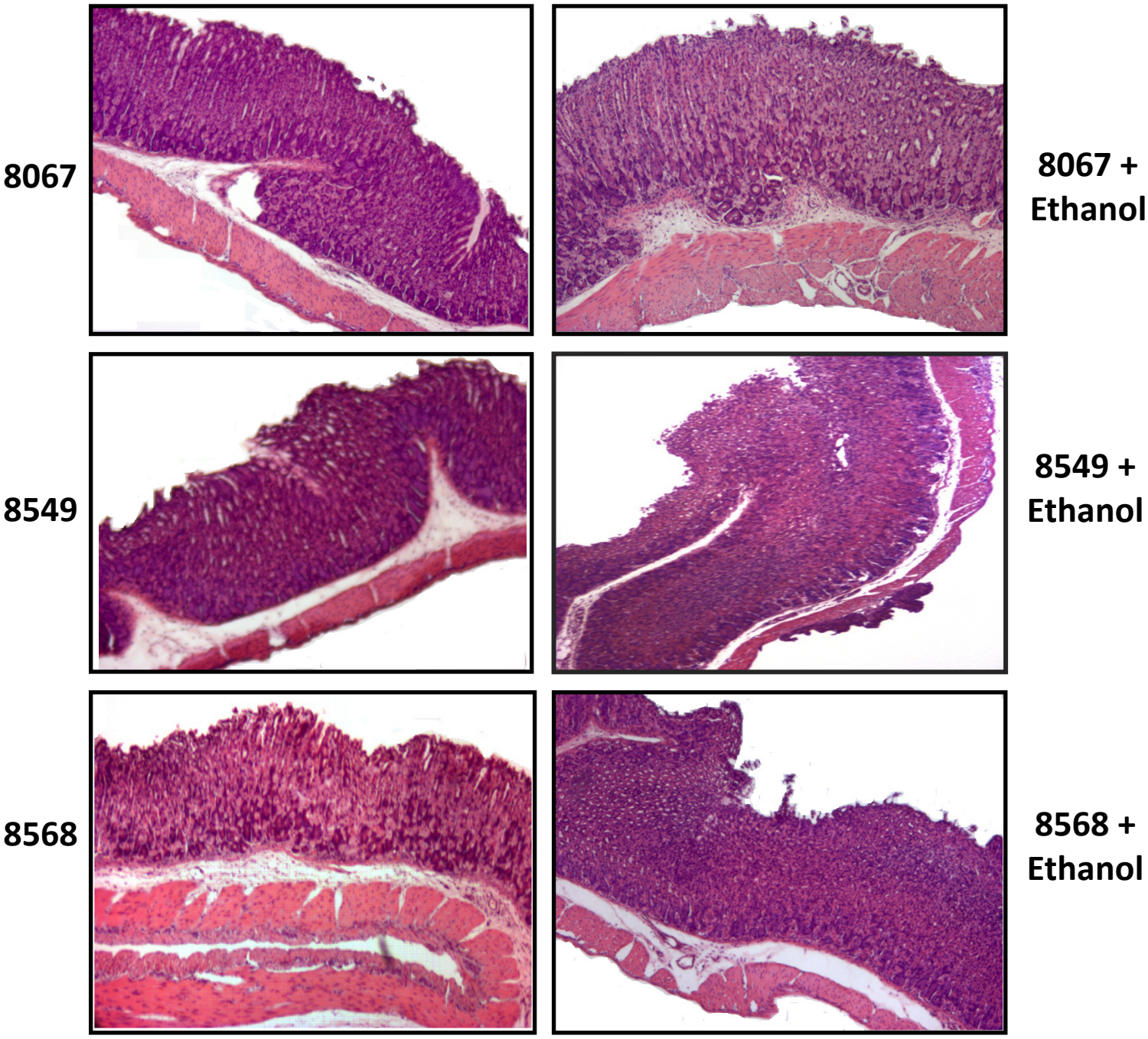


Fig.9



5. DISCUSSÃO

Neste trabalho, primeiramente, foi padronizada uma metodologia que servirá de ferramenta para o campo da pesquisa de novos antagonistas, ou agonistas, para o P2X7R. Essa nova metodologia está baseada na propriedade desse receptor de abrir um poro seletivo para moléculas de até 900 Da quando exposto a concentrações de ATP extracelular acima de 100 µM (Burnstock 2009). Ainda não se sabe a origem desse poro, se é o canal iônico que se dilata, ou uma proteína diferente do canal, porém, estão interligados de alguma forma. Também, alguns autores relatam a ligação desse poro com a Panexina-1 (Locovei et al. 2007), se são poros com mecanismos distintos e seletivos para moléculas catiônicas ou aniônicas (Schachter et al. 2008). Porém nos nossos dados observamos que nossa metodologia não evidenciou a correlação entre o poro ativado pelo P2X7R e o canal de Panexina1, de acordo com um experimento de permeabilização, onde utilizamos um antagonista para Panexina 1, a mefloquina. Esse dado corrobora com um trabalho publicado por Cankurtaran-Sayar et al. (2009), onde observaram a permeabilização de corantes em macrófagos nocautes para Panexina 1 quando tratados com o ATP, o que evidenciou a atividade do poro formado pelo P2X7R na ausência do canal de Panexina 1. Além disso, eles utilizaram células HEK-293 transfectadas com o P2X7R e o mesmo perfil foi obtido. Também cabe ressaltar que apenas a ativação do poro formado pelo P2X7R ativa vias de sinalização intracelular, que leva à produção e liberação de citocinas e mediadores pró-inflamatórios (Pelegrin 2011).

Atualmente, baseando-se na propriedade de formação do poro desse receptor, existem diversas técnicas já padronizadas, ou sendo padronizadas, para determinação do IC_{50} de moléculas com possível atividade antagonista.

As mais utilizadas, porém, são a análise de incorporação de corantes de até 900 Da através da microscopia de fluorescência ou por citometria de fluxo. Essas técnicas não permitem uma análise de vários compostos ao mesmo tempo com rapidez na obtenção e análise dos resultados. Logo, para a montagem de uma metodologia baseada no "high throughput screening" (HTS) triagem de alta eficiência, essas técnicas não poderiam ser utilizadas. Pensando nisso, padronizamos uma metodologia que pode ser aplicada numa plataforma de HTS, a qual foi validada quando os dados obtidos através do experimento com esse novo protocolo demonstrou resultados semelhantes aos obtidos com a técnica de microscopia de fluorescência, como demonstrado no nosso primeiro manuscrito.

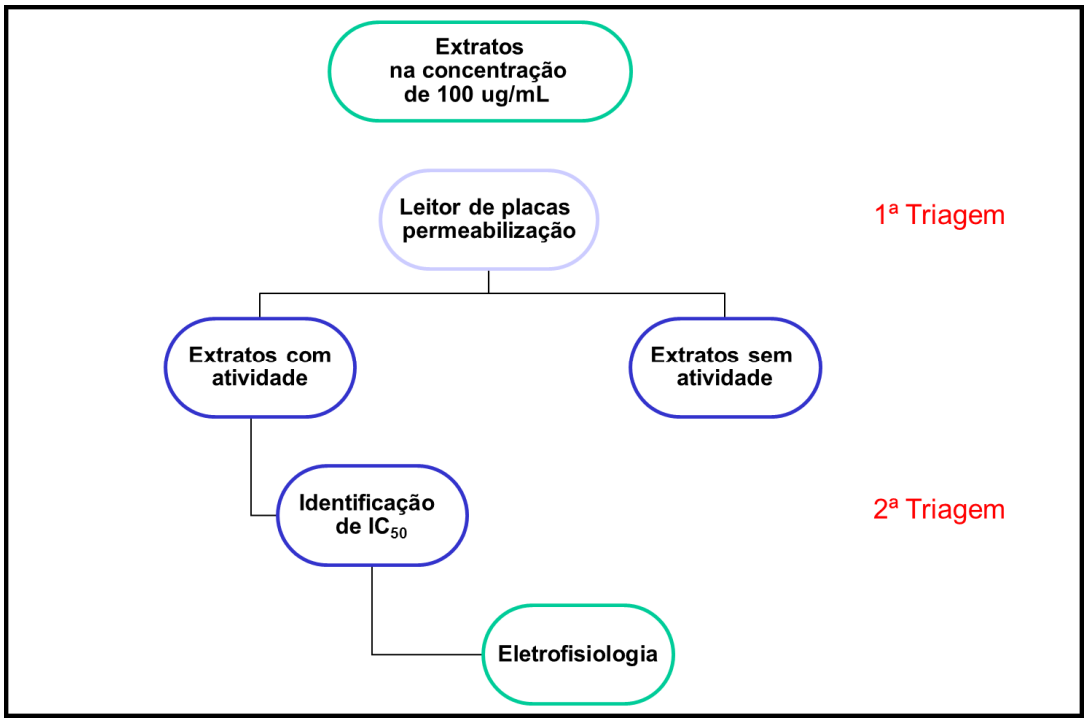
Além disso, observando o quadro 1 do primeiro manuscrito, os valores de IC_{50} e EC_{50} obtidos para os respectivos antagonistas e agonista utilizados neste trabalho, ficaram dentro do valor descrito na literatura para inibição específica do P2X7R. Adicionalmente, nosso resultado de microscopia de fluorescência prova que, na presença do antagonista específico para o P2X7R (ATP Oxidado), não houve a incorporação do corante catiônico pelas células tratadas com 5mM de ATP, assim como houve a inibição significativa da incorporação na presença do outro antagonista reversível na dose correspondente à utilizada para inibir o P2X7R. Esses dados confirmam o perfil agonista e antagonista desses compostos para o P2X7R, como já descrito na literatura (Burnstock 2009), o que permite a aplicabilidade desse novo método para avaliar a possível inibição deste receptor.

Logo, podemos concluir que esta técnica é mais accessível, pois dispensa um investimento pesado na aquisição de equipamentos e permite a

análise simultânea de vários compostos ao mesmo tempo em relação a outras metodologias, pois se baseia na técnica de espectrofotometria com a utilização de uma placa opaca de 96 poços. Dessa forma, acreditamos que a publicação desse manuscrito poderá ajudar na pesquisa por novos e seletivos agonistas e/ou antagonistas para o P2X7R, que possam ser futuramente aplicados na clínica para o tratamento de doenças relacionadas à este receptor, o que têm sido o objetivo de muitos grupos de pesquisa e laboratórios farmacêutico pelo mundo (Gunosewoyo et al. 2010).

Após a padronização da metodologia, testamos 1800 extratos cedidos através de colaboração científica com o Laboratório de Química de Produtos Naturais (LPQN) – CPqRR – FIOCRUZ - MG. O LPQN possui uma extratoteca com mais de 12.000 extratos cedidos no regime de confidencialidade. Os extratos foram plaqueados em placas de polipropileno de fundo “v” na concentração de 2 mg por poço. Ao chegarem, os extratos foram ressuspendidos em solução salina extracelular com 1% DMSO para que fossem utilizados nos tratamentos em células J774.G8, na concentração de 100 µg/mL. Os extratos que demonstraram atividade nessa concentração foram eleitos para a segunda fase da triagem, para determinação dos valores de IC_{50}, seguindo o organograma abaixo:

Quadro 2: Organograma da metodologia utilizada



Dos 1800 extratos testados, apenas três extratos apresentaram atividade nessa faixa de corte, inibindo significativamente a captação de iodeto de propídeo, em relação ao controle tratado com 5 mM de ATP (agonista) e não mostrando significativa diferença com o controle tratado apenas com o corante. Os três extratos ativos foram obtidos de fungos, dois originários da Antártica (8549 = *Cryptococcus vitoriae* e 8568 = *Metschnikowia australis*) e um do sul do Brasil (8067 = fungo algícola a ser identificado). Esse resultado possui uma característica altamente inovadora, pois até o momento só existe o trabalho de Santiago et al. (2012), que demonstrou as atividades dos extratos de fungos antárticos pertencentes aos gêneros *Alternaria, Antarctomyces, Cadophora, Davidiella, Helgardia, Herpotrichia, Microdochium, Oculimacula, Phaeosphaeri,* em modelos usando promastigotas de *Leishmania amazonensis* e células tumorais UCC-62 (melanoma), MCF-7 (mama) e TK-10 (renal).

Porém, não há, até o presente momento, nenhum trabalho na literatura que relate a ação biológica dos fungos estudados no presente trabalho.

Para determinação dos valores de IC_{50} utilizamos, além de células J774.G8, células de linhagem humana (U937), para avaliar a possível diferença no perfil inibitório desses extratos fúngicos entre espécies diferentes, e se eles seriam ativos sobre a linhagem humana, o que despertaria o interesse no prosseguimento na pesquisa destes com possibilidade de futura aplicabilidade clínica. Os valores de IC_{50} obtidos nos testes *in vitro* com células de linhagem humana U937 foram menores dos que encontramos nos testes com a linhagem de células macrofágicas murinas J774.G8, conforme demonstrado no segundo manuscrito e na tabela abaixo. Esse dado prova que células humanas são mais sensíveis ao tratamento com esses extratos; sendo assim, serão necessárias doses mínimas para se obter o efeito desejado, característica vital que um bioproduto ou composto sintético deve ter para atrair a atenção da pesquisa clínica e da indústria farmacêutica.

Tabela 4: IC_{50} dos fungos estudados nesse trabalho.

FUNGO	**J774.G8 (Camundongo)**	**U937 (Humano)**
Cryptococcus vitoriae	3.8 μg/mL	1.5 μg/mL
Metschnikowia australis	2.6 μg/mL	0.92 μg/mL
fungo Algícola (UFMG-3270)	2.1 μg/mL	0.69 μg/mL

Vale ressaltar que há estudos na literatura que também relatam a diferença de IC_{50} entre espécies diferentes, como por exemplo, o trabalho de Hibell et al.2001, no qual o autor faz uma comparação entre respostas de

células transfectadas com P2X7R de rato, camundongo ou humano ao tratamento com os principais antagonistas utilizados para esse receptor. Como conclusão, ele relata as diferenças de potência desses antagonistas (IC_{50}) entre essas espécies. O mesmo perfil de diferença de potência entre espécies foi observado em outros artigos, inclusive, quando tratam de moléculas novas com promissora ação sobre o P2X7R. (Coddou et al. 2011).

Após a determinação dos $IC´s_{50}$, foi avaliado se o extrato também poderia inibir a passagem de moléculas aniônicas pelo poro do P2X7R ativado, na concentração de ATP extracelular acima de 100 µM, no nosso caso utilizamos 5mM de ATP. Esse protocolo serviu para respondermos duas questões. A primeira era saber se a nossa metodologia padronizada serviria também para detectar a atividade de um possível segundo poro seletivo para moléculas aniônicas, assim como funcionou para detectar a inibição ou não da captação do corante catiônico (iodeto de propídeo). A segunda questão era saber se os extratos tinham ação inibitória sobre os dois poros, ou somente sobre o poro catiônico, conforme sugerido por uma das teorias acerca deste poro (Schachter et al. 2008).

A possibilidade do P2X7R modular vias diferentes para captação de corantes aniônicos e catiônicos foi postulada em 2008 (Schachter et al. 2008). Desde então, diversos trabalhos têm sido publicados abordando experimentalmente essa possível origem do poro, inclusive citando a diferença na modulação da captação de moléculas catiônicas e aniônicas durante processos inflamatórios e infecciosos, como por exemplo, no trabalho de Marques-da-Silva et al. 2011, onde os autores relatam a diferença na permeabilização entre corantes aniônicos e catiônicos, durante a infecção de

macrófagos murinos por *Leishmania amazonensis*. Os autores observaram que durante esse processo infecioso, a captação de corantes aniônicos aumentou enquanto que a de corantes catiônicos diminuiu surpreendentemente. Dessa forma, foi comprovada a modulação diferencial na captação de moléculas catiônicas e aniônicas promovida pelo P2X7R durante um processo infeccioso.

Contudo, esse padrão pode mudar de acordo com o tipo de processo inflamatório ou infeccioso, o que ainda cabe ser avaliado (Marques-da-Silva et al. 2011). Entretanto, nossos resultados mostrados no segundo manuscrito, mostram que os extratos fúngicos inibiram a captação do corante aniônico (lucifer yellow), no mesmo padrão de inibição observado em relação ao corante catiônico (iodeto de propídeo). Foi demonstrado o mesmo perfil de inibição da captação do corante aniônico por células J774.G8 tratadas previamente com os extratos 8067, 8549 e 8568 em seus respectivos IC_{50}, em relação ao controle tratado com ATP e o corante somente. Dessa forma, conseguimos comprovar o envolvimento de um possível poro aniônico, além de mostrar que os extratos fúngicos testados também agem inibindo a captação de corantes aniônicos.

Para confirmar os resultados obtidos até essa fase e comprovar a ação antagonista dos extratos fúngicos sobre o P2X7R, se fez necessária a avaliação da ação desses extratos sobre a ativação de funções fisiológicas relacionadas a esse receptor.

O papel da sinalização purinérgica nas respostas inflamatória e imune, mediado pelo ATP, tem sido amplamente descrito na literatura, assim como a sua correlação com o P2X7R, em especial no processo de maturação e liberação de IL-1β (Pelegrin et al. 2009; Clark et al. 2010; Gunosewoyo et al. 2010; Coddou et al. 2011). Além da IL-1β, também está descrita na literatura a

ação do P2X7R sobre a maturação e liberação de outras citocinas pró-inflamatórias, como por exemplo, a IL-8 (Idzko et al. 2003), IL-18 (Mehta et al. 2001) e o TNF-α (Suzuki et al. 2004) por vários tipos de células, incluindo macrófagos, monócitos, células da micróglia e outros (Gourine et al. 2005).

Experimento com camundongos que não expressavam o P2X7R, após silenciamento gênico, mostrou que os macrófagos peritoneais retirados desses camundongos não liberavam IL-1β depois de estimulados com ATP (Solle et al. 2001). Quando estes mesmos camundongos foram submetidos a um processo de artrite induzida por colágeno, houve uma significativa redução no perfil de resposta inflamatória sistêmica (Labasi et al. 2002). Esses dados sugeriram a participação do P2X7R na via de liberação desta citocina.

Também já é descrita a relação do P2X7R com a liberação de citocinas pirogênicas (Gourine et al. 2005), assim como macrófagos, linfócitos e outras células do sistema imune liberam grandes quantidades de ATP no espaço extracelular (Dubyak et al. 1993), o que promove a ativação deste receptor durante um evento inflamatório, ativando assim, a liberação de citocinas relacionadas à atividade do P2X7R.

Com base nesses achados, esse receptor foi eleito um alvo promissor para o tratamento de doenças inflamatórias que afligem a população, como por exemplo, a artrite reumatoide, uma doença incapacitante, onde o paciente sofre um processo inflamatório crônico nas articulações. Esse processo gera deformações, e implica na qualidade de vida do paciente devido ao constante estado doloroso desencadeado pela inflamação intensa.

Devido à participação do P2X7R no processo inflamatório (Coddou et al. 2011), a pesquisa de antagonistas seletivos e com baixo índice de efeitos

adversos, poderá gerar compostos ativos a serem utilizados, futuramente, para o tratamento clínico de doenças inflamatórias, principalmente da artrite reumatoide. Atualmente, já existem compostos sintéticos sendo testados para o tratamento da artrite reumatoide via antagonismo desse receptor (Gunosewoyo et al. 2010; Coddou et al. 2011). Porém, até o momento nenhum composto passou da fase clínica III.

Com base no papel do P2X7R na via de liberação da IL-1β, uma citocina pró-inflamatória relacionada com a evolução da artrite reumatoide, analisamos o efeito dos extratos fúngicos sobre a liberação desta citocina, em células de linhagem J774.G8 ou U937 tratadas com os IC_{50} destes extratos, na presença ou não de ATP. Utilizando os sobrenadantes foram analisados os níveis de IL-1β por ELISA (R&D Systems; Mineápolis; EUA). Os resultados obtidos mostraram um significativo efeito inibitório desses extratos sobre a liberação desta citocina, na presença do agonista (ATP), em relação ao controle tratado com ATP somente. Isto comprova a possível ação destes extratos sobre o P2X7R, pois mesmo na presença do seu agonista fisiológico (ATP), não ocorreu um aumento na liberação desta citocina. Embora existam outras vias fisiológicas de maturação desta citocina, como por exemplo a via clássica através da ativação do Toll Like Receptor 4 (TLR-4) por lipopolissacarídeos de bactérias gram-negativas, neste experimento nos utilizamos o ATP, visando a liberação da IL-1β via P2X7R. Esses dados confirmaram o perfil antagonista e anti-inflamatório desses extratos fúngicos via P2X7R, o que sinaliza para a candidatura desses como possíveis bioprodutos com possível atividade terapêutica, este dado consta no segundo manuscrito.

Na continuidade do nosso entendimento da relevância dos extratos em estudo, avaliamos os seus potenciais efeitos sobre otras vias moduladas pelo P2X7R, a fim de confirmar essa atividade antagonista observada via inibição desse receptor.

A via de produção de NO por células macrofágicas do sistema imune e sua correlação com o P2X7R, também já esta bem descrita (Sperlagh et al. 1998). Com relação aos radicais nitrogenados produzidos pela célula fagocítica, o principal é o óxido nítrico. A formação do NO, além disso, requer um estímulo de um agente indutor, tais como algumas citocinas: TNF, IFN-γ ou IL-1 para sua atividade, que são formados quando o macrófago é ativado (Cho et al. 1992). Recentemente, foi descoberta a via de modulação da produção e liberação do NO via complexo inflamassoma com a participação do P2X7R (Bours et al. 2011).

O NO é um importante e versátil mediador nos sistemas biológicos. Esta molécula é conhecida por executar importantes funções na regulação de uma ampla gama de processos fisiológicos. Este radical é biossintetizado a partir da L-arginina pela enzima óxido nítrico sintase (NOS), em uma reação de duas etapas de oxidação, sendo os produtos formados a L-citrulina e o NO, este último com uma vida extremamente curta (Stamler et al. 1992).

A localização de uma NOS no interior da célula pode influenciar a função biológica do NO produzido (Kelm et al. 1992). O papel fisiológico do radical NO envolve imunidade celular, inibição da agregação plaquetária e neurotransmissão (Bredt et al. 1994; Wynn et al. 1994). Em células do sistema imune o NO tem ação citotóxica ou citostática na destruição de patógenos e células tumorais, num mecanismo importante na imunidade não específica

(Wynn et al. 1994). A ativação de macrófagos por IFN-γ, lipopolissacarídeos bacterianos (LPS), IL-1, fator de necrose tumoral α (TNF α) leva à indução de uma isoforma de NOS para produzir altos níveis (micromol) de NO continuamente por algumas horas (Green et al. 1994).

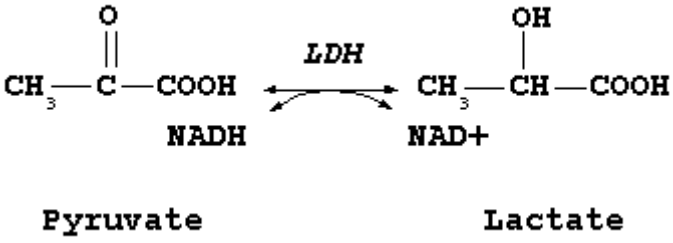
Uma vez que o NO formado em baixas concentrações é altamente reativo com agentes fisiológicos como tióis e radicais de oxigênio, a dosagem direta em amostras biológicas é difícil (Marzinzig et al. 1997). Após cumprir sua função biológica, ele desaparece espontaneamente, transformando-se em nitrito (NO_2^-) e nitrato (NO_3^-). Dessa forma, um método para a determinação indireta de NO é a dosagem espectrofotométrica de seus produtos de oxigenação. Algumas células produzem diretamente NO_3^- que precisa ser reduzido a NO_2^- e esta redução pode ser enzimática (através da nitrato redutase), sendo o produto da reação, o NO_2^-, detectado pelo reagente de Griess (Green et al. 1994).

Para avaliar se os extratos fúngicos aqui testados também agiriam sobre a via do NO, os sobrenadantes das culturas de células J774.G8 e U937, tratadas com os extratos nos respectivos IC`s$_{50}$ foram submetidos ao método descrito por Green et al. (1994), utilizando o reagente de Griess. Os resultados obtidos também foram promissores, mostrando significativa inibição na liberação de NO pelas células tratadas com estes extratos na presença do ATP em relação ao controle tratado com ATP, o que pode estar diretamente relacionada com o perfil de antagonismo sobre o P2X7R, desses extratos fúngicos. Esse efeito possivelmente relacionado a inibição da ativação do complexo Inflamassoma e liberação da IL-1β via antagonismo deste receptor, como observado no resultado anterior, o que levou a inibição de outras

respostas inflamatórias, como por exemplo, a liberação de óxido nítrico. Cabe ressaltar que o percentual de inibição das vias funcionais relacionadas ao P2X7R, observado através dos resultados dos ensaios de ELISA para IL-1β, Liberação de NO e ROS, demonstraram serem diferentes dos percentuais de inibição dos extratos sobre a abertura do poro relacionado ao P2X7R devido a estas vias não serem exclusivamente ativadas por este receptor. Existem outras vias intracelulares que ativam estas funções, como por exemplo, a via da MAPK, PKC, TLR-4, entre outras. Porém quando comparamos os resultados de cada experimento isoladamente com seus respectivos controles, notamos que foi confirmada a inibição do P2X7R com inibição das funções a ele relacionadas.

Com o intuito de fazermos uma caracterização farmacológica do efeito inibitório destes extratos sobre a formação do poro associado ao P2X7R, nós realizamos um experimento de eletrofisiologia com o IC_{50} de cada um deles, utilizando células J774.G8. Por ser um receptor ionotrópico, abre um canal catiônico seletivo quando ativado, o uso dessa técnica permite o registro das correntes iônicas da membrana da célula. Logo, se este receptor estiver inibido, haverá uma diminuição dessa corrente iônica. Utilizamos a configuração “Whole-Cell”, o que permitiu a aquisição dos dados de todos os canais iônicos ativados, ou não, na membrana das células. Nossos resultados confirmaram o efeito antagonista destes sobre a formação dessa corrente, além de mostrar que esse perfil inibitório ocorre também em doses acima e abaixo do valor das suas concentrações inibitórias, mostrando um efeito farmacológico dose-dependente com perfil específico para o P2X7R.

Com este conjunto de resultados que apontaram a atividade inibitória sobre funções biológicas relacionadas ao P2X7R, buscamos avaliar se os extratos seriam tóxicos para as células utilizadas nesse trabalho. Embora nossa metodologia padronizada também seja capaz de detectar compostos citotóxicos, através da leitura de incorporação de corantes na ausência de ATP, utilizamos o ensaio de detecção de LDH para avaliação da citotoxicidade (Doles; Goiania; GO; Brasil). A LDH, lactato desidrogenase, é uma enzima localizada no interior das células, na mitocôndria, e catalisa a interconversão de piruvato e lactato com uma concomitante interconversão de NADH e NAD^+. Converte o piruvato, o produto final da glicólise em ácido láctico quando o oxigênio está ausente ou em pequenas quantidades, e realiza a reação reversa durante o ciclo de Cori, via glicolítica anaeróbica, no fígado. Existem isoformas dessas enzimas distribuídas pelo organismo humano: LDH-1: músculo cardíaco e eritrócitos; LDH-2: miócitos cardíacos e leucócitos; LDH-3: pneumócitos; LDH-4: rins, placenta e pâncreas; LDH-5: hepatócitos e miócitos esqueléticos.



Via da Lactato Desidrogenase (KIT LDH; Doles; Go; Brasil)

Os resultados obtidos mostraram que os três extratos (8067,8549 e 8568) não demonstraram atividade citotóxica após o tratamento por 24 h em

concentrações quatro vezes maiores ou menores que os seus respectivos IC`s_{50}, mesmo na presença do tratamento com ATP (agonista do P2X7R) por 1 h. mostrando que foram capazes de antagonizar o evento de morte celular via P2X7R assim como os efeitos deletérios do ATP. Os resultados obtidos foram estatisticamente semelhantes ao controle composto somente por células. Além disso, os valores obtidos foram significativamente menores do que o controle tratado com ATP somente. Esse resultado foi semelhante tanto em células J774.G8, quanto em células U937, demonstrando assim uma faixa de segurança destes extratos, o que viabilizou os testes *in vivo*.

Nossos testes *in vivo* demonstraram a potencial atividade analgésica dos três extratos fúngicos estudados aqui, conforme demonstrado no terceiro manuscrito. Utilizamos para a avaliação do efeito destes extratos sobre a dor de origem neuropática e inflamatória, o experimento do desafio com formalina, o qual já se encontra estabelecido em nosso grupo de pesquisa e inclusive têm sido utilizado por diversos grupos de pesquisa como parâmetro para estas análises (Onasanwo et al. 2012; Pinheiro et al. 2013 & Silva et al. 2010). Os extratos 8067 e 8549 demonstraram inibição na dor neuropática nos primeiros 5 minutos do experimento com formalina (um agente indutor da dor), porém os três extratos (8067,8549 e 8568) apresentaram inibição da dor nos 15 a 30 minutos finais desse experimento, indicando uma analgesia também sobre a dor de origem inflamatória. Demonstraram, inclusive, serem mais ativos que o fármaco comercialmente disponível (Diclofenaco de Sódio) e o BBG (antagonista reversível do P2X7R). Esse dado confirma os nossos resultados anteriores, que mostraram a inibição da liberação de uma citocina pró-inflamatória (IL-1β) e mediadores inflamatórios (NO e ROS), através do

tratamento *in vitro* com esses extratos, sinalizando, assim, uma ação anti-inflamatória destes extratos via inibição do P2X7R. O extrato 8568 demosntrou efeito anti-inflamatório, tanto *in vitro* quanto *in vivo*, entretanto não apresentou ação sobre a dor neuropática, o que o diferencia dos outros dois extratos, essa diferença pode se dever ao fato desse extrato não apresentar princípios ativos capazes de interferir com a transdução da dor neuropática, que é conhecidamente diferente da dor inflamatória (Toulme et al. 2010), o que talvez tenha refletido uma seletividade de ação deste composto sobre somente um tipo de dor.

No experimento de gastroproteção *in vivo* através da indução de úlcera por Etanol 70%, o tratamento prévio dos animais com os três extratos demonstrou significativa inibição sobre a formação de úlceras, inclusive sendo mais eficaz que o antagonista para o P2X7R (BBG) e o fármaco utilizado na clínica (Lansoprazol). Nesse experimento, também observamos uma possível participação deste receptor no contexto da úlcera, visto que o antagonista utilizado (BBG) também promoveu gastroproteção semelhante ao Lansoprazol. O que necessita de estudos futuros mais aprofundados, para confirmar a participação do P2X7R nesta patologia.

Com o objetivo de obter uma maior quantidade de informações sobre a ação destes extratos *in vivo,* utilizamos os animais submetidos ao protocolo de úlcera induzida por etanol, para avaliarmos se o tratamento com estes extrados, via oral, poderia levar a um quadro de toxicidade aguda, pois para termos um dado seguro sobre uma possível toxicidade crônica, os animais teriam que receber o tratamento por um período mais prologando e em doses maiores. Porém devido a quantidade que possuíamos de cada extrato, foi

possível somente avaliar uma possível toxicidade aguda. Com base nos resultados do terceiro manuscrito sobre a avaliação dos parâmetros bioquímicos da função hepática, através da análise sérica dos níveis de transaminases (AST e ALT), podemos comprovar que os extratos não promoveram uma toxicidade hepática aguda. A toxicidade hepática pode ocorrer, pois os fármacos orais são biotransformados pelo Fígado, podendo assim, causar alguma toxicidade neste órgão. Também avaliamos os níveis séricos de creatinina, que esta relacionada com a função renal, importante para a excreção de grande parte dos fármacos. Como demostrado também no terceiro manuscrito, estes extratos não promoveram toxicidade renal aguda.

Com base nos resultados *in vitro* e *in vivo* obtidos no nosso trabalho, podemos concluir que os 3 extratos fúngicos (8067, 8549 e 8568), demonstraram atividade antagonista no nosso método padronizado de análise da atividade do P2X7R, confirmaram este perfil de antagonismo do P2X7R, tanto nos testes funcionais *in vitro*, quanto nos experimentos *In vivo*, inclusive sinalizando uma participação deste receptor no contexto da úlcera. Portanto, há um potencial inovador que poderá despertar a atenção do campo clínico e farmacêutico, o que poderá sinalizar a formulação de novos fitoterápicos com base nos princípios ativos obtidos a partir desses extratos.

Nesse intuito, os resultados obtidos nesse trabalho geraram três pedidos de registro de patente, um relacionado com cada extrato fúngico aqui testado, os quais se encontram na fase de análise de patenteabilidade pelo GESTEC-FIOCRUZ. Porém, devido a lei da propriedade industrial (9279/96) – Art.10, não se permite, no Brasil, patentear extratos obtidos de produtos naturais, nem suas frações ou princípios dela isolados. Por isso iremos requerer a patente

internacional desses extratos nos Estados Unidos da América, onde se permite o patenteamento de produtos naturais [FD&C 201(g)(1)(b)].

6. CONCLUSÕES

Mediante os resultados obtidos nesta Tese, pode-se concluir que:

a) Baseado nos resultados obtidos foi padronizado um método rápido e mais acessível, que permite fazer a triagem de quantidades maiores de extratos (cerca de 60 por placa) ao mesmo tempo em relação aos métodos atuais;

b) Essa nova metodologia padronizada permite a avaliação da atividade do receptor $P2X_7$ utilizando-se corantes catiônicos e aniônicos, contemplando as teorias acerca da abertura do poro membranar pelo receptor $P2X_7$;

c) Com a aplicação dessa nova metodologia padronizada foi possível identificar a atividade antagonista de três extratos testados: 8067, 8549 e 8568;

d) A ação inibitória dos extratos sobre uma função atribuída ao receptor $P2X_7$ foi observada através do ensaio de ELISA para IL-1β, sobre a expressão e liberação do NO (através do método de Griess) e eletrofisiologia, confirmando a provável ação antagonista desses extratos sobre esse receptor;

e) Os extratos que demonstraram atividade antagonista "*in vitro"* não foram significativamente citotóxicos;

f) Os extratos demonstraram atividade analgésica "*in vivo"*, tanto na dor neuropática quanto na inflamatória;

g) Os extratos demonstraram atividade gastroprotetora "in vivo", no modelo de úlcera induzida por etanol;

h) Nossos resultados apontam a obtenção de três bioprodutos patenteáveis que possam contribuir futuramente na formulação de possíveis novos fitofármacos.

7. BIBLIOGRAFIA

Abbracchio MP, Burnstock G. Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther 64, no. 3 919940: 445-75.

Al-Shukaili, A., J. Al-Kaabi, and B. Hassan. "A Comparative Study of Interleukin-1beta Production and P2x7 Expression after Atp Stimulation by Peripheral Blood Mononuclear Cells Isolated from Rheumatoid Arthritis Patients and Normal Healthy Controls." Inflammation 31, no. 2 (2008): 84-90.

Al-Sohaibani, S., and K. Murugan. "Anti-Biofilm Activity of Salvadora Persica on Cariogenic Isolates of Streptococcus Mutans: In Vitro and Molecular Docking Studies." Biofouling 28, no. 1 (2012): 29-38.

Barbosa-Filho, J. M., P. C. K. Medeiros, F. M. Diniz, M. L. Batista, F. P. Athalayde-Filho, S. M. Silva, V. L. E. Da-Cunha, S. R. G. J. Almeida, and J. L. Quintans-J£nior. "Natural Products Inhibitors of Achetylcholinesterase." Br.J.Pharmacol. 2, (2016): 258-285.

Bours, M. J., P. C. Dagnelie, A. L. Giuliani, A. Wesselius, and F. Di Virgilio. "P2 Receptors and Extracellular Atp: A Novel Homeostatic Pathway in Inflammation." Front Biosci (Schol Ed) 3, (2011): 1443-56.

Bredt, D. S., and S. H. Snyder. "Nitric Oxide: A Physiologic Messenger Molecule." Annu Rev Biochem 63, (1994): 175-95.

Brown, S. G., A. Townsend-Nicholson, K. A. Jacobson, G. Burnstock, and B. F. King. "Heteromultimeric P2x(1/2) Receptors Show a Novel Sensitivity to Extracellular Ph." J Pharmacol Exp Ther 300, no. 2 (2002): 673-680.

Buchanan, M. S., A. R. Carroll, R. Addepalli, V. M. Avery, J. N. Hooper, and R. J. Quinn. "Natural Products, Stylissadines a and B, Specific Antagonists of the

P2x7 Receptor, an Important Inflammatory Target." J Org Chem 72, no. 7 (2007): 2309-17.

Burnstock, G. "Purinergic Signalling: Past, Present and Future." Braz J Med Biol Res 42, no. 1 (2009): 3-8.

Burnstock, G., and G. E. Knight. "Cellular Distribution and Functions of P2 Receptor Subtypes in Different Systems." Int.Rev Cytol. 240, (2004): 31-304.

Butinar, L., T. Strmole, and N. Gunde-Cimerman. "Relative Incidence of Ascomycetous Yeasts in Arctic Coastal Environments." Microb Ecol 61, no. 4 (2011): 832-43.

Calixto, J. B. "Twenty-Five Years of Research on Medicinal Plants in Latin America: A Personal View." J Ethnopharmacol. 100, no. 1-2 (2005): 131-134.

Cankurtaran-Sayar, S., K. Sayar, and M. Ugur. "P2x7 Receptor Activates Multiple Selective Dye-Permeation Pathways in Raw 264.7 and Human Embryonic Kidney 293 Cells." Mol.Pharmacol. 76, no. 6 (2009): 1323-1332.

Caporali, F., P. L. Capecchi, A. Gamberucci, P. E. Lazzerini, G. Pompella, M. Natale, S. Lorenzini, E. Selvi, M. Galeazzi, and F. Laghi Pasini. "Human Rheumatoid Synoviocytes Express Functional P2x7 Receptors." J Mol Med (Berl) 86, no. 8 (2008): 937-49.

Chang, K., K. Hanaoka, M. Kumada, and Y. Takuwa. "Molecular Cloning and Functional Analysis of a Novel P2 Nucleotide Receptor." J Biol Chem 270, no. 44 (1995): 26152-8.

Cho, H. J., Q. W. Xie, J. Calaycay, R. A. Mumford, K. M. Swiderek, T. D. Lee, and C. Nathan. "Calmodulin Is a Subunit of Nitric Oxide Synthase from Macrophages." J Exp Med 176, no. 2 (1992): 599-604.

Clark, A. K., A. A. Staniland, F. Marchand, T. K. Kaan, S. B. McMahon, and M. Malcangio. "P2x7-Dependent Release of Interleukin-1beta and Nociception in the Spinal Cord Following Lipopolysaccharide." J Neurosci 30, no. 2 (2010): 573-82.

Coddou, C., Z. Yan, T. Obsil, J. P. Huidobro-Toro, and S. S. Stojilkovic. "Activation and Regulation of Purinergic P2x Receptor Channels." Pharmacol Rev 63, no. 3 (2011): 641-83.

Coutinho-Silva, R., and P. M. Persechini. "P2z Purinoceptor-Associated Pores Induced by Extracellular Atp in Macrophages and J774 Cells." Am J Physiol 273, no. 6 Pt 1 (1997): C1793-C1800.

Craig, D. B., and C. J. Wallace. "Atp Binding to Cytochrome C Diminishes Electron Flow in the Mitochondrial Respiratory Pathway." Protein Sci 2, no. 6 (1993): 966-76.

Donnelly-Roberts, D. L., M. T. Namovic, C. R. Faltynrk, and M. F. Jarvis. "Mitogen-Activated Protein Kinase and Caspase Signalling Pathways Are Required for P2x7 Receptor (P2x7r)-Induced Pore Formation in Human Thp-1." J Pharmacol Exp Ther 308, (2004): 1053-1061.

Drury, A. N. "The Physiological Activity of Adenine Compounds with Especial Reference to Their Action Upon the Mammalian Heart." J Physiol 68, (1929): 213-237.

Dubyak, G. R., and C. el-Moatassim. "Signal Transduction Via P2-Purinergic Receptors for Extracellular Atp and Other Nucleotides." Am.J Physiol 265, no. 3 Pt 1 (1993): C577-C606.

Faria, R. X., F. P. de Farias, and L. A. Alves. "Are Second Messengers Crucial for Opening the Pore Associated with P2x7 Receptor?" Am J Physiol Cell Physiol 288, no. 2 (2005): C260-C271.

Fernando, S. L., B. M. Saunders, R. Sluyter, K. K. Skarratt, H. Goldberg, G. B. Marks, J. S. Wiley, and W. J. Britton. "A Polymorphism in the P2x7 Gene Increases Susceptibility to Extrapulmonary Tuberculosis." Am J Respir Crit Care Med 175, no. 4 (2007): 360-6.

Fountain, S. J., L. Cao, M. T. Young, and R. A. North. "Permeation Properties of a P2x Receptor in the Green Algae Ostreococcus Tauri." J Biol Chem 283, no. 22 (2008): 15122-6.

Friedle, S. A., V. M. Brautigam, M. Nikodemova, M. L. Wright, and J. J. Watters. "The P2x7-Egr Pathway Regulates Nucleotide-Dependent Inflammatory Gene Expression in Microglia." Glia 59, no. 1 (2011): 1-13.

Gillespie, J. H. "The Biological Significance of the Linkages in Adenosine Triphosphoric Acid." J Physiol 80, no. 4 (1934): 345-59.

Gourine, A. V., D. M. Poputnikov, N. Zhernosek, E. V. Melenchuk, R. Gerstberger, K. M. Spyer, and V. N. Gourine. "P2 Receptor Blockade Attenuates Fever and Cytokine Responses Induced by Lipopolysaccharide in Rats." Br J Pharmacol 146, no. 1 (2005): 139-45.

Green, I. C., J. M. Cunningham, C. A. Delaney, M. R. Elphick, J. G. Mabley, and M. H. Green. "Effects of Cytokines and Nitric Oxide Donors on Insulin

Secretion, Cyclic Gmp and DNA Damage: Relation to Nitric Oxide Production." Biochem Soc Trans 22, no. 1 (1994): 30-7.

Gunosewoyo, H., M. J. Coster, and M. Kassiou. "Molecular Probes for P2x7 Receptor Studies." Curr Med Chem 14, no. 14 (2007): 1505-23.

Gunosewoyo, H., M. J. Coster, M. R. Bennett, and M. Kassiou. "Purinergic P2x(7) Receptor Antagonists: Chemistry and Fundamentals of Biological Screening." Bioorg Med Chem 17, no. 14 (2009): 4861-5.

Gunosewoyo, Hendra, and Michael Kassiou. "P2x Purinergic Receptor Ligands: Recently Patented Compounds." Expert opinion on therapeutic patents 20, no. 5 (2010): 625-46.

Guo, L., J. Z. Wu, T. Han, T. Cao, K. Rahman, and L. P. Qin. "Chemical Composition, Antifungal and Antitumor Properties of Ether Extracts of Scapania Verrucosa Heeg. And Its Endophytic Fungus Chaetomium Fusiforme." Molecules 13, no. 9 (2008): 2114-25.

Idzko, Marco, Elisabeth Panther, Hinrich C. Bremer, Stephan Sorichter, Werner Luttmann, Christian J. Virchow, Francesco Di Virgilio, Yared Herouy, Johannes Norgauer, and Davide Ferrari. "Stimulation of P2 Purinergic Receptors Induces the Release of Eosinophil Cationic Protein and Interleukin-8 from Human Eosinophils." British Journal of Pharmacology 138, no. 7 (2003): 1244-50.

Kelm, M., M. Feelisch, T. Krebber, W. Motz, and B. E. Strauer. "The Role of Nitric Oxide in the Regulation of Coronary Vascular Resistance in Arterial Hypertension: Comparison of Normotensive and Spontaneously Hypertensive Rats." J Cardiovasc Pharmacol 20 Suppl 12, (1992): S183-6.

Kennedy, C., and G. Burnstock. "Evidence for Two Types of P2-Purinoceptor in Longitudinal Muscle of the Rabbit Portal Vein." Eur.J.Pharmacol. 111, no. 1 (1985): 49-56.

Khakh, B. S., and R. A. North. "Neuromodulation by Extracellular Atp and P2x Receptors in the Cns." Neuron 76, no. 1 (2012): 51-69.

Klapperstuck, M., C. Buttner, T. Bohm, G. Schmalzing, and F. Markwardt. "Characteristics of P2x7 Receptors from Human B Lymphocytes Expressed in Xenopus Oocytes." Biochim Biophys Acta 1467, no. 2 (2000): 444-456.

Labasi, J. M., N. Petrushova, C. Donovan, S. McCurdy, P. Lira, M. M. Payette, W. Brissette, J. R. Wicks, L. Audoly, and C. A. Gabel. "Absence of the P2x7 Receptor Alters Leukocyte Function and Attenuates an Inflammatory Response." J Immunol. 168, no. 12 (2002): 6436-6445.

Lister, M. F., J. Sharkey, D. A. Sawatzky, J. P. Hodgkiss, D. J. Davidson, A. G. Rossi, and K. Finlayson. "The Role of the Purinergic P2x7 Receptor in Inflammation." J Inflamm (Lond) 4, (2007): 5.

Locovei, S., E. Scemes, F. Qiu, D. C. Spray, and G. Dahl. "Pannexin1 Is Part of the Pore Forming Unit of the P2x(7) Receptor Death Complex." FEBS Lett. 581, no. 3 (2007): 483-488.

Mann, J. "Natural Products in Cancer Chemotherapy: Past, Present and Future." Nat Rev Cancer 2, (2002): 143-148.

Marques-da-Silva, C., M. M. Chaves, J. C. Rodrigues, S. Corte-Real, R. Coutinho-Silva, and P. M. Persechini. "Differential Modulation of Atp-Induced P2x7-Associated Permeabilities to Cations and Anions of Macrophages by Infection with Leishmania Amazonensis." PloS one 6, no. 9 (2011): e25356.

Marques-da-Silva, C., M. M. Chaves, N. G. Castro, R. Coutinho-Silva, and M. Z. Guimaraes. "Colchicine Inhibits Cationic Dye Uptake Induced by Atp in P2x2 and P2x7 Receptor-Expressing Cells: Implications for Its Therapeutic Action." Br J Pharmacol 163, no. 5 (2011): 912-26.

Marzinzig, M., A. K. Nussler, J. Stadler, E. Marzinzig, W. Barthlen, N. C. Nussler, H. G. Beger, S. M. Morris, Jr., and U. B. Bruckner. "Improved Methods to Measure End Products of Nitric Oxide in Biological Fluids: Nitrite, Nitrate, and S-Nitrosothiols." Nitric Oxide 1, no. 2 (1997): 177-89.

Mehta, V. B., J. Hart, and M. D. Wewers. "Atp-Stimulated Release of Interleukin (Il)-1beta and Il-18 Requires Priming by Lipopolysaccharide and Is Independent of Caspase-1 Cleavage." J Biol Chem 276, (2001): 3820-3826.

Mohandas, S. P., S. Ravikumar, S. J. Menachery, G. Suseelan, S. S. Narayanan, H. Nandanwar, and K. M. Nampoothiri. "Bioactives of Microbes Isolated from Western Ghat Belt of Kerala Show Beta-Lactamase Inhibition Along with Wide Spectrum Antimicrobial Activity." Appl Biochem Biotechnol, (2012).

Nelson, D. W., R. J. Gregg, M. E. Kort, A. Perez-Medrano, E. A. Voight, Y. Wang, G. Grayson, M. T. Namovic, D. L. Donnelly-Roberts, W. Niforatos, P. Honore, M. F. Jarvis, C. R. Faltynek, and W. A. Carroll. "Structure-Activity Relationship Studies on a Series of Novel, Substituted 1-Benzyl-5-Phenyltetrazole P2x7 Antagonists." J Med.Chem 49, no. 12 (2006): 3659-3666.

Njuguna, N. M., C. Masimirembwa, and K. Chibale. "Identification and Characterization of Reactive Metabolites in Natural Products-Driven Drug Discovery." J Nat Prod, (2012).

North, R. A. "Molecular Physiology of P2x Receptors." Physiol.Rev. 82, no. 4 (2002): 1013-1067.

Onasanwo, S. A., T. D. Fabiyi, F. S. Oluwole, and S. B. Olaleye. "Analgesic and Anti-Inflammatory Properties of the Leaf Extracts of Anacardium Occidentalis in the Laboratory Rodents." Niger J Physiol Sci 27, no. 1 (2012): 65-71.

Pelegrin, P. "Many Ways to Dilate the P2x7 Receptor Pore." Br J Pharmacol 163, no. 5 (2011): 908-11.

Pelegrin, Pablo, and Annmarie Surprenant. "The P2x(7) Receptor-Pannexin Connection to Dye Uptake and Il-1beta Release." Purinergic Signalling 5, no. 2 (2009): 129-37.

Persechini, P. M., R. C. Bisaggio, J. L. Alves-Neto, and R. Coutinho-Silva. "Extracellular Atp in the Lymphohematopoietic System: P2z Purinoceptors Off Membrane Permeabilization." Braz.J.Med.Biol.Res. 31, no. 1 (1998): 25-34.

Pinheiro, M. M., S. B. Fernandes, C. E. Fingolo, F. Boylan, and P. D. Fernandes. "Anti-Inflammatory Activity of Ethanol Extract and Fractions from Couroupita Guianensis Aublet Leaves." J Ethnopharmacol, (2013).

Ralevic, V., and G. Burnstock. "Receptors for Purines and Pyrimidines." Pharmacol Rev 50, no. 3 (1998): 413-492.

Said, T., M. Dutot, R. Christon, J. L. Beaudeux, C. Martin, J. M. Warnet, and P. Rat. "Benefits and Side Effects of Different Vegetable Oil Vectors on Apoptosis, Oxidative Stress, and P2x7 Cell Death Receptor Activation." Invest Ophthalmol Vis Sci 48, no. 11 (2007): 5000-6.

Santos J.A.A., Fidalgo-Neto A.A., Faria R.X., Simões A.P.S., Calheiros A.S., Bérenger A.L., Castro-Faria-Neto H.C., Frutuoso V.S., Alves L.A. . "Effect of Rheedia Longifolia Aqueous Leaves Extract and Fractions from Methanol Extract on P2x7 Receptor in Vitro: Novel Antagonists? ." Journal of Medicinal food submetido, (2011).

Schachter, J., A. P. Motta, A. de Souza Zamorano, H. A. da Silva-Souza, M. Z. Guimaraes, and P. M. Persechini. "Atp-Induced P2x7-Associated Uptake of Large Molecules Involves Distinct Mechanisms for Cations and Anions in Macrophages." J Cell Sci 121, no. Pt 19 (2008): 3261-70.

Silva, J. P., R. S. Rodarte, A. S. Calheiros, C. Z. Souza, F. C. Amendoeira, M. A. Martins, P. M. Silva, V. S. Frutuoso, and E. Barreto. "Antinociceptive Activity of Aqueous Extract of Bowdichia Virgilioides in Mice." J Med Food 13, no. 2 (2010): 348-51.

Skaper, Stephen D., Patrizia Debetto, and Pietro Giusti. "P2x(7) Receptors in Neurological and Cardiovascular Disorders." Cardiovascular psychiatry and neurology 2009, no. Figure 1 (2009): 861324-861324.

Solle, M., J. Labasi, D. G. Perregaux, E. Stam, N. Petrushova, B. H. Koller, R. J. Griffiths, and C. A. Gabel. "Altered Cytokine Production in Mice Lacking P2x(7) Receptors." J Biol Chem 276, no. 1 (2001): 125-32.

Sperlagh, B., G. Hasko, Z. Nemeth, and E. S. Vizi. "Atp Released by Lps Increases Nitric Oxide Production in Raw 264.7 Macrophage Cell Line Via P2z/P2x7 Receptors." Neurochem.Int. 33, no. 3 (1998): 209-215.

Stamler, J. S., D. J. Singel, and J. Loscalzo. "Biochemistry of Nitric Oxide and Its Redox-Activated Forms." Science 258, no. 5090 (1992): 1898-902.

Starzl, T. E., S. Iwatsuki, G. Klintmalm, G. P. Schroter, R. Weil, 3rd, L. J. Koep, and K. A. Porter. "Liver Transplantation, 1980, with Particular Reference to Cyclosporin-A." Transplant Proc 13, no. 1 Pt 1 (1981): 281-5.

Surprenant, A., F. Rassendren, E. Kawashima, R. A. North, and G. Buell. "The Cytolytic P2z Receptor for Extracellular Atp Identified as a P2x Receptor (P2x7)." Science. 272, no. 5262 (1996): 735-738.

Suzuki, Tomohisa, Izumi Hide, Katsutoshi Ido, Shinichi Kohsaka, Kazuhide Inoue, and Yoshihiro Nakata. "Production and Release of Neuroprotective Tumor Necrosis Factor by P2x7 Receptor-Activated Microglia." The Journal of neuroscience : the official journal of the Society for Neuroscience 24, no. 1 (2004): 1-7.

Teplyakova, T. V., N. V. Psurtseva, T. A. Kosogova, N. A. Mazurkova, V. A. Khanin, and V. A. Vlasenko. "Antiviral Activity of Polyporoid Mushrooms (Higher Basidiomycetes) from Altai Mountains (Russia)." Int J Med Mushrooms 14, no. 1 (2012): 37-45.

Torigoe DY, Laurindo IMM. "Artrite Reumatóide E Doenças Cardiovasculares = Rheumatoid Arthritis and Cardiovascular Disease." Rev Bras Reumatol, no. 46 (2006): 60-66.

Toulme, E., M. Tsuda, B. S. Khakh, and K. Inoue. "On the Role of Atp-Gated P2x Receptors in Acute, Inflammatory and Neuropathic Pain." In Translational Pain Research: From Mouse to Man, edited by L. Kruger and A. R. Light. Boca Raton, FL, 2010.

Vial, C., J. A. Roberts, and R. J. Evans. "Molecular Properties of Atp-Gated P2x Receptor Ion Channels." Trends Pharmacol Sci 25, no. 9 (2004): 487-93.

Wynn, T. A., I. P. Oswald, I. A. Eltoum, P. Caspar, C. J. Lowenstein, F. A. Lewis, S. L. James, and A. Sher. "Elevated Expression of Th1 Cytokines and Nitric Oxide Synthase in the Lungs of Vaccinated Mice after Challenge Infection with Schistosoma Mansoni." J Immunol 153, no. 11 (1994): 5200-9.

Zeng, P. Y., J. G. Wu, L. M. Liao, T. Q. Chen, J. Z. Wu, and K. H. Wong. "In Vitro Antioxidant Activities of Endophytic Fungi Isolated from the Liverwort Scapania Verrucosa." Genet Mol Res 10, no. 4 (2011): 3169-79.

8. ANEXOS

Artigo Publicado

Molecules 2012, 17, 1-x manuscripts; doi:10.3390/molecules170x0000x

molecules
ISSN 1420-3049
www.mdpi.com/journal/molecules

Review

Action of Natural Products on P2 Receptors: A Reinvented Era for Drug Discovery

Faria, R.X.[1], Ferreira, L.G.B.[1], Soares-Bezerra, R.J.[1], Frutuoso, V. S.2 and Alves, L.A. [1], *

1 Laboratory of Cellular Communication, Oswaldo Cruz Foundation, AV. Brazil, 4365, Rio de Janeiro, Brazil; E-Mail: robson.xavier@gmail.com; leonardobraga12@gmail.com; romulo@ioc.fiocruz.br; alveslaa@gmail.com

2 Laboratory of Immnopharmacology, Oswaldo Cruz Foundation, AV. Brazil 4365, Rio de Janeiro, Brazil; E-mail: frutuoso@ioc.fiocruz.br

Molecules **2012**, *17*, 13009-13025; doi:10.3390/molecules171113009

OPEN ACCESS

molecules

ISSN 1420-3049

www.mdpi.com/journal/molecules

Review

Action of Natural Products on P2 Receptors: A Reinvented Era for Drug Discovery

Robson Faria [1], Leonardo Ferreira [1], Rômulo Bezerra [1], Valber Frutuoso [2] and Luiz Alves [1,*]

[1] Laboratory of Cellular Communication, Oswaldo Cruz Foundation, 4365 Rio de Janeiro, Brazil; E-Mails: robson.xavier@gmail.com (R.F.); leonardobraga12@gmail.com (L.F.); romulo@ioc.fiocruz.br (R.B.)

[2] Laboratory of Immnopharmacology, Oswaldo Cruz Foundation, 4365 Rio de Janeiro, Brazil; E-Mail: frutuoso@ioc.fiocruz.br

* Author to whom correspondence should be addressed; E-Mail: alveslaa@gmail.com; Tel.: +55-21-2562-1809; Fax: +55-21-2562-1772.

Received: 7 September 2012; in revised form: 12 October 2012 / Accepted: 24 October 2012 / Published: 1 November 2012

Abstract: Natural products contribute significantly to available drug therapies and have been a rich source for scientific investigation. In general, due to their low cost and traditional use in some cultures, they are an object of growing interest as alternatives to synthetic drugs. With several diseases such as cancer, and inflammatory and neuropathic diseases having been linked to the participation of purinergic (P2) receptors, there has been a flurry of investigations on ligands within natural products. Thirty-four different sources of these compounds have been found so far, that have shown either agonistic or antagonistic effects on P2 receptors. Of those, nine different plant sources demonstrated effects on P2X2, P2X3, P2X7, and possibly P2Y12 receptor subtypes. Microorganisms, which represent the largest group, with 26 different sources, showed effects on both receptor subtypes, ranging from P2X1 to P2X4 and P2X7, and P2Y1, P2Y2, P2Y4, and P2Y6. In addition, there were seventeen animal sources that affected P2X7 and P2Y1 and P2Y12 receptors. Natural products have provided some fascinating new mechanisms and sources to better understand the P2 receptor antagonism. Moreover, current investigations should clarify further pharmacological mechanisms in order to consider these products as potential new medicines.

Keywords: P2 receptors; natural products; antagonists

1. Introduction

Natural products have long been used in traditional Western and Eastern medicine, and they are being actively pursued for drug therapies today. Natural products, which are derived not only from plants, but also from fungi, bacteria, and marine organisms, present distinctive characteristics in their secondary metabolites [1]. The unique properties of these secondary metabolites are often involved in defense and interaction with the environment, and as such they have often given rise to extraordinarily useful drugs, such as penicillin and morphine. These secondary metabolites, which the body can properly digest and process, have been and will likely continue to be, a rich source of alternatives to synthetic drugs [2]. Several factors have facilitated the search for natural products as potential new drug therapies. These factors include, but are not limited to, isolation techniques, such as spectroscopic, chromatographic, biosynthetic, and synthetic methods, and the fact that these isolates can now be obtained by synthetic or combinatorial chemistry and molecular modeling [3–5]. These advances have allowed for the investigation of natural products to become much easier and more time efficient as a source of therapeutic strategies.

One relevant area of investigation is the group of ionotropic (P2X) and metabotropic (P2Y) receptors, which have been found in all studied cells so far. These purinergic receptors, also known as P2 receptors, are crucial for the normal function of an organism, and they have been associated with some disease processes, such as rheumatoid arthritis [6], pain [7,8], and cancer [9] development. In the search for understanding the role of P2 receptors in these diseases, selective agonistic and antagonistic ligands have been designed and used as therapeutic agents and pharmacological tools [10]. In this context, many effective ligands have been found, but selective targeting of some receptor subtypes still remains elusive. This is the case for P2Y4, P2X2, and P2X5 receptors, where no such ligands have been discovered. This article focuses on some of the basic properties of these P2 receptors and the natural products that act on them as either agonists or antagonists. Herein, we discuss and give evidence of the large potential of natural products as possible modulators of P2X and P2Y receptors.

2. The P2—ATP Activated Receptors

Since the 1970s, it has been clearly demonstrated that ATP and other analogues are extracellular messengers, acting on purinergic receptors. ATP can be released without cell lysis by specific mechanisms such as: exocytosis, ABC transporters, and membrane channels (e.g., pannexins, connexins, maxi-anion channel, and others) [11,12]. Once in the extracellular fluid, nucleotides can activate the P2X and P2Y families of purinergic receptors. The P2X group comprises of seven inotropic receptors found in mammalian cells: P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, and P2X7. The P2Y family is composed of metabotropic receptors, having eight subtypes in mammalian cells: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14. The P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors are coupled to Gq, activating phospholipase C-β. The P2Y12, P2Y13, and P2Y14 receptors are coupled to Gi, inhibiting adenylyl cyclase. The P2Y11 receptor has the unique property of coupling with both Gq and Gs.

In general, the P2X receptors are more structurally restrictive than P2Y in agonist selectivity. There is a cross-reactivity of P2Y receptor probes with P2X receptors, meaning that some of these probes are

not altogether selective. More specifically, ATP acts as the active ligand for P2X receptors, whereas in addition to ATP, P2Y receptors respond to naturally occurring nucleotides such as UDP, ADP, UTP, and UDP glucose. Among P2X receptors, subtypes P2X2, P2X4, and P2X7, in the presence of millimolar ATP concentrations and prolonged activation time, form a nonselective pore with a cut of up to 900 Da, depending on cellular type and specie analyzed [13]. So far, it is an open question whether these ion channels dilate or activate another protein responsible for high conductance channels to allow the passage of small molecules, such as Lucifer yellow, Yo-pro, and propidium iodide. Up until now, there have not been specific compounds to discriminate between low and high conductance channels [13,14].

So far, less than one hundred natural products have been identified that act on P2 receptors. They are divided by species and classified as being of animal, plant, or microorganism origin as described in next sections.

3. Natural Products from Plant Sources Acting on P2 Receptors

Among all types of natural products, plant extracts have received the greatest attention until now. In this section, we describe and discuss plant extracts that act by modulating the activity of P2X and P2Y receptors. As can be seen in Table 1, there are three purified compounds and seven crude extracts derived from from plants. As their number is small, we will provide a more detailed description of their effects.

Table 1. Natural products from plant sources.

Compound	Receptor Type	Effects (IC_{50}/EC_{50}) *	Tested Model	Reference
Mustard oil	P2X3	Participation in sensitization in nociceptive neurons following MO application to the tooth pulp. ND	Male Sprague-Dawley adult rats	[15]
Sodium Ferulate	P2X3	Decreases participation of these receptors in pain after primary sensory afferent chronic injury ND	Rat dorsal root ganglion	[16]
Tetramethylpyrazine	P2X3	Inhibition of depolarization, burn injury pain and neuropathic pain induced by α,β-methylene-ATP ND	Rat dorsal root ganglion	[17–20]
Puerarin	P2X3	Impairment of neuropathic pain ND	Dorsal root ganglion neurons	[21,22]
Emodin	P2X2/3	Inhibition of the transmission of neuropathic pain stimuli ND	Sprague-Dawley male rats	[23]
Emodin	P2X7	Inhibits ATP/BzATP-activated P2X7 receptor IC_{50} = 200 nM (cell death)	Rat peritoneal macrophages	[24]
Emodin	P2X7	Inhibits ATP/BzATP-activated P2X7 receptor IC_{50} = 500 nM (BzATP- and induced dye uptake)	Rat peritoneal macrophages	[24]
Emodin	P2X7	Inhibits ATP/BzATP-activated P2X7 receptor IC_{50} = 3.4 μM (BzATP-evoked current)	HEK 293	[24]

Table 1. *Cont.*

Compound	Receptor Type	Effects (IC_{50}/EC_{50}) *	Tested Model	Reference
Rheedia.longifolia methanol extract	P2X7	Inhibits P2X7 receptor-associated pore opening, currents and dye uptake functional assay IC_{50} = 2 μg/mL (functional assay)	Mouse peritoneal macrophages	[25]
Flavonoid molecules	P2Y2	Potent antagonism and inhibition of intracellular calcium release ND	NG108-15 cells	[26]
Trigonella foenum leaf extract	P2Y12 (?)	Inhibition of ADP-induced platelets aggregation IC_{50} = 1.28 mg/mL	Rabbit platelets	[27]
Trigonella foenum leaf extract	P2X	Inhibits α,β-methylene-ATP (30 μM) induced isometric contraction IC_{50} = 1.57 mg/mL	Mouse vas deferens	[27]
Colchicine	P2X7	Inhibits P2X7 receptor-associated pore opening EC_{50} = 290 μM	Xenopus laevis oocytes	[28]
Colchicine	P2X7	Inhibits P2X7 receptor-associated pore opening EC_{50} = 540 μM	Peritoneal mouse macrophages	[28]

* EC_{50} = half maximal effective concentration; IC_{50} = half maximal inhibitory concentration; ND = Not Determinated; MO = Mustard oil; BzATP = 3'-*O*-(4-benzoyl)benzoyl adenosine 5'-triphosphate.

P2X2/3 (heterometric receptor) and P2X3 receptors in trigeminal subnucleus caudalis are involved in the initiation and maintenance of central sensitization in subnucleus oralis nociceptive neurons induced by mustard oil application to the tooth pulp in anesthetized rats, this effect is possible associated with the neuroplastic changes in receptors NMDA (*N*-methyl-D-aspartate receptors) [15]. In chronic pain mediated by P2X3 receptors, sodium ferulate, an active principle from Chinese herbal medicine with anti-inflammatory activities, inhibited the nociceptive facilitation of the primary sensory afferent neurons after chronic constriction injury [16,29]. In this context, there are several papers describing the effects of Chinese herbal medicines on P2X3 and P2X2/3 receptors on reducing pain. The ligustrazine alkaloid tetramethylpyrazine has been studied with analgesic purposes in the context of nociceptive responses [17,18], burn injury pain [19], and neuropathic pain [20] induced by α,β-methylene-ATP. The burn injury pain transmission mediated by P2X3 receptor may be reduced by puerarin, which is one of the three major isoflavonoid compounds and has been widely used in treatment of myocardial and cerebral ischemia [30]. Puerarin may also impair the neuropathic pain mediated by P2X3 receptor in dorsal root ganglion neurons [22].

In 2004, Shemon observed that chelerythrine, a benzophenanthridine alkaloid, blocks the ATP-induced cation fluxes mediated by the P2X7 receptor, as well as the ATP induced stimulation of phospholipase D in human B lymphocytes [31]. Said, in 2007, described the *in vitro* and *in vivo* toxicity of four vegetable oils compared to castor oil to validate their use as vehicles of lipophilic drugs in eye drops [32]. P2X7 receptor activation was a parameter analyzed to assess the cytotoxicity induced by these oils. They observed that only castor oil promoted P2X7 receptor activation. In addition, Coutinho-Silva's group investigated the effect of mineral oil and thioglycolate, substances capable of recruiting macrophages into mouse peritoneal cavity, on P2X7 receptor expression and function. They found that mineral oil induced P2X7 down regulation associated with reduced

functional activity of this receptor [33]. In the paper published by Liu and colleagues in 2010, the authors studied the anti-inflammatory and immunosuppressive mechanisms of emodin (1,3,8-trihydroxy-6-methylanthraquinone), an anthraquinone derivative from *Rheum officinale Baill*. The P2X7 receptor activities induced by ATP or BzATP were inhibited by emodin pre-treatment in native macrophages or transfected HEK-293 cells with P2X7R [24]. Interestingly, emodin was able to impair P2X2/3 receptor role in transmission of neuropathic pain stimuli of primary sensory neurons in Sprague-Dawley rats [23]. Santos and coworkers have demonstrated that the extract and fractions of *Rheedia longifolia* inhibited the P2X7 receptor-induced dye uptake and ionic currents. After chromatography analysis, they identified the bisflavonoids as the most probable active compounds responsible for P2X7 inhibitory effects present in the *R. longifolia* extract and fractions [25].

The *Colchicum* sp. secondary metabolite colchicine is traditionally associated with gout treatment because of its ability to disturb cytoskeletal microfilaments, inhibiting inflammatory cell activation. Marques-da-Silva and colleagues showed that *in vitro* dolchicine was able to inhibit pore opening, but not the P2X7 receptor low-conductance channel. Furthermore, dolchicine also diminished the maturation and release of IL-1β and production of nitric oxide and reactive oxygen species induced by ATP. Interestingly, these effects were specific to dolchicine and were not found with other mitotic inhibitors, such as taxol and vincristine [28].

In relation to P2Y receptors, Mendes and colleagues, in 2003, observed P2Y1 or P2Y2 receptors' participation in the mechanism of the vascular relaxation produced by polyphenolic substances from red wine [34]. Kaulich and colleagues evaluated a series of 40 flavonoids as antagonists at P2Y2 receptors expressed in NG108-15 cells. By measuring the inhibition of UTP-stimulated intracellular calcium release, they identified diverse flavonoids as potent antagonists at P2Y2 receptors, with IC_{50} values in the low micromolar range and potency similar or higher than the standard P2Y2 antagonists Reactive Blue 2 and Suramin [26]. Polyphenolic compounds extracted from *Aronia melanocarpa* fruits have been reported to be cardioprotective agents. The Luzak group examined the ability of *Aronia melanocarpa* extract to increase the efficacy of human umbilical vein endothelial cells to inhibit platelet functions *in vitro*. They observed that only at low concentrations (5 μg/mL) did *Aronia melanocarpa* extract significantly improve antiplatelet action of human umbilical vein endothelial cells towards ADP-activated platelets in the aggregation test [35]. In another work, ADP-activated platelet aggregation was inhibited by ethyl acetate extract from *Opuntia humifusa raf*. This extract inhibited ADP-induced intracellular calcium mobilization and ATP release [36].

Parvizpur and collaborators observed that *Trigonella foenum* (TFG) leaf extract can exert analgesic effects in both formalin and tail flick tests [37,38]. In another paper [27], they studied the involvement of purinergic receptors in the formalin and tail flick tests. The TFG extract [0.5, 1, 1.5, 3 mg/mL] inhibited ADP [10^{-5} mol] induced platelet aggregation [IC_{50} = 1.28 mg/mL]. α,β-methylene-ATP [30 mM] induced isometric contraction in the vas deferens was inhibited by Suramin, a P2 receptor antagonist or TFG extract [IC_{50} were 91.07 μM and 1.57 mg/mL, respectively].

4. Natural Products from Animal Sources Acting on P2 Receptors

Animal sources of natural products have received less attention in the search for discovering new medicines. Nevertheless, new medicines, most of which are of invertebrate origin, have been approved

for human use. According to Table 2, there are sixteen purified compounds from animal origin, which are discussed below.

Table 2. Natural products from animal sources.

Compound	Receptor Type	Source	Effects (IC_{50}/EC_{50}) *	Tested Model	Reference
Halistanol sulfate	P2Y12	*Topsentia* sp.	Binds to P2Y12 receptor IC_{50} = 0.48 μM	1321N cells	[39]
Sterol sulfate Sch 572423			Binds to P2Y12 receptor IC_{50} = 2.2 μM	1321N cells	[39]
Iso-iantheran-A	P2Y11	*Ianthella quadrangulata*	Activates P2Y11 receptor EC_{50} = 1.29 μM	1321N1 wild-type cells	[40]
Iso-iantheran-B	P2Y11	*Ianthella quadrangulata*	Activates P2Y11 receptor EC_{50} = 0.48 μM	1321N1 wild-type cells	[40]
Stylissadines A	P2X7	*Stylissa flabellata*	Inhibits BzATP-induced pore formation IC_{50} = 0.7 μM	THP-1 cells. (Human Monocytes)	[41]
Stylissadines B			Inhibits BzATP-induced pore formation IC_{50} = 1.8 μM	THP-1 cells	[41]
Niphatoxin C	P2X7	*Callyspongia* sp.	Impairment of P2X7 receptor activity ND	THP-1 cells	[42]
LL37	P2X7	Human neutrophils and epithelial cells	Induces IL-1β maturation and release in LPS-primed monocytes ND	Human monocytes	[43]
rCRAMP	P2Y	*Ratus novergicus*	Induction of IL-6 expression and ERK 1/2 in glial cells, blocked by P2Y receptor antagonists ND	Rat glial cells	[44]
CRAMP	P2X7	*Mus musculus*	Inhibition of all responses related to P2X7 activation ND	Peritoneal macrophages	[45]
Cellular prion protein	P2X4	*Homo sapiens*	Prevents and reverses Copper-inhibited ATP-evoked current EC_{50} = 4.6 μM	*Xenopus laevis* oocyte	[46]
Melittin	P2X2/3 and P2X3	Apitoxin (bee venom)	Antagonists of both receptor suppressed melittin-evoked persistent spontaneous nociception ND	Male Sprague-Dawley albino rats weighing 180–250 g	[47]
Alphadefensin 1–3	P2Y6	Human $CD14^-/CD24^+$ cells	Inhibits M-CSF-induced differentiation of $CD14^-/CD24^+$ cells through P2Y6 receptor ND	$CD14^+/CD24^-$ monocytes human cells	[48]
Ω-Conotoxin GVIA	P2X2/3	*Conus* sp.	Inhibits P2X2/3 receptor response IC_{50} = 3.84 μM	Rat dorsal root ganglion neurons	[49]
	P2X3		Inhibits P2X3 receptor response IC_{50} = 21.2 nM	Rat dorsal root ganglion neurons	[49]
Purotoxin-1	P2X	*Lycosa* spider	Inhibition of ionic currents in the sensory neurons of rats ND	Rat dorsal root ganglion neurons	[50]
Purotoxin-1	P2X3	*Geolycosa* sp. spider venom	Potent inhibitory effects ND	Sensory neurons	[51]

* EC_{50} = half maximal effective concentration; IC_{50} = half maximal inhibitory concentration; ND = Not Determinated.

Several research groups consider marine organisms as a reliable source of new drugs. On par with this idea, a diverse array of compounds obtained from marine animals are under clinical trials [52]. Bioassay-guided fractionation of an active fraction of the marine sponge *Topsentia* sp. (*Halichondriidae*) obtained from a marine fraction library led to the isolation and identification of halistanol sulfate and of a new sterol sulfate compound, denominated Sch 572423. Both compounds inhibited the P2Y12 receptor [39]. Greve and collaborators isolated three new iantherans (iso-iantheran A, 8-carboxy-iso-iantheran A, and iso-iantheran B) composed of a rare dimeric benzofuran skeleton, including a 2,3-dihydroxy-1,3-butadiene disulfate moiety, obtained from the marine sponge, *Ianthella quadrangulata*. Biological assays demonstrated an agonist effect of iso-iantheran-A and iso-iantheran-B on P2Y11 receptors with EC_{50} values of 1.29 μM and 0.48 μM, respectively [40].

In 2007, Buchanan and colleagues [41] published three papers on P2X7 receptor function, based on a natural product high throughput screening effort to discover selective P2X7 receptor antagonists using marine sponge derivatives. Initially, they observed that the Australian marine sponge *Stylissa flabellata* was related to the blockage on the cationic current trough the P2X7 receptors by benzophrenatridins alkaloids (stylissadines A and B). This action was not selective however, and they noted the inhibition of some enzymes by this phytochemistry compound, such as protein kinase C and alanine aminotransferase. Both compounds inhibited P2X7 receptor function with IC_{50} values of 0.7 μM and 1.8 μM, respectively [41]. In another paper, these authors used the Australian marine sponge *Callyspongia sp*. (*Callyspongiidae*) and isolated the bioactive constituents the Niphatoxin C, which belongs to the 3-alkylpyridinium class of alkaloids. This constituent impaired the P2X7 receptor activity on THP-1 cells [42].

Melittin is the principal active component of apitoxin (bee venom) and is a powerful stimulator of phospholipase A2. The subcutaneous injection of melittin could induce persistent spontaneous nociception and primary thermal or mechanical hyperalgesia, but the exact peripheral mechanisms remain unclear. Post-treatment of the primary injury site with subcutaneous injection of A-317491 (P2X3 and P2X2/3 receptor antagonist) and Reactive Blue 2 (general P2Y receptor antagonist) suppressed the melittin-evoked persistent spontaneous nociception and hypersensitivity (thermal and mechanical) responses [47].

Grishin and colleagues demonstrated that the spider venom purotoxin-1 can inhibit P2X3 receptor function and delay the recovery rate from its desensitization [51]. Moreover, this 35-amino acid single-chain peptide also down regulates primary afferent sensory neurons leading to antinociceptive states with attractive 12 nM concentration. It is noteworthy that the concentration of purotoxin-1 was 3-fold lower than the P2X3 and P2X2/3 receptor antagonist A-317491, which encourages development of novel pain killer drugs. In 2009, Savchenko and colleagues studied the modulatory effect of peptide compounds of *Lycosa* spider venom on the ionic currents in the sensory neurons of rats through P2X receptors in rat dorsal root ganglion neurons [50].

Antimicrobial peptides (also called host defense peptides) are an evolutionarily conserved component of the innate immune response and are found among all classes of organisms. In general, these peptides are potent, broad-spectrum antibiotics, but in some cases, as we describe below, these peptides may modulate the ionic channels.

The human cathelicidin-derived peptide LL37 is a potent antimicrobial peptide produced predominantly by neutrophils and epithelial cells. LPS-primed monocytes stimulated with LL37 lead

to the maturation and release of interleukin-1beta (IL-1beta) via the P2X7 receptor. IL-1beta release and cell permeability were suppressed by pretreatment with the P2X7 receptor inhibitors oxidized ATP, KN04, and KN62 [43]. LPS-primed monocytes, stimulated with LL37, resulted in P2X7 receptor maturation and release of IL-beta.

Another peptide, the antibacterial cathelicidin rCRAMP (homologue of the human LL-37), not only exhibits potent bactericidal activities in rats, but also functions as a chemoattractant for immune cells. Brandenburg and colleagues [44] showed that rCRAMP-induced IL-6 expression and ERK1/2 phosphorylation in glial cells. This effect might be mediated by P2Y11 and was not mediated by P2X receptors since those that block the P2X receptors did not affect the production of IL-6. On the other hand, Seil and collaborates described that CRAMP, also in mice, inhibited all the responses coupled to P2X7 receptors in macrophages [45].

Another family of peptides denominated as conotoxins, which belong to a group of neurotoxic peptides isolated from the venom of the marine cone snail, genus *Conus*, was found to modulate several types of ionic channels, including N-type calcium channels, which selectively act in the ascending pain pathway [53,54]. Extracted from *Conus* snails, Ω-conotoxin GVIA inhibits P2X3 and P2X2/3 receptor-mediated responses with IC_{50} values of 21.2 nM and 3.84 μM, respectively [49].

The human alpha-defensin 1–3, which is a small arginine-rich peptide, participates in the host immune defense, and is secreted by $CD14^{-}/CD24^{+}$ cells. This peptide has been shown to inhibit macrophage-colony stimulating factor induced differentiation of $CD14^{-}/CD24^{+}$ cells, at least in part through P2Y6, a receptor involved in macrophage differentiation [48].

Cellular prion protein physiological function remains unknown, but there is evidence supporting its role in copper homeostasis. Lorca and his group have shown that the perfusion of this domain prevents and reverses the inhibition by Cu^{2+} of ATP-evoked currents of the P2X4 receptor subtype, highlighting a modulatory role for cellular prion protein in synaptic transmission through regulation of Cu^{2+} levels [46].

5. Natural Products from Microorganisms Acting on P2 Receptors

Substances produced by microorganisms that modulate tissues, cell types, and protein function, have long been known as shown in Table 3. To this effect, ivermectin, a semisynthetic derivative of the natural fermentation products of *Streptomyces avermitilis*, is widely used in human and veterinary medicine as an antiparasitic agent [55]. In 1999, Khakh showed that ivermectin is a specific positive allosteric effector of heterologously expressed P2X4, but not of P2X2, P2X3, P2X2/3, or P2X7 receptor channels in rats [56]. This result was confirmed by other groups in humans [57], mice [58], and rats [59]. Recently, however, Nörenberg *et al*. 2012 [60] described a positive allosteric effector of ivermectin also on human P2X7 receptor.

The natural peptide polymyxin B is a well-known and potent antibiotic that binds and neutralizes bacterial endotoxin lipopolysaccharide. Ferrari demonstrated that polymyxin B increased the responses mediated by the P2X7 receptor in HEK293 and K562 cell lines transfected with P2X7 receptor cDNA, as well as in mouse and human macrophages [61].

Table 3. Natural products from microorganism product sources.

Compound	Receptor Type	Source	Effects (IC_{50}/EC_{50}) *	Tested Model	Reference
Ivermectin	P2X4	*Streptomyces avermitilis*	Positive allosteric effect EC_{50} = 250 nM	*Xenopus laevis* oocyte	[56]
Ivermectin	P2X4	*Streptomyces avermitilis*	Blockage of ethanol-inhibitory effects ND	*Xenopus laevis* oocyte	[62]
Ivermectin	P2X7	*Streptomyces avermitilis*	Positive allosteric effect EC_{50} = 50 nM (EC_{50} from high affinity-binding site)	Macrophage from humans	[60]
Polymyxin B	P2X7	*Bacillus polymyxa*	Enhanced P2X7 responses in transfected-HEK293 and K562 cells ND	Mouse and human macrophage cells	[61]
Pfiesteriatoxin	P2X7	*Pfiesteriapiscicida*	Activation of cell permeabilization similarly to ATP activation ND	GH4C1 rat pituitary cells	[63]
Pfiesteriatoxin	P2X7	*Pfiesteriapiscicida*	Induction of toxic and c-*fos* luciferase that is blocked by oxATP and PPADS ND	GH4C1 rat pituitary cells	[64]
HlyA	P2X1 and P2X7	*Escherichia coli*	Antagonists of both receptor blocked HlyA induced hemolysis ND	Human, mouse and equine Erythrocytes	[65]
Cytotoxic factors	P2X7	*Pseudomonas aeruginosastrain 808*	P2X7 receptor participation in ATP-dependent pathway ND	J774 macrophage cell line	[66]
Leukotoxin	P2X7	*Aggregatibacter actinomycetemcomitans*	Leukotoxin-induced proinflammatory responses, release of IL-1β and IL-18 are blocked by oxATP ND	Human macrophages	[67]
Oxo-AHL	P2Y2 and P2Y4	*Pseudomonasaeruginosa*	Inhibits P2Y2 and P2Y4 expression in cystic fibrosis IC_{50} = 0.3 pM	HTGS cell line MM39	[68]
LPS	P2X7	Gram-negative bacteria	P2X7 receptor modulates LPS-induced responses ND	Murine Peritoneal Macrophages	[69]
LPS	P2X7	Gram-negative bacteria	P2X7 receptor inhibition in TLR-4-defficient cell ND	HEK293 cells	[70]
LPS	P2Y6	Gram-negative bacteria	Vascular inflammation following selective induction of endothelial P2Y6 receptor ND	HMEC-1	[71]
LOS	P2X	Gram-negative bacteria	Inhibition of P2X receptor decrease LOS-induced caspase-8 activation and apoptosis ND	Primary bovine pulmonary artery endothelial cells	[72]

* EC_{50} = half maximal effective concentration; IC_{50} = half maximal inhibitory concentration; ND = Not Determinated; LOS = Lipooligosaccharide.

Pfiesteria piscicida is a dinoflagellate that produces the putative bioactive substance *Pfiesteria* toxin, which displays toxicity in fishes and humans. This substance induced cell permeabilization in a similar manner as ATP in culture of GH4C1 rat pituitary cells expressing functional P2X7 receptors.

This effect was inhibited by the P2X7 receptor antagonist oxidized ATP [63]. In addition, Kimm-Brinson examined the pharmacological activity of the *Pfiesteria* toxin on the signaling pathway that induces the *c-fos* luciferase construct in GH4C1 rat pituitary cells. ATP-, BZATP-, and *Pfiesteria* toxin-induced cytotoxicity, and *c-fos* luciferase activity was inhibited by pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid and the P2X7 irreversible antagonist oxidized-ATP [64].

Escherichia coli, which exhibits facultative and invasive strains, is the dominant facultative bacterium in the normal intestinal flora, but it is also responsible for the majority of serious extraintestinal infections. Alpha-hemolysin (HlyA) is a virulence factor produced by invasive *E. coli* strains, which causes hemolysis by forming pores in the erythrocyte membrane and triggers purinergic receptor activation to mediate the full hemolytic action. General P2 antagonists (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid and suramin) and ATP scavengers (apyrase, hexokinase) inhibited HlyA-induced lysis of equine, murine, and human erythrocytes. P2X1 and P2X7 receptors antagonists indicated both receptors to be involved in the HlyA-induced hemolysis [65].

Cytotoxic factors released by a nonmucoid clinical isolate of *Pseudomonas aeruginosa*, strain 808, produced ATP-dependent and -independent responses. The ATP dependent pathway utilizes the P2X7 receptor to exert its effects, in contrast to the ATP-independent pathway [66].

Aggregatibacter (Actinobacillus) actinomycetemcomitans is a facultative anaerobic Gram-negative bacterium associated with severe forms of periodontitis and several virulence factors. Among those, the leukotoxin is suggested to have an important role in its pathogenicity [73,74]. This toxin is able to lyse human immune cells and induces significant secretion of the pro-inflamatory cytokines IL-1 and IL-18 in human macrophages [67,75]. This pro-inflammatory response was inhibited by oxidized ATP, which indicates the involvement of the P2X7 receptor [67].

Cystic fibrosis is a genetic disease characterized by the hypersecretion of mucus, inflammation in the airways, and especially by persistent severe bacterial infections, generally by the gram-negative bacterium *Pseudomonas aeruginosa* [76]. ATP or UTP analogues were shown to induce chloride secretion by cystic fibrosis epithelial cells [77] and also to induce bronchial relaxation [78]. *P. aeruginosa* virulence factors can be regulated by two unique quorum sensing systems [79] composed by a small diffusible signal molecule (*N*-acylhomoserine lactone [AHL]) and a transcriptional activator protein [80]. In this work, the authors tested the effects of AHLs on HTGS cells regarding its action upon P2 receptors. Oxo-AHLs treatments repressed the stimulatory effects of secretory leukocyte proteinase inhibitor secretion by nucleotides, possibly due to the repression of P2Y2 and P2Y4 receptor expression, in cystic fibrosis but not normal HTGS cells [68].

Components of gram-negative bacteria have been studied in the context of the purinergic signaling in inflammation processes that promote an increase of nucleotide concentrations. The bacterial endotoxin LPS is one of the strongest stimuli for the immune response, and several papers have shown that P2X7 receptor activation can modulate LPS-induced responses [69,81]. Interestingly, a binding site for LPS within the P2X7 receptor C-terminus has been proposed. Accordingly, Leiva-Salcedo and colleagues investigated if LPS can directly modulate the activity of the P2X7 receptor. They found that LPS alone was unable to induce any P2X7 receptor-related activity, suggesting that the receptor is not directly activated by the endotoxin. On the other hand, pre-application of LPS inhibited P2X7 receptor ionic channel and pore function in HEK293 cells [70]. Compellingly, another bacterial product lipooligosacharide also is able to promote the release of ATP and its derivatives, which can enhance

the processes of cell activation and apoptosis. These lipid A-containing compounds stimulate endothelial cells to take part in several inflammatory steps, such as production and release of reactive oxygen species, cytokine, and again, the "universal" danger-associated molecule pattern, ATP [72].

Some microorganisms, such as yeast and bacteria, are capable of producing alcohols; as such, the effect of short alcohols was described [82,83]. ATP-activated ionic currents in presynaptic and postsynaptic membranes, due to P2X4 and P2X2 receptor activation, were reduced by ethanol treatment [83]. In 2011, Ostrovskaya demonstrated that ethanol inhibits ATP-gated currents mediated by P2X4 receptor in a rapid manner, and the inhibition does not depend on voltage or ATP concentration [84].

In another paper, Asatryan showed that ivermectin may reverse the inhibitory effects of ethanol in P2X4 receptor in dose-dependent manner [62]. Fischer and collaborates demonstrated that P2X3, a receptor widely expressed in dorsal root ganglion neurons, seems to be distinctly modulated by 2,2,2-thicloroethanol. As compared to ethanol, 2,2,2-thicloroethanol inhibited current responses and intracellular calcium elevation of ATP and its analogue α,β-methylene-ATP [85]. Davies, in 2005, transfected the hP2X3 and hP2X4 receptors to *Xenopus* oocytes and studied the ionic current after ATP treatment. Ethanol reduced P2X4 receptor responses and increased the maximal response of P2X3 receptor ionic currents [86].

During a systemic inflammatory response, endothelial-expressed surface molecules have been strongly implicated in regulating immune responses. Based on previous studies about exhibiting enhanced extracellular nucleotide released during acute inflammation, Riegel postulated that endothelial nucleotide receptors could play a role in vascular inflammation. Pharmacologic or genetic *in vivo* studies showed attenuated inflammatory responses in *P2Y6*$^{-/-}$ mice or after P2Y6 antagonist treatment during LPS-induced vascular inflammation [71].

6. Conclusions

The association of diseases with expression of purinergic receptors should prompt further search for pharmacological compounds with selective action on P2X and P2Y receptors. Accordingly, the results described here revealed that several promising natural products present significant agonist or antagonist activities for P2 receptors. A better understanding of the role of P2 receptors in physiological and pathological processes will be a key element in the discovery of new medicines for several pathological conditions such as cancer, rheumatoid arthritis, and pain.

Acknowledgments

This work was supported by grants from FAPERJ, CNPq, PAPES V/Fiocruz-CNPq, and Oswaldo Cruz Institute. We are grateful to Vinicius Cotta de Almeida for helpful discussions and suggestions.

References

1. Musselman, L. *Encyclopedia of Common Natural Ingredients Used in Food, Drugs, and Cosmetics*; John Wiley & Sons: New York, NY, USA, 1996; p. 422.

2. Butler, M.S. The role of natural product chemistry in drug discovery. *J. Nat. Prod.* **2004**, *67*, 2141–2153.
3. Ley, S.V.; Baxendale, I.R. New tools and concepts for modern organic synthesis. *Nat. Rev. Drug Discov.* **2002**, *1*, 573–586.
4. Geysen, H.M.; Schoenen, F.; Wagner, D.; Wagner, R. Combinatorial compound libraries for drug discovery: an ongoing challenge. *Nat. Rev. Drug Discov.* **2003**, *2*, 222–230.
5. Lombardino, J.G.; Lowe, J.A. The role of the medicinal chemist in drug discovery—Then and now. *Nat. Rev. Drug Discov.* **2004**, *3*, 853–862.
6. Portales-Cervantes, L.; Niño-Moreno, P.; Doníz-Padilla, L.; Baranda-Candido, L.; García-Hernández, M.; Salgado-Bustamante, M.; González-Amaro, R.; Portales-Pérez, D. Expression and function of the P2X(7) purinergic receptor in patients with systemic lupus erythematosus and rheumatoid arthritis. *Hum. Immunol.* **2010**, *71*, 818–825.
7. Inoue, K. P2 receptors and chronic pain. *Purinergic Signal.* **2007**, *3*, 135–144.
8. Tsuda, M.; Tozaki-Saitoh, H.; Inoue, K. Pain and purinergic signaling. *Brain Res. Rev.* **2010**, *63*, 222–232.
9. White, N.; Burnstock, G. P2 receptors and cancer. *Trends Pharmacol. Sci.* **2006**, *27*, 211–217.
10. Guile, S.D.; Alcaraz, L.; Birkinshaw, T.N.; Bowers, K.C.; Ebden, M.R.; Furber, M.; Stocks, M.J. Antagonists of the P2X(7) receptor. From lead identification to drug development. *J. Med. Chem.* **2009**, *52*, 3123–3141.
11. Boeynaems, J.M.; Communi, D.; Gonzalez, N.S.; Robaye, B. Overview of the P2 receptors. *Semin. Thromb. Hemost.* **2005**, *31*, 139–149.
12. Lazarowski, E.R.; Boucher, R.C.; Harden, T.K. Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol. Pharmacol.* **2003**, *64*, 785–795.
13. Virginio, C.; MacKenzie, A.; Rassendren, F.A.; North, R.A.; Surprenant, A. Pore dilation of neuronal P2X receptor channels. *Nat. Neurosci.* **1999**, *2*, 315–321.
14. Faria, R.X.; Defarias, F.P.; Alves, L.A. Are second messengers crucial for opening the pore associated with P2X7 receptor? *Am. J. Physiol. Cell Physiol.* **2005**, *288*, C260–C271.
15. Hu, B.; Chiang, C.Y.; Hu, J.W.; Dostrovsky, J.O.; Sessle, B.J. P2X receptors in trigeminal subnucleus caudalis modulate central sensitization in trigeminal subnucleus oralis. *J. Neurophysiol.* **2002**, *88*, 1614–1624.
16. Zhang, A.; Gao, Y.; Zhong, X.; Xu, C.; Li, G.; Liu, S.; Lin, J.; Li, X.; Zhang, Y.; Liu, H.; *et al*. Effect of sodium ferulate on the hyperalgesia mediated by P2X3 receptor in the neuropathic pain rats. *Brain Res.* **2010**, *1313*, 215–221.
17. Liang, S.D.; Gao, Y.; Xu, C.S.; Xu, B.H.; Mu, S.N. Effect of tetramethylpyrazine on acute nociception mediated by signaling of P2X receptor activation in rat. *Brain Res.* **2004**, *995*, 247–252.
18. Liang, S.D.; Xu, C.S.; Zhou, T.; Liu, H.Q.; Gao, Y.; Li, G. L. Tetramethylpyrazine inhibits ATP-activated currents in rat dorsal root ganglion neurons. *Brain Res.* **2005**, *1040*, 92–97.
19. Gao, Y.; Xu, C.; Yu, K.; Li, G.; Wan, F.; Liu, S.; Lin, J.; Liu, H.; Zhang, J.; Li, X.; *et al*. Effect of tetramethylpyrazine on DRG neuron P2X3 receptor involved in transmitting pain after burn. *Burns* **2010**, *36*, 127–134.

20. Gao, Y.; Liang, S.D.; Shao, L.J.; Mu, S.N.; Xu, C.S.; Zhang, C.P. Effect of tetramethylpyrazine on neuropathic pain mediated by P2X3 receptor. *Acta Pharmacol. Sin.* **2006**, *77*, 27–32.
21. Xu, C.; Li, G.; Gao, Y.; Liu, S.; Lin, J.; Zhang, J.; Li, X.; Liu, H.; Liang, S. Effect of puerarin on P2X3 receptor involved in hyperalgesia after burn injury in the rat. *Brain Res. Bull.* **2009**, *80*, 341–346.
22. Xu, C.; Xu, W.; Xu, H.; Xiong, W.; Gao, Y.; Li, G.; Liu, S.; Xie, J.; Tu, G.; Peng, H.; *et al*. Role of puerarin in the signalling of neuropathic pain mediated by P2X3 receptor of dorsal root ganglion neurons. *Brain Res. Bull.* **2012**, *87*, 37–43.
23. Gao, Y.; Liu, H.; Deng, L.; Zhu, G.; Xu, C.; Li, G.; Liu, S.; Xie, J.; Liu, J.; Kong, F.; *et al*. Effect of emodin on neuropathic pain transmission mediated by P2X2/3 receptor of primary sensory neurons. *Brain Res. Bull.* **2011**, *84*, 406–413.
24. Liu, L.; Zou, J.; Liu, X.; Jiang, L.H.; Li, J. Inhibition of ATP-induced macrophage death by emodin via antagonizing P2X7 receptor. *Eur. J. Pharmacol.* **2010**, *640*, 15–19.
25. Santos, J.A.; Fidalgo-Neto, A.A.; Faria, R.X.; Simões, A.; Calheiros, A.S.; Bérenger, A.L.; Faria-Neto, H.C.; Figueiredo, M.R.; Frutuoso, V.S.; Alves, L.A. Effect of Rheedia longifolia leaf extract and fractions on the $P2X_7$ receptor in vitro: novel antagonists? *J. Med. Food* **2011**, *14*, 920–929.
26. Kaulich, M.; Streicher, F.; Mayer, R.; Müller, I.; Müller, C.E. Flavonoids—Bovel lead compounds for the development of P2Y2 receptor antagonists. **2003**, *59*, 72–81.
27. Parvizpur, A.; Ahmadiani, A.; Kamalinejad, M. Probable role of spinal purinoceptors in the analgesic effect of Trigonella foenum (TFG) leaves extract. *J. Ethnopharmacol.* **2006**, *104*, 108–112.
28. Marques-da-Silva, C.; Chaves, M.M.; Castro, N.G.; Coutinho-Silva, R.; Guimaraes, M.Z. Colchicine inhibits cationic dye uptake induced by ATP in P2X2 and P2X7 receptor-expressing cells: implications for its therapeutic action. *Br. J. Pharmacol.* **2011**, *163*, 912–926.
29. Zhang, A.; Xu, C.; Liang, S.; Gao, Y.; Li, G.; Wei, J.; Wan, F.; Liu, S.; Lin, J. Role of sodium ferulate in the nociceptive sensory facilitation of neuropathic pain injury mediated by P2X(3) receptor. *Neurochem. Int.* **2008**, *53*, 278–282.
30. Gao, Y.; Xu, C.; Liang, S.; Zhang, A.; Mu, S.; Wang, Y.; Wan, F. Effect of tetramethylpyrazine on primary afferent transmission mediated by P2X3 receptor in neuropathic pain states. *Brain Res. Bull.* **2008**, *77*, 27–32.
31. Shemon, A.N.; Sluyter, R.; Conigrave, A.D.; Wiley, J.S. Chelerythrine and other benzophenanthridine alkaloids block the human P2X7 receptor. *Br. J. Pharmacol.* **2004**, *142*, 1015–1019.
32. Said, T.; Dutot, M.; Christon, R.; Beaudeux, J.L.; Martin, C.; Warnet, J.M.; Rat, P. Benefits and side effects of different vegetable oil vectors on apoptosis, oxidative stress, and P2X7 cell death receptor activation. *Invest. Ophthalmol. Vis. Sci.* **2007**, *48*, 5000–5006.
33. Marques da Silva, C.; Miranda Rodrigues, L.; Passos da Silva Gomes, A.; Mantuano Barradas, M.; Sarmento Vieira, F.; Persechini, P.M.; Coutinho-Silva, R. Modulation of P2X7 receptor expression in macrophages from mineral oil-injected mice. *Immunobiology* **2008**, *213*, 481–492.
34. Mendes, A.; Desgranges, C.; Chèze, C.; Vercauteren, J.; Freslon, J.L. Vasorelaxant effects of grape polyphenols in rat isolated aorta. Possible involvement of a purinergic pathway. *Fundam. Clin. Pharmacol.* **2003**, *17*, 673–681.

35. Luzak, B.; Golanski, J.; Rozalski, M.; Krajewska, U.; Olas, B.; Watala, C. Extract from Aronia melanocarpa fruits potentiates the inhibition of platelet aggregation in the presence of endothelial cells. *Arch. Med. Sci.* **2010**, *6*, 141–144.
36. Oh, W.-J.; Endale, M.; Park, J.-Y.; Kwak, Y.-S.; Kim, S.; Kim, G.-S.; Rhee, M.H. The inhibitory effect of *Opuntia humifusa* Raf. Ethyl acetate extract on platelet aggregation. *J. Med. Plants Res.* **2011**, *5*, 1418–1424.
37. Ahmadiani, A.; Javan, M.; Semnanian, S.; Barat, E.; Kamalinejad, M. Anti-inflammatory and antipyretic effects of Trigonella foenum-graecum leaves extract in the rat. *J. Ethnopharmacol.* **2001**, *75*, 283–286.
38. Javan, M.; Ahmadiani, A.; Semnanian, S.; Kamalinejad, M. Antinociceptive effects of Trigonella foenum-graecum leaves extract. *J. Ethnopharmacol.* **1997**, *58*, 125–129.
39. Yang, S.W.; Buivich, A.; Chan, T.M.; Smith, M.; Lachowicz, J.; Pomponi, S.A.; Wright, A.E.; Mierzwa, R.; Patel, M.; Gullo, V.; *et al*. A new sterol sulfate, Sch 572423, from a marine sponge, Topsentia sp. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1791–1794.
40. Greve, H.; Meis, S.; Kassack, M.U.; Kehraus, S.; Krick, A.; Wright, A.D.; König, G.M. New iantherans from the marine sponge Ianthella quadrangulata: novel agonists of the P2Y(11) receptor. *J. Med. Chem.* **2007**, *50*, 5600–5607.
41. Buchanan, M.S.; Carroll, A.R.; Addepalli, R.; Avery, V.M.; Hooper, J.N.; Quinn, R.J. Natural products, stylissadines A and B, specific antagonists of the P2X7 receptor, an important inflammatory target. *J. Org. Chem.* **2007**, *72*, 2309–2317.
42. Buchanan, M.S.; Carroll, A.R.; Addepalli, R.; Avery, V.M.; Hooper, J.N.; Quinn, R.J. Niphatoxin C, a cytotoxic tripyridine alkaloid from Callyspongia sp. *J. Nat. Prod.* **2007**, *70*, 2040–2041.
43. Elssner, A.; Duncan, M.; Gavrilin, M.; Wewers, M.D. A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. *J. Immunol.* **2004**, *172*, 4987–4994.
44. Brandenburg, L.O.; Jansen, S.; Wruck, C.J.; Lucius, R.; Pufe, T. Antimicrobial peptide rCRAMP induced glial cell activation through P2Y receptor signalling pathways. *Mol. Immunol.* **2010**, *47*, 1905–1913.
45. Seil, M.; Kabré, E.; Nagant, C.; Vandenbranden, M.; Fontanils, U.; Marino, A.; Pochet, S.; Dehaye, J.P. Regulation by CRAMP of the responses of murine peritoneal macrophages to extracellular ATP. *Biochim. Biophys. Acta* **2010**, *1798*, 569–578.
46. Lorca, R.A.; Chacón, M.; Barría, M.I.; Inestrosa, N.C.; Huidobro-Toro, J.P. The human prion octarepeat fragment prevents and reverses the inhibitory action of copper in the P2X4 receptor without modifying the zinc action. *J. Neurochem.* **2003**, *85*, 709–716.
47. Lu, Z.M.; Xie, F.; Fu, H.; Liu, M.G.; Cao, F.L.; Hao, J.; Chen, J. Roles of peripheral P2X and P2Y receptors in the development of melittin-induced nociception and hypersensitivity. *Neurochem. Res.* **2008**, *33*, 2085–2091.
48. Droin, N.; Jacquel, A.; Hendra, J.B.; Racoeur, C.; Truntzer, C.; Pecqueur, D.; Benikhlef, N.; Ciudad, M.; Guery, L.; Jooste, V.; *et al*. Alpha-defensins secreted by dysplastic granulocytes inhibit the differentiation of monocytes in chronic myelomonocytic leukemia. *Blood* **2010**, *115*, 78–88.

49. Lalo, U.V.; Pankratov, Y.V.; Arndts, D.; Krishtal, O.A. Omega-conotoxin GVIA potently inhibits the currents mediated by P2X receptors in rat DRG neurons. *Brain Res. Bull.* **2001**, *54*, 507–512.
50. Savchenko, H.A.; Vasylevs'kyĭ, A.A.; Pluzhnykov, K.A.; Korol'kova, I.V.; Mamenko, M.V.; Volkova, T.M.; Maksymiuk, O.P.; Boĭchuk, I.A.; Hrishyn, I.V.; Kryshtal', O.O. Peptide components of Geolycosa spider venom modulate P2X receptor activity of rat sensory neurons. *Fiziol. Zh.* **2009**, *55*, 11–16.
51. Grishin, E.V.; Savchenko, G.A.; Vassilevski, A.A.; Korolkova, Y.V.; Boychuk, Y.A.; Viatchenko-Karpinski, V.Y.; Nadezhdin, K.D.; Arseniev, A.S.; Pluzhnikov, K.A.; Kulyk, V.B.; *et al*. Novel peptide from spider venom inhibits P2X3 receptors and inflammatory pain. *Ann. Neurol.* **2010**, *67*, 680–683.
52. Faulkner, D.J. Marine natural products. *Nat. Prod. Rep.* **2001**, *18*, 1–49.
53. Lewis, R.J.; Dutertre, S.; Vetter, I.; Christie, M.J. Conus venom peptide pharmacology. *Pharmacol. Rev.* **2012**, *64*, 259–298.
54. Lewis, R.J.; Nielsen, K.J.; Craik, D.J.; Loughnan, M.L.; Adams, D.A.; Sharpe, I.A.; Luchian, T.; Adams, D.J.; Bond, T.; Thomas, L.; *et al*. Novel omega-conotoxins from Conus catus discriminate among neuronal calcium channel subtypes. *J. Biol. Chem.* **2000**, *275*, 35335–35344.
55. Burkhart, C.N. Ivermectin: an assessment of its pharmacology, microbiology and safety. *Vet. Hum. Toxicol.* **2000**, *42*, 30–35.
56. Khakh, B.S.; Proctor, W.R.; Dunwiddie, T.V.; Labarca, C.; Lester, H.A. Allosteric control of gating and kinetics at P2X(4) receptor channels. *J. Neurosci.* **1999**, *19*, 7289–7299.
57. Priel, A.; Silberberg, S.D. Mechanism of ivermectin facilitation of human P2X4 receptor channels. *J. Gen. Physiol.* **2004**, *123*, 281–293.
58. Sim, J.A.; Park, C.K.; Oh, S.B.; Evans, R.J.; North, R.A. P2X1 and P2X4 receptor currents in mouse macrophages. *Br. J. Pharmacol.* **2007**, *152*, 1283–1290.
59. Bowler, J.W.; Bailey, R.J.; North, R.A.; Surprenant, A. P2X4, P2Y1 and P2Y2 receptors on rat alveolar macrophages. *Br. J. Pharmacol.* **2003**, *140*, 567–575.
60. Nörenberg, W.; Sobottka, H.; Hempel, C.; Plötz, T.; Fischer, W.; Schmalzing, G.; Schaefer, M. Positive allosteric modulation by ivermectin of human but not murine P2X7 receptors. *Br. J. Pharmacol.* **2012**, *167*, 48–66.
61. Ferrari, D.; Pizzirani, C.; Adinolfi, E.; Forchap, S.; Sitta, B.; Turchet, L.; Falzoni, S.; Minelli, M.; Baricordi, R.; di Virgilio, F. The antibiotic polymyxin B modulates P2X7 receptor function. *J. Immunol.* **2004**, *173*, 4652–4660.
62. Asatryan, L.; Popova, M.; Perkins, D.; Trudell, J.R.; Alkana, R.L.; Davies, D.L., Ivermectin antagonizes ethanol inhibition in purinergic P2X4 receptors. *J. Pharmacol. Exp. Ther.* **2010**, *334*, 720–728.
63. Melo, A.C.; Moeller, P.D.; Glasgow, H.; Burkholder, J.M.; Ramsdell, J.S. Microfluorimetric analysis of a purinergic receptor (P2X7) in GH4C1 rat pituitary cells: effects of a bioactive substance produced by Pfiesteria piscicida. *Environ. Health Perspect.* **2001**, *109 (Suppl 5)*, 731–737.
64. Kimm-Brinson, K.L.; Moeller, P.D.; Barbier, M.; Glasgow, H.; Burkholder, J.M.; Ramsdell, J.S. Identification of a P2X7 receptor in GH(4)C(1) rat pituitary cells: a potential target for a bioactive substance produced by Pfiesteria piscicida. *Environ. Health Perspect.* **2001**, *109*, 457–462.

65. Skals, M.; Jorgensen, N.R.; Leipziger, J.; Praetorius, H.A. Alpha-hemolysin from Escherichia coli uses endogenous amplification through P2X receptor activation to induce hemolysis. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 4030–4035.
66. Zaborina, O.; Dhiman, N.; Ling Chen, M.; Kostal, J.; Holder, I.A.; Chakrabarty, A.M. Secreted products of a nonmucoid Pseudomonas aeruginosa strain induce two modes of macrophage killing: external-ATP-dependent, P2Z-receptor-mediated necrosis and ATP-independent, caspase-mediated apoptosis. *Microbiology* **2000**, *146*, 2521–2530.
67. Kelk, P.; Abd, H.; Claesson, R.; Sandström, G.; Sjöstedt, A.; Johansson, A. Cellular and molecular response of human macrophages exposed to Aggregatibacter actinomycetemcomitans leukotoxin. *Cell Death Dis.* **2011**, *2*, e126.
68. Saleh, A.; Figarella, C.; Kammouni, W.; Marchand-Pinatel, S.; Lazdunski, A.; Tubul, A.; Brun, P.; Merten, M.D. Pseudomonas aeruginosa quorum-sensing signal molecule *N*-(3-oxododecanoyl)-L-homoserine lactone inhibits expression of P2Y receptors in cystic fibrosis tracheal gland cells. *Infect. Immun.* **1999**, *67*, 5076–5082.
69. Le Feuvre, R.A.; Brough, D.; Iwakura, Y.; Takeda, K.; Rothwell, N.J. Priming of macrophages with lipopolysaccharide potentiates P2X7-mediated cell death via a caspase-1-dependent mechanism, independently of cytokine production. *J. Biol. Chem.* **2002**, *277*, 3210–3218.
70. Leiva-Salcedo, E.; Coddou, C.; Rodríguez, F.E.; Penna, A.; Lopez, X.; Neira, T.; Fernández, R.; Imarai, M.; Rios, M.; Escobar, J.; *et al*. Lipopolysaccharide inhibits the channel activity of the P2X7 receptor. *Mediators Inflamm.* **2011**, *2011*, 152625.
71. Riegel, A.K.; Faigle, M.; Zug, S.; Rosenberger, P.; Robaye, B.; Boeynaems, J.M.; Idzko, M.; Eltzschig, H.K. Selective induction of endothelial P2Y6 nucleotide receptor promotes vascular inflammation. *Blood* **2011**, 117, 2548–2555.
72. Sylte, M.J.; Kuckleburg, C.J.; Inzana, T.J.; Bertics, P.J.; Czuprynski, C.J. Stimulation of P2X receptors enhances lipooligosaccharide-mediated apoptosis of endothelial cells. *J. Leukoc. Biol.* **2005**, *77*, 958–965.
73. Kachlany, S.C. Aggregatibacter actinomycetemcomitans leukotoxin: from threat to therapy. *J. Dent. Res.* **2010**, *89*, 561–570.
74. Guthmiller, J.M.; Lally, E.T.; Korostoff, J. Beyond the specific plaque hypothesis: Are highly leukotoxic strains of Actinobacillus actinomycetemcomitans a paradigm for periodontal pathogenesis? *Crit. Rev. Oral Biol. Med.* **2001**, *12*, 116–124.
75. Johansson, A.; Sandström, G.; Claesson, R.; Hänström, L.; Kalfas, S. Anaerobic neutrophil-dependent killing of Actinobacillus actinomycetemcomitans in relation to the bacterial leukotoxicity. *Eur. J. Oral Sci.* **2000**, *108*, 136–146.
76. Hata, J.S.; Fick, R.B. Pseudomonas aeruginosa and the airways disease of cystic fibrosis. *Clin. Chest Med.* **1988**, *9*, 679–689.
77. Knowles, M.R.; Clarke, L.L.; Boucher, R.C. Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis. *N. Engl. J. Med.* **1991**, *325*, 533–538.
78. Aksoy, M.O.; Kelsen, S.G. Relaxation of rabbit tracheal smooth muscle by adenine nucleotides: mediation by P2-purinoceptors. *Am. J. Respir. Cell Mol. Biol.* **1994**, *10*, 230–236.

79. Latifi, A.; Foglino, M.; Tanaka, K.; Williams, P.; Lazdunski, A. A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol. Microbiol.* **1996**, *21*, 1137–1146.
80. Swift, S.; Throup, J.P.; Williams, P.; Salmond, G.P.; Stewart, G.S. Quorum sensing: A population-density component in the determination of bacterial phenotype. *Trends Biochem. Sci.* **1996**, *21*, 214–219.
81. Chiao, C.W.; Tostes, R.C.; Webb, R.C. P2X7 receptor activation amplifies lipopolysaccharide-induced vascular hyporeactivity via interleukin-1 beta release. *J. Pharmacol. Exp. Ther.* **2008**, *326*, 864–870.
82. Ho, N.W.; Chen, Z.; Brainard, A.P.; Sedlak, M. Successful design and development of genetically engineered Saccharomyces yeasts for effective cofermentation of glucose and xylose from cellulosic biomass to fuel ethanol. *Adv. Biochem. Eng. Biotechnol.* **1999**, *65*, 163–192.
83. Davies, D.L.; Machu, T.K.; Guo, Y.; Alkana, R.L. Ethanol sensitivity in ATP-gated P2X receptors is subunit dependent. *Alcohol. Clin. Exp. Res.* **2002**, *26*, 773–778.
84. Ostrovskaya, O.; Asatryan, L.; Wyatt, L.; Popova, M.; Li, K.; Peoples, R.W.; Alkana, R.L.; Davies, D.L., Ethanol is a fast channel inhibitor of P2X4 receptors. *J. Pharmacol. Exp. Ther.* **2011**, *337*, 171–179.
85. Fischer, W.; Wirkner, K.; Weber, M.; Eberts, C.; Köles, L.; Reinhardt, R.; Franke, H.; Allgaier, C.; Gillen, C.; Illes, P. Characterization of P2X3, P2Y1 and P2Y4 receptors in cultured HEK293-hP2X3 cells and their inhibition by ethanol and trichloroethanol. *J. Neurochem.* **2003**, *85*, 779–790.
86. Davies, D.L.; Kochegarov, A.A.; Kuo, S.T.; Kulkarni, A.A.; Woodward, J.J.; King, B.F.; Alkana, R.L. Ethanol differentially affects ATP-gated P2X(3) and P2X(4) receptor subtypes expressed in Xenopus oocytes. *Neuropharmacology* **2005**, *49*, 243–253.

Natural Compounds (Publicado)

The Role of Natural Products in the Search for a P2X7R Antagonist

Soares-Bezerra R. J.[1],Ferreira N.C.S[1],Faria,R.X[1],Ferreira LG.B.[1], Frutuoso V.S.[2] & Alves L.A.[1+]

1. Laboratory of Cellular Communication, Oswaldo Cruz Institute – FIOCRUZ, Brazil;

2. Laboratory of Immunopharmacology, Oswaldo Cruz Institute – FIOCRUZ, Brazil.

The Role of Natural Products in the Search for a P2X7R Antagonist

Soares-Bezerra R. J.[1],Ferreira N.C.S[1],Faria,R.X[1],Ferreira LG.B.[1], Frutuoso V.S.[2] & Alves L.A.[1+]

1. Laboratory of Cellular Communication, Oswaldo Cruz Institute – FIOCRUZ, Brazil;
2. Laboratory of Immunopharmacology, Oswaldo Cruz Institute – FIOCRUZ, Brazil.

+ Author Correspondence
e-mail: alveslaa@ioc.fiocruz.br; alveslaa@gmail.com
Address: Laboratório de Comunicação Celular – sala 39 Pav 108 IOC – Fiocruz
Avenida Brasil 4365, Manguinhos, RJ – Brazil - CEP-21045-900

Abstract

Abstract

P2 receptors, purinergic receptors, are expressed in all human cell types and are activated by extracellular purines and pyrimidines. They have been found in all mammalian cells studied thus far. P2 receptors are subdivided into two groups: the P2Y receptors, which are metabotropic receptors, i.e. they activate intracellular metabolic pathways, and P2X receptors, which are ionotropic (promote the opening of an ion channel in the cell membrane). Among the P2X receptors has the P2X7 subtype, which is involved in many important physiological and metabolic functions, as well as in several diseases, especially inflammatory diseases. Based on this, the P2X7 receptor is considered a possible therapeutic target. However, there are no selective antagonists for P2X7 that could be useful in clinical treatments of a variety of diseases, which are related to this receptor. In this context, natural products emerge as a source of bioproducts, that can be utilized to obtain new molecules with antagonistic activity, or to become the base for synthesis of new antagonistic compounds. These new molecules may be used in clinical trials, to discover new selective antagonists for P2X7 receptors. In this chapter, we will discuss the use of natural products as a source for screening of new antagonists to P2X7 receptors.

Key Words: Natural Products, Antagonism, Purinergic, P2X7R.

Introduction

Antagonists for P2X7R: Why have they become necessary?

At times, the bridge between basic research and clinical practice is a long road that is difficult to imagine. However, in the case of P2X7R, Burnstock, an Australian researcher, discovered the P2 receptors in 1970's, however only in the 1980's they were differentiated as P2X and P2Y receptors. These receptors were further delineated into subtypes, of which P2X7 has been the focus of growing research. More than 500 articles were published in the previous 5 years about this receptor alone.

Within the immunological system, the P2X7R appears to be a key component to mediate responses related to inflammatory disease. These receptors are found in diverse species. In humans, more specifically in the immune system, P2X7R was found in mastocytes, erythrocytes, monocytes, macrophages, peripheral dendritic cells, T and B lymphocytes, Langerhans cells, microglia, Schwann cells, and astrocytes (Skaper; Debetto; Giusti, 2010). The activation of this receptor has been implied in ischemia, diabetes, cancer, Alzheimer's, cardiovascular disease, neuropathic pain, protozoa and bacterial diseases, acute pancreatitis and colitis (Coddou *et al.*, 2011). The P2X7 receptor is found at the center of much of the regulation of these diseases. It has become the target for not only treatment, but also the understanding of the mechanisms involved in the processes.

Current researches on P2X7 receptor have focused on the development of antagonists in both basic and therapeutic applications. The goal of this research is to combat side effects while creating more directed and safer intervention with minimal side effects, which can be applied in clinical practice. In turn, the search includes all involved antagonists, from both synthetic and natural products. The key challenge regarding a P2X7R antagonist remains passing from the level of *in vitro* to *in vivo,* due to the drastic ramifications that an intervention at this molecular level would imply within a living system. As synthetic antagonists have hit several roadblocks, hopes have turned to natural products to discover new molecules with innate natural antagonist properties of this receptor.

Possible Implications of an Antagonist in Medicine

The relevance of the study of an antagonist's usefulness in clinic is based on its biological characteristics: an antagonist is a molecule that will bind to a receptor without inducing any physiological response. Generally, an antagonist has more affinity to the receptor than an agonist, and high concentrations of an antagonist lead to a blockage on the agonist binding to the receptor. However, this inhibition effect can be reversible with a decrease of the antagonist's concentration. Otherwise, if the affinity between an antagonist and a receptor is high, the inhibitory effect becomes irreversible, which prevents the binding with an agonist molecule. Summarizing, an antagonist can be reversible or irreversible, depending on the affinity and/or its concentration (Katzung 9^{th} edition).

The P2X7 receptor is involved in many diseases, and approximately 317 articles have been published relating this correlation as of June of 2012. There seems to be an effective participant along with the evolution of these pathologies. As such, the antagonist inhibition of its receptor emerges as a promising target to treat these diseases, calling special attention to the scientific community.

The majority of the aforementioned articles is linked to inflammatory diseases and the P2X7R. The participation of the P2X7R in the IL-1β release pathway, a pro-inflammatory cytokine, has been studied as a potential target for the treatment of pathologies in relation with the inflammatory process, i.e.: Rheumatoid Arthritis, Neuropathic and Inflammatory Pain. Recently, there has been an international joint effort between researchers and pharmaceutical industries to discover compounds that could be potential antagonists for the P2X7R, with selective action and possible clinical applications.

Other possible clinical applications include diseases such as diabetes, which became a public health problem in many countries around the world, leading to secondary incapacitating pathologies or death. Current treatments are based on subcutaneous injections of insulin and/or the administration of anti-diabetogenic drugs that cause several side effects including: insomnia, hypoglycemia, headaches, and anorexia. The P2X7R was found in pancreatic alpha cells (Coutinho-Silva *et al.,* 2007), and it the correlates with the increase in the expression of this receptor during diabetes

in pancreatic alpha cells. Another disease that showed an increase in the expression of the P2X7R receptor was cancer, signaling a promising new target for the discovery of antagonist molecules, given 75% of anti-cancer drugs originate from natural products (Mann *et al.* 2002).

The location of P2X7R in the nervous system has been described as well (Tsuda *et al.*, 2012), and it became a recent target for the treatment of neuropathic pain. Injuries in a peripheral nerve lead to an up-regulation in the expression of its receptor in dorsal horn microglia (Tsuda *et al.*, 2012). In addition, the expression of the P2X7R in microglia was implicated with the neuronal damage in the cortex by the release of ROS, which could be a cause leading to Alzheimer's disease (Lee *et al.*, 2011). Alzheimer's is still without a cure and has expensive and irresolute treatment, which leads to many side effects. Recently, the P2X7R has been looked to as a promising target for treatment and research has been growing with the support of many companies industry-wide, which are searching for an antagonist with clinical applicability.

Pharmacology of P2X7R

Of all agonists, ATP is the only one that is physiological and activates the P2X7R under physiological conditions, and normally in the extracellular, its concentration is low (in the nanomolar range) (Chen *et al.*, 2009). However, in pathological conditions, cells that store ATP in the cytoplasm at higher concentrations than the extracellular medium tend to release ATP, which will activate the P2X7R. The activation of this receptor represents a danger signal, since it occurs in situations of high exposure to extracellular ATP, which is typically found in the processes of cytolysis, tissue trauma and hypoxia/ischemia caused by inflammation. Although, the P2X7R is the only receptor that is activated in the millimolar range of concentration of ATP, in despite of the other P2X receptors, which are activated at micromolar concentration levels (Lister *et al.*, 2007; Skaper; Debetto; Giuski, 2010; Coddou *et al.*, 2011).

Once activated, the P2X7R triggers an intracellular signaling cascades, which result in the release of inflammatory mediators such as cytokines IL-1β, IL-6, IL-18, NO,

ROS, and tumor necrosis factor-α (TNF-α), mediates apoptosis in polymorph nuclear cells, and induces the formation of multinucleated giant cells. Furthermore, a prolonged stimulation of this receptor results in the opening of a membrane pore, which allows passage of molecules up to 900 Da, as shown in Figure 1 (Lister *et al.*, 2007).

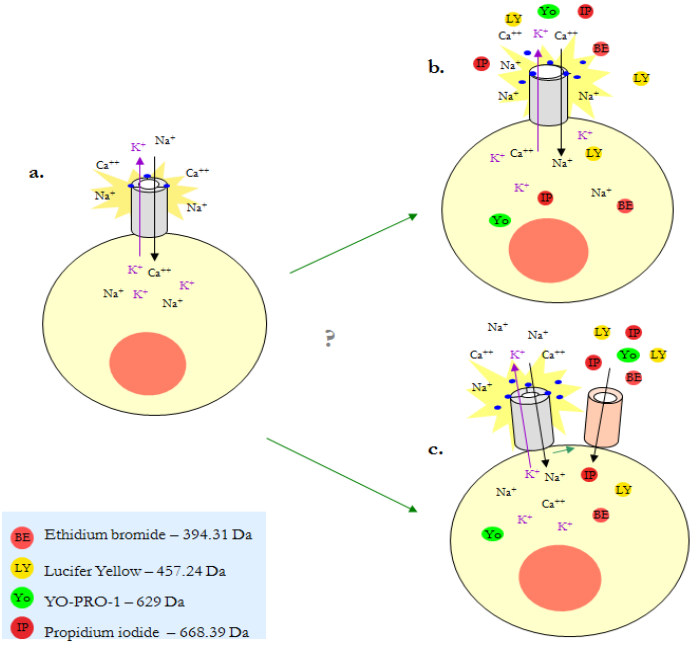


Figure 1: Expression of the P2X7 receptor on the cell membrane. In (a) the receptor is activated by its agonist, ATP and then opens an ion channel (b), which allows the passage of cations such as Na + and Ca2 +. If this stimulation is extended, it is capable of forming a membrane pore (c) allowing the passage of high molecular weight molecules such as ethidium bromide, Lucifer Yellow, propidium iodide and YO-PRO-1

Currently there some molecules described with agonist or antagonist activity on the P2X7R, between the agonists for this receptor. These include BzATP (EC_{50}: 10 nM), and ATP (EC_{50}: 3-4 mM), the first being more potent than the last, since 50% can promote the biological effect in a nanomolar concentration (Coddou et al., 2011).

In despite of the antagonists, a selective antagonist for P2X7R is not yet known, because many antagonists interact with other receptors of the P2X family and the few that shows specificity to P2X7R, they still in the trial phase (Gunosewoyo et al., 2010) (as shown in the Table 1).

Table 1: Agonists and antagonists to P2X7R.

Classification	Concentration	Relation with others P2X receptors	Citation
Powerfull Agonists	EC_{50}		
ATP	2-4 mM	$P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ e $P2X_6$.	Coddou *et al*., 2011
BzATP	10 μM	$P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ e $P2X_6$.	Gunosewoyo *et al*., 2010
Parcial Agonists	EC_{50}		
αβ-meATP	> 300 μM	$P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ e $P2X_6$.	Gunosewoyo *et al*., 2010
βγ-meATP	> 300 μM	$P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$ e $P2X_5$.	Coddou *et al*., 2011
2-meSATP	200 μM	$P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ e $P2X_6$.	Friedle *et al*., 2010
Antagonists	IC_{50}		
A438079	0,2-1 μM	$P2X_7$	Gunosewoyo *et al*., 2010
A740003	20-700 nM	$P2X_7$	Gunosewoyo *et al*., 2010
A804598	10 nM	$P2X_7$	Gunosewoyo *et al*., 2010
AZ10606120	10-200 nM	$P2X_7$	Gunosewoyo *et al*., 2010
AZ11645373	5-10 nM	$P2X_7$	Gunosewoyo *et al*., 2010
BBG	15-250 nM	$P2X_4$ e $P2X_5$.	Coddou *et al*., 2011
KN-62	10 μM	$P2X_7$	Gunosewoyo *et al*., 2010
MRS2159	5 μM	$P2X_1$ e $P2X_3$.	Coddou *et al*., 2011
NF279	3-20 μM	$P2X_1$, $P2X_2$ e $P2X_3$.	Coddou *et al*., 2011
oATP	100-300 μM	irreversible antagonist	Coddou *et al*., 2011
PPADS	10-45 μM	$P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ e $P2X_6$.	Gunosewoyo *et al*., 2010
PPNDS	1-10 μM	$P2X_1$.	Gunosewoyo *et al*., 2010
Suramin	> 300 μM	$P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ e $P2X_6$.	Coddou *et al*., 2011

Current research for P2X7R antagonists:

Synthetic Compounds:

Recently, the discovery of synthetic selective antagonists for the P2X7R have been related with SAR (structure-activity relationship) studies and synthesis of semisynthetic compounds, based on molecules obtained from natural compounds (Matasi *et al*., 2011).

Currently, some compounds have been described with potential antagonistic activity, such as, the cyanoguanidines, disubstituted tetrazoles, (Nelson, Gregg *et al.* 2006), A-438 079, 3 - ((5 - (2,3-dichlorophenyl) 1H-tetrazol-1-yl) methyl pyridine, a selective and competitive antagonist with inhibition on submicromolar concentrations to rP2X7R, hP2X7R and mP2X7R (Nelson, Gregg *et al.*, 2006). Another compound, A-740 003 (N-cyanoguanidine) (5-quinolinilamino) methyl] amino} -2,2-dimethylpropyl) -2 - (3,4-dimethoxyphenyl) acetamide), other selective and competitive antagonists with IC's50 to rP2X7R = 18nM and 40 nM and the hP2X7R with submicromolar concentration to mP2X7R. Other compounds showed antagonistic activity as well, such as the compound A804598 (2-cyano-1-[(1S)-1-phenylethyl]-3-quinoline-5-guanidine), which demonstrated inhibition at low concentrations [9-11nM] on receptors: rP2X7R, hP2X7R and mP2X7R (Coddou Yan *et al*., 2011). Recently, a study published by Stock *et al*., 2012, showed the efficacy and safety of a compound (CE-224,535) in the treatment of patients with Rheumatoid Arthritis, but this compound demonstrated to be less potent than the usual clinical drugs.

Research on the discovery of selective inhibitory molecules to P2X7R has offered new insights into the pharmacology of the P2X7R and its therapeutic potential (Donnelly-Roberts, Namovic *et al.*, 2004).

Natural Products

Natural products from plants, animals, and microorganisms have been used for thousands years for the treatment of various human diseases, becoming the known basis of the traditional medicine throughout the world. The traditional Chinese medicine and Japanese-Chinese Medicine in Asia, as well as alternative medicine in the Americas, are examples of its use (Koehn, Carter, 2005; Balunas; Kinghorn, 2005; Itokawa *et al.*, 2008).

Information about the medicinal use of plants was acquired and transmitted over centuries. These properties piqued scientific curiosity, and at the turn of the nineteenth century in 1805, the first pure form of a natural compound with pharmacological activity was isolated: morphine. Since then, other numerous molecules have been discovered and utilized in many pharmacological treatments, such as aspirin, digitoxin, pilocarpine, and quinine (Butler, 2004; Njuguna; Masimirembwa; Chibale, 2012).

Nowadays, medicine has some other examples of drugs provided from natural products, as the discovery and clinical use of quinine and artemisin, which represent just two of many natural compounds that have activity against *Plasmodium falciparum*, and have been used as a first-line drug treatment until today. Additionally, they serve as the basis for research and synthesis of various synthetic antimalarial compounds (Njuguna, Masimirembwa *et al.*, 2012). The discovery of penicillin by Fleming in 1928 from the fungus *Penicillium chrysogenum* can also be cited. Since then, there have been advancements and discoveries in the identification of biological activity of microorganisms and their secondary metabolites for synthesis of numerous antibiotics (Mohandas *et al.*, 2012).

Subsequently, another important discovery emerged, Cyclosporine in 1979 from the fungus *Tolypocladium inflatum*, leading to a fundamental improvement on the organ transplantation. Before the discovery of this drug, the durability of a graft was short, generally only few hours after surgery due to the intense onslaught of recipient's immune system against the graft, promoting rejection. Due to the immunosuppressive action on T lymphocytes, cyclosporine acts preventing the recipient's immune system

from attacking the organ graft, thus preventing the rejection, an undesirable factor for the success of the transplant (Starzl, Iwatsuki *et al.*, 1981).

Currently, it's estimated there are roughly 200,000 known compounds isolated from natural products, and many are used in modern medicine. Interesting examples of this applicability are morphine and salicylate (Marienhagen *et al.*, 2012; Calixto *et al.*, 2005). Antineoplastic agents can also be included, as around 75% come from natural products, i.e., taxol and vincristine (Mann, 2002) as shown in Table 2. These natural products are not obtained exclusively from plants, but also from fungi, marine organisms, and bacteria. Based on the enormous biological wealth worldwide, Brazil accounts for about 15 to 20% of naturally-based pharmaceuticals, being the country with largest number of endemic plant species. Knowledge of this pharmaceutical potential however, remains low.

Finally, research of natural products leads to an increased knowledge about their potential properties. Research in this area shows to be promising, offering many highlights to be explored.

Table 2: Drugs from Natural Products.

DRUG	USE	DISEASE	SOURCE	REFERENCE
Resveratrol	Antiplatelet	Cardiovascular	Plant	Vasanthi et al.,2012
Galantamine	Cholinesterase Inhibitor	Alzheimer´s Disease	Plant	Yanagisawa., 2012
Mefloquine	Antimalarial	Parasitic	Plant	Barreto., 2009
Magnolol	Modulate $GABA_AR$	Neurological	Plant	Alexeev et al., 2012
Taxol	Antimitotyc agent	Oncologycal	Plant	Mann et al.,2002
Codeine	Analsegic	Pain	Plant	Eban K., 2011
Isoflavone	Estrogen Therapy	Metabolic	Plant	Eden J.A., 2012
Cyclosporine	Antiproliferative activity on T cells	Graft Disease	Fungus	Starzl, Iwatsuki et al. 1981
Penicillin	Antibiotic	Bacterial infections	Fungus	Mohandas et al.2012

Natural Products and P2X7R antagonists: An overview

Currently there are a few articles published that make a correlation between P2X7 receptors and natural products as a source of new possible antagonists, as shown in Table 3. One of these articles, Shemon *et al.*, 2004, related the block on the cationic

efflux trough the P2X7 receptors by benzophenanthridine alkaloids, but this action was not selective. They noted the inhibition of some enzymes by this Phytochemistry compound, as well as PKC and alanine aminotransferase. Buchanan *et al.*, 2007 noted the specific antagonistic effect on P2X7 by a class of tretameric pirrazolyc imidazoles (Stilissadines A e B). In the same year, this scientific group also isolated the Niphatoxin C from a marine sponge (*Callyspongia sp*).

Further, the Said *et al.*, 2007 study can also be cited, as it related the action of vegetable oils on activation of cell death through P2X7R. These oils were obtained from: *Olea europaea L ; Zea mays; camelina sativa,* and *Aleurites moluccan.* Only the Castor oil, which is currently used as a eye drops vehicle, showed an agonistic action leading to an intense cell death process by the activation of the P2X7 receptor.

Gunosewoyo *et al.*, 2009 suggested the study of chemical properties of synthetic or natural compounds and the biological screening to effort the discovery of new antagonists for P2X7. Liu *et al.*, 2010 described the antagonistic activity of Emodin, an antraquinonic compound obtained from *Rheum officinale Baill* on this receptor. The antagonistic effect of the methanolic extract of *Rheedia logifolia* was described as promoting an analgesic activity on inflammatory pain (Santos *et al.*, 2011).

Since these bioproducts have a great demand for testing, it becomes necessary to develop techniques which are capable of solving the problem of screening the samples, of which only between 10-15% are used for therapy. In addition, some screening processes consume time and can lead to many months before implementation. Therefore, the development of new techniques for screening should be focused on one specific molecular target, allowing the analysis of many samples quickly and efficiently (Balunas; Kinghorn, 2005; Saklani; Kutty, 2008).

Table 3: Natural compounds with antagonistic activity on P2X7R

Article	Biological specie	Isolated compound/*IC_{50}
Shemon *et al*., 2004	*Chelidonium majus* (*Papaveraceae*)	Chelerytrine (benzophenanthridine alkaloid) $IC_{50 =}$ 10 µM
Buchanan *et al*., 2007	*Stylissa flabellata* (Marine sponge)	Stilissadines A and B $IC_{50 =}$ 0.7µM and 1.8 µM
Buchanan *et al*., 2007	*Callyspongia SP* (Marine Sponge)	Niphatoxin C (Alkaloids) $IC_{50 =}$ ND
Said *et al*., 2007	*Olea europaea L; Capsicum sp; Zea mays; camelina sativa*; *Aleurites moluccana*	Vegetable oils $IC_{50 =}$ ND
Gunosewoyo *et al*., 2009	Alkaloids obtained from SAR studies	Massadine (IC_{50} = ND) and Protoberberine (IC_{50} = 0.3 µM)
Liu et al., 2010	*Rheum officinale Baill*	Emodin $IC_{50 =}$ 500 nM
Santos *et al*., 2011	*Rheedia longifólia* (Guttiferae)	Bisflavonoids $IC_{50 =}$ 2µg/mL

*IC_{50} = half maximal inhibitory concentration; ND = Not Determined

Concluding Remarks

As shown previously, natural products can be a rich resource in the search of new medicines, or other active compounds that can be useful in the clinical treatment of various diseases. Focusing on the P2X7 receptor, some published works regard the activity of natural compounds as a potent and selective antagonist, which could be used for the treatment of pathologies that are in relationship with the activation of P2X7R.

Many trial studies have been conducted to discover new drugs that act on P2X7R, namely those based on synthetic compounds. These studies are primarily on trial phase one or three and have used patients with Rheumatoid Arthritis, demonstrating the importance of the quest to find a new compound that could act on inflammatory diseases by P2X7R (Coddou *et al.*, 2011). These studies are occurring in the scientific centers of pharmaceutical companies, such as Merck, Astra Zeneca, and Glaxo, which shows the great interest that the pharmaceutical industry has in this research area.

Under this scenario, natural products can emerge as a source to discovering new molecules with antagonistic activity, or those that could be used as a base for the synthesis of new synthetic compounds with P2X7R activity.

Acknowledgements: This work was supported by grants from FAPERJ, CAPES, CNPq, and the Oswaldo Cruz Institute.

References

1. Adinolfi E, Raffaghello L, Giuliani AL, Cavazzini L, Capece M, Chiozzi P, Bianchi G, Kroemer G, Pistoia V, Di Virgilio F. (2012). Expression of P2X7 Receptor Increases In Vivo Tumor Growth. Cancer Res, **72**: 2957-69.

2. Alexeev M, Grosenbaugh DK, Mott DD, Fisher JL. (2012). The natural products magnolol and honokiol are positive allosteric modulators of both synaptic and extra-synaptic GABA(A) receptors. *Neuropharmacology*, **62**: 2507-14. Epub 12 Mar 2012.

3. Balunas MJ, Kinghorn AD. (2005). Drug discovery from medicinal plants. Life Sciences, **78**: 431-41.

4. Barreto Miranda I, Löscher T. (2009). Malaria--current diagnosis and therapy. *Med Klin (Munich)*, **104**: 543-52. [Article in German].

5. Buchanan MS, Carroll AR, Addepalli R, Avery VM, Hooper JN, and Quinn RJ. (2007). "Niphatoxin C, a cytotoxic tripyridine alkaloid from Callyspongia sp." [In eng]. J Nat Prod, **70**: 2040-1.

6. Buchanan, M. S., A. R. Carroll, et al. (2007). "Natural products, stylissadines A and B, specific antagonists of the P2X7 receptor, an important inflammatory target." *J Org Chem*, **72**: 2309-2317.

7. Butler MS. (2004). The role of natural product chemistry in drug discovery. J Nat Prod, **4**: 2141-53.

8. Calixto, J. B. (2005). "Twenty-five years of research on medicinal plants in Latin America: a personal view." *J Ethnopharmacol,* **100**: 131-134.

9. Chen P, Liu Z, Liu S, Xie Z, Aimiuwu J, Pang J, Klisovic R, Blum W, Grever MR, Marcucci G, Chan KK. (2009). A LC-MS/MS method for the analysis of intracellular nucleoside triphosphate levels. Pharm Res, **26**: 1504-15.

10. Chen P, Liu Z, Liu S, Xie Z, Aimiuwu J, Pang J, Klisovic R, Blum W, Grever MR, Marcucci G, Chan KK. (2009). A LC-MS/MS method for the analysis of intracellular nucleoside triphosphate levels. Pharm Res, **26**:1504-15.

11. Coddou C, Yan Z, Obsil T, Huidobro-Toro JP, Stojilkovic SS. (2011). Activation and regulation of purinergic P2X receptor channels. Pharmacol Rev, **63**: 641-83.

12. Coutinho-Silva R, Robson T, Beales PE, Burnstock G. (2007). Changes in expression of P2X7 receptors in NOD mouse pancreas during the development of diabetes. *Autoimmunity*, **40**:108-16.

13. Donnelly-Roberts, D. L., M. T. Namovic, et al. (2004). "Mitogen-activated protein kinase and caspase signalling pathways are required for P2X7 receptor

(P2X7R)-induced pore formation in human THP-1." *J Pharmacol Exp Ther*, **308**: 1053-1061.

14. Eban K. Painful medicine. *Fortune*. (2011), **164**: 142-5, 147-8, 150.

15. Eden JA. (2012). Phytoestrogens for menopausal symptoms: a review. *Maturitas*, **72**: 157-9.

16. Gunosewoyo, H. and M. Kassiou (2010). "P2X purinergic receptor ligands: recently patented compounds." *Expert opinion on therapeutic patent,* **20**: 625-646.

17. Gunosewoyo, H., M. J. Coster, et al. (2009). "Purinergic P2X(7) receptor antagonists: Chemistry and fundamentals of biological screening." *Bioorg Med Chem*, **17**: 4861-4865.

18. Itokawa H, Morris-Natschke SL, Akiyama, T, Lee KH. (2008). Plant-derived natural product research aimed at new drug discovery. J Nat Med, **62**: 263-80.

19. Katzung BG. (2005). Basic and Clinic Pharmacology. 9ª ed. Rio de Janeiro: Guanabara Koogan.

20. Koehn FE, Carter GT. (2005). The evolving role of natural products in drug discovery. Nature Rev, **4**: 206-20.

21. Lee HG, Won SM, Gwag BJ, Lee YB. (2011). Microglial $P2X_7$ receptor expression is accompanied by neuronal damage in the cerebral cortex of the APPswe/PS1dE9 mouse model of Alzheimer's disease. *Exp Mol Med*, **43**:7-14.

22. Lister MF, Sharkey J, Sawatzky DA, Hodgkiss JP, Davidson DJ, Rossi AG. (2007). The role of the purinergic P2X7 receptor in inflammation. J Inflamm, **16**: 4-5.

23. Liu L, Zou J, Liu X, Jiang LH, Li J. (2010). Inhibition of ATP-induced macrophage death by emodin via antagonizing P2X7 receptor. Eur J Pharmacol, **640**: 15-9.

24. Mann, J. (2002). "Natural products in cancer chemotherapy: past, present and future." *Nat Rev Cancer*, **2**: 143-148.

25. Marienhagen J, Bott M. (2012). Metabolic engineering of microorganisms for the synthesis of plant natural products. *J biotechnol*, **9**: in press.

26. Matasi JJ, Brumfield S, Tulshian D, Czarnecki M, Greenlee W, Garlisi CG, Qiu H, Devito K, Chen SC, Sun Y, Bertorelli R, Geiss W, Le VD, Martin GS, Vellekoop SA, Haber J, Allard ML. (2011). Synthesis and SAR development of novel P2X7 receptor antagonists for the treatment of pain: part 1. *Bioorg Med Chem Lett,* 21:3805-8.

27. Mohandas, SP, S. Ravikumar, et al. (2012). "Bioactives of Microbes Isolated from Western Ghat Belt of Kerala Show beta-Lactamase Inhibition along with Wide Spectrum Antimicrobial Activity." *Appl Biochem Biotechnol*, **167**: 1753-62.

28. Njuguna NM, Masimirembwa C, Chibale K. (2012). Identification and characterization of reactive metabolites in natural products-driven drug discovery. J Nat Prod, **75**: 507-13.

29. Said, T., M. Dutot, et al. (2007). "Benefits and side effects of different vegetable oil vectors on apoptosis, oxidative stress, and P2X7 cell death receptor activation." *Invest Ophthalmol Vis Sci*, **48**: 5000-5006.

30. Saklani A, Kutty SK (2008). Plant-derived compounds in clinical trials. *Drug Discov Today*, **13**: 161-71.

31. Santos JAA, Fidalgo-Neto AA, Faria RX, Simões A, Calheiros AS, Bérenger AL.(2011). Effect of Rheedia longifolia leaf extract and fractions on the P2X7 receptor in vitro: novel antagonists? *J Med Food*, **14**: 920-9.

32. Shemon, A. N., R. Sluyter, et al. (2004). "Chelerythrine and other benzophenanthridine alkaloids block the human P2X7 receptor." *Br J Pharmacol*, **142**: 1015-1019.

33. Skaper SD, Debetto P, Giusti P. (2010). The P2X7 purinergic receptor: from physiology to neurological disorders. FASEB J, **24**: 337-45.

34. Starzl, TE, S. Iwatsuki. (1981). "Liver transplantation, 1980, with particular reference to cyclosporin-A." *Transplant Proc*, **13**: 281-285.

35. Stock TC, Bloom BJ, Wei N, Ishaq S, Park W, Wang X, Gupta P, Mebus CA. (2012). Efficacy and safety of CE-224,535, an antagonist of P2X7 receptor, in treatment of patients with rheumatoid arthritis inadequately controlled by methotrexate. *J Rheumatol*, **39**:720-7.

36. Tsuda M, Tozaki-Saitoh H, Inoue K. (2012). Purinergic system, microglia and neuropathic pain. *Curr Opin Pharmacol*, **12**:74-9.

37. Vasanthi HR, ShriShriMal N, Das DK. (2012). Phytochemicals from plants to combat cardiovascular disease. *Curr Med Chem*, **19**: 2242-51.

38. Yanagisawa K. Strategy and perspectives for development of Alzheimer disease-modifying drugs. (2012). Seishin Shinkeigaku Zasshi, **114**: 262-7.

Resultado de Alguns dos 1800 Extratos Triados Através da Metodologia Padronizada Neste Trabalho

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,635	4,38	4,447	5,606	10,981	6,842	3,168	3,088	5,24	4,34	4,104	CÉL
B	1,677	5,328	7,447	4,212	2,437	2,185	3,208	3,056	4,101	4,881	2,567	CÉL+ATP
C	5,851	3,026	2,624	4,029	3,354	2,073	4,299	3,858	3,331	2,728	3,223	TRITON 0.1%
D	0,682	5,819	6,464	4,063	4,65	2,646	2,322	2,854	4,157	3,465	2,798	BBG
E	0,784	8,767	5,47	4,511	5,311	2,231	2,726	4,354	3,269	2,287	6,837	BBG+ATP
F	0,734	5,364	5,087	5,767	5,412	3,581	2,397	2,21	2,35	3,858	9,79	DMSO 0.05%
G		5,994	4,498	4,86	4,225	2,899	2,748	2,678	2,503	2,408	10,981	
H		6,879	8,214	2,767	3,363	5,512	3,223	3,371	2,413	4,463	4,443	
A	0,873	1,395	42,564	10,012	11,063	21.119	26,626	28,972	28,961	23,333	5,993	CÉL
B	2,931	73,791	45,528	43,382	49,322	49,454	22,57	38,405	41,645	42,244	7,536	CÉL+ATP
C	20,515	97,898	61,008	48,696	52,107	64,311	44,444	22,72	40,929	36,097	9,269	TRITON 0.1%
D	0,995	19,957	26,453	19,986	9,006	11,643	21,19	21,121	10,859	10,863	10,068	BBG
E	1,307	29,28	4,474	5,309	11,574	11,647	11,06	10,853	10,964	10,953	10,92	BBG+ATP
F	0,982	38,953	17,673	4,265	4,075	22,578	41,202	31,152	31,26	11,119	21,118	DMSO 0.05%
G		110,274	21,584	5,092	22,464	41,875	21,239	31,238	5,228	10,965	10,808	
H		21,022	6,999	31,066	5,044	9,964	10,654	11,899	12,917	9,818	9,897	Extrato c/ Atividade
A	0,587	4,366	3,324	4,355	3,377	5,327	3,703	3,83	3,871	2,972	3,406	CÉL
B	1,702	5,633	4,206	3,268	3,339	4,673	3,463	4,051	4,178	2,261	2,396	CÉL+ATP
C	12,428	4,515	4,543	5,829	2,191	4,975	5,391	3,266	3,951	3,348	2,347	TRITON 0.1%
D	0,608	5,256	4,772	7,42	2,898	3,166	4,223	6,392	3,779	3,422	5,316	BBG
E	0,693	4,228	4,531	4,278	4,399	4,371	4,301	3,733	3,793	4,612	4,388	BBG+ATP
F	0,621	5,152	5,764	3,851	2,559	3,678	3,708	3,258	3,71	5,402	3,31	DMSO 0.05%
G		4,718	6,115	3,162	2,136	4,211	2,944	3,433	2,76	3,085	2,367	
H		5,327	5,334	4,824	3,934	3,945	4,176	3,198	3,447	3,814	5,349	
A	0,607	4,384	5,382	3,358	5,385	4,399	3,635	11,721	10,918	2,943	4,307	CÉL
B	1,801	5,632	4,292	3,548	3,607	4,696	3,68	4,165	3,994	2,298	3,348	CÉL+ATP
C	8,107	4,462	4,594	5,61	12,141	4,972	5,46	3,287	3,863	3,371	4,393	TRITON 0.1%
D	0,716	5,036	4,574	7,407	3,187	2,966	3,816	6,568	3,941	3,303	4,335	BBG
E	0,818	3,96	4,59	3,921	5,436	4,279	4,425	3,817	3,615	4,249	4,344	BBG+ATP
F	0,62	5,196	6,04	4,008	12,5	3,881	3,841	3,354	3,531	5,26	4,383	DMSO 0.05%
G		4,677	5,944	3,199	20,013	3,861	2,923	3,318	2,779	3,093	2,395	
H		5,651	5,11	4,673	3,976	3,775	4,261	3,229	3,339	3,543	3,376	
A	0,286	2,793	1,756	1,419	2,78	1,896	1,286	1,792	1,758	1,569	1,516	CÉL
B	0,642	2,272	1,6	1,251	1,473	1,614	1,755	1,351	2,358	1,946	1,633	CÉL+ATP
C	3,326	2,426	1,75	1,444	1,351	1,406	2,517	1,793	2,05	2,134	1,929	TRITON 0.1%
D	0,295	2,843	1,357	1,442	1,401	1,318	1,293	1,494	2,913	1,579	2,461	BBG
E	0,306	1,336	1,474	1,748	1,456	1,404	1,703	1,509	1,921	2	2,056	BBG+ATP
F	0,33	1,383	1,567	1,45	1,649	1,413	1,485	1,709	2,191	1,631	1,689	DMSO 0.05%
G		1,377	1,214	1,321	1,353	1,287	1,333	1,653	1,629	1,7	1,088	
H		1,359	2,962	1,503	1,285	1,291	1,53	1,482	1,574	1,432	1,642	
A	0,211	2,799	3,966	1,805	1,468	1,921	1,18	1,61	1,712	1,43	1,539	CÉL

B	0,865	2,3	1,369	1,389	1,488	1,638	1,699	1,21	2,352	1,902	1,708	CÉL+ATP
C	2,472	O.489	1,743	1,547	1,385	1,461	2,343	1,844	2,176	2,208	1,891	TRITON 0.1%
D	0,29	2,819	1,416	1,487	1,453	1,352	1,115	1,537	3,223	1,559	2,325	BBG
E	0,302	1,316	1,554	1,795	1,344	1,422	1,664	1,646	1,923	1,952	2,078	BBG+ATP
F	0,283	1,396	1,876	0.509	1,473	1,195	1,361	1,699	2,068	1,751	1,556	DMSO 0.05%
G		1,266	1,355	1,341	1,385	1,333	1,253	1,693	1,659	1,776	1,155	
H		1,306	2,97	1,501	1,436	1,14	1,55	1,545	1,518	1,45	1,649	Extrato c/ Atividade
A	0,153	5,971	3,03	1,794	1,489	1,583	1,26	1,519	1,744	1,495	1,528	CÉL
B	0,451	2,42	1,461	1,296	1,532	1,678	1,792	1,347	2,458	1,84	1,798	CÉL+ATP
C	2,405	2,357	1,599	1,351	1,467	1,442	2,635	1,913	2,19	2,176	1,944	TRITON 0.1%
D	0,205	2,944	1,475	1,544	1,319	1,324	1,226	1,504	3,057	1,63	2,447	BBG
E	0,26	1,426	1,431	1,691	1,407	1,331	1,769	1,465	1,654	2,082	2,088	BBG+ATP
F	0,19	1,394	1,53	1,455	1,332	1,225	1,447	1,643	2,349	1,715	1,701	DMSO 0.05%
G		1,206	1,239	1,353	1,375	1,358	1,177	1,701	1,584	1,581	1,029	
H		1,246	1,386	1,558	1,363	1,235	1,488	1,352	1,63	1,427	1,465	
A	0,608	4,603	4,603	6,084	4,941	6,557	2,638	3,078	5,756	4,464	2,851	CÉL
B	1,391	3,152	8,402	3,973	2,218	2,414	3,427	3,182	3,892	4,926	2,592	CÉL+ATP
C	12,537	3,529	2,758	4,265	3,236	2,872	4,915	4,395	3,439	2,917	3,533	TRITON 0.1%
D	0,77	7,225	7,267	4,505	4,709	3,915	3,177	4,119	4,372	4,228	3,388	BBG
E	0,808	9,448	6,519	4,869	4,203	2,969	3,677	5,224	4,386	2,535	7,298	BBG+ATP
F	0,685	5,62	4,797	6,325	4,371	4,822	2,504	2,714	2,239	3,698	10,639	DMSO 0.05%
G		8,127	6,454	5,8	4,335	2,243	4,688	3,474	2,818	2,51	2,385	
H		6,952	9,448	2,587	3,694	5,326	3,54	3,253	2,223	4,732	4,393	
A	0,402	4,448	5,768	4,729	6,526	5,77	2,446	2,991	5,711	4,104	3,508	CÉL
B	1,229	3,405	9,065	3,838	2,215	2,528	3,595	3,319	4,078	4,819	2,666	CÉL+ATP
C	12,419	3,941	2,809	4,192	3,807	2,409	4,494	4,136	3,355	2,789	3,102	TRITON 0.1%
D	0,577	6,717	7,615	4,533	4,643	3,393	3,119	3,332	4,465	3,672	3,11	BBG
E	0,608	9,2	6,025	4,602	4,484	2,661	3,321	4,97	4,406	2,137	7,104	BBG+ATP
F	0,447	5,633	4,857	6,345	4,689	4,452	2,416	2,429	2,273	3,933	10,267	DMSO 0.05%
G		7,83	6,79	5,278	4,551	2,212	3,923	3,076	2,909	2,436	2,581	
H		7,35	8,806	2,581	3,49	6,382	3,346	3,64	2,348	4,852	4,706	