UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

O papel dos nucleotídeos e nucleosídeos da adenina e do receptor P2X7 no controle da proliferação e morte celular tumoral

PAOLA DE ANDRADE MELLO

PORTO ALEGRE, 2015

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

O papel dos nucleotídeos e nucleosídeos da adenina e do receptor P2X7 no controle da proliferação e morte celular tumoral

Tese apresentada por **Paola de Andrade Mello** para obtenção do TÍTULO DE DOUTOR em Ciências Farmacêuticas

Orientador: Prof[a]. Dra. Andréia Buffon
Coorientador: Prof. Dr. Guido Lenz
Coorientador no exterior: Prof. Dr. Simon Robson

PORTO ALEGRE, 2015

Tese apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas, em nível de Doutorado Acadêmico da Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul e aprovada em 10 de Dezembro de 2015, pela Banca Examinadora constituída por:

Profa. Dr. Ana Maria Oliveira Battastini
Universidade Federal do Rio Grande do Sul

Profa. Dr. Cristina Beatriz Cazabuena Bonorino
Pontifícia Universidade Católica do Rio Grande do Sul

Profa. Dr. Elizandra Braganhol
Universidade Federal de Ciências da Saúde de Porto Alegre

CIP - Catalogação na Publicação

Mello, Paola de Andrade
O papel dos nucleotídeos e nucleosídeos da adenina e do receptor P2X7 no controle da proliferação e morte celular tumoral / Paola de Andrade Mello. -- 2015.
146 f.

Orientadora: Andréia Buffon.
Coorientador: Guido Lenz.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Farmácia, Programa de Pós-Graduação em Ciências Farmacêuticas, Porto Alegre, BR-RS, 2015.

1. ATP. 2. Adenosina. 3. P2X7. 4. Câncer. 5. Alvos Terapêuticos. I. Buffon, Andréia, orient. II. Lenz, Guido, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Esta tese foi desenvolvida no Laboratório de Análises Bioquímicas e Citológicas (LABC) do Departamento de Análises da Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul, no Laboratório de Sinalização e Plasticidade Celular (Labsinal) do Departamento de Biofísica do Instituto de Biociências da Universidade Federal do Rio Grande do Sul e no Department of Medicine, Gatroenterology & Transplatation of Beth Israel Deaconess Medical Center, a teaching hospital of Harvard Medical School. A estudante recebeu bolsa de estudos da CAPES.

Aos meus pais,

a quem devo a vida e a minha formação moral.

Meu reconhecimento e gratidão pelo amor, paciência, compreensão e apoio constante nesta jornada da vida.

AGRADECIMENTOS

A prof[a]. Dra. Andréia Buffon e ao prof. Dr. Guido Lenz, exemplo de profissionais competentes, dedicados, éticos e humanos, pela orientação, ensinamentos, disponibilidade, apoio, compreensão e empolgação. Pela amizade e convívio.

Aos meus colegas do Laboratório de Análise Bioquímica e Citológica, em especial aos meus bolsistas de iniciação Jessica Nascimento e Camilo D' Amore. Agradeço imensamente a todo o apoio, a ajuda e a disponibilidade durante a realização deste trabalho.

Ao doutor Eduardo Chiela por todo o apoio, orientação, disponibilidade e amizade durante esta etapa. Você foi fundamental no desenvolvimento do meu trabalho.

A professora Márcia Wink e seu grupo de pesquisa pelo apoio, ajuda e troca de experiências.

Ao meu supervisor no exterior, Dr. Simon Robson e a também a Dra. Yan Wu, pela orientação, amizade e dedicação durante o meu doutorado sanduíche na Harvard Univesity.

A todos os colaboradores que participaram do desenvolvimento desta tese, muito obrigada pelo auxílio e colaboração.

Ao Programa de Pós-Graduação em Ciências Farmacêuticas pela oportunidade.

A CAPES, pelo auxílio financeiro tanto no Brasil quanto no exterior.

Aos meus pais, Celso e Sirlei, agradeço pela educação, apoio, incentivo, conforto, compreensão e amor incondicional.

RESUMO

Estudos têm demonstrado que o microambiente tumoral é rico em ATP e adenosina, sugerindo o envolvimento da sinalização purinérgica no desenvolvimento e/ou manutenção do câncer. Ainda, o receptor purinérgico P2X7, conhecido pelo seu papel na indução de apoptose, encontra-se reduzido em alguns tecidos tumorais em comparação aos tecidos saudáveis, indicando que a sua redução possa ser um mecanismo de resistência celular à apoptose. Dessa forma, compreender o papel da sinalização purinérgica no contexto do câncer se torna indispensável e permite que novas abordagens terapêuticas sejam implementadas. Nesse trabalho, avaliamos a função dos nucleotídeos e nucleosídeos da adenina, bem como do receptor P2X7 na indução da morte celular em células de câncer cervical. Também verificamos o efeito do *heat shock* na potencialização da atividade do receptor P2X7 frente à curta exposição ao ATP em células de câncer de cólon. De acordo com os nossos resultados, o efeito citotóxico do ATP extracelular nas linhagens de câncer cervical é mediado principalmente pela ação do seu metabólito adenosina, que ao entrar no interior das células, promove o aumento dos níveis intracelulares de AMP, ativação de AMPK, aumento da p53 e indução de autofagia. O papel do receptor P2X7 nesse contexto parece ser apenas coadjuvante, visto que o seu bloqueio ou silenciamento impediu em apenas 20% a morte celular. Além disso, utilizando células de câncer de cólon, nós demonstramos que o *heat shock* aumenta a funcionalidade do receptor P2X7, independente da interação com *heat shock proteins* ou canais do tipo conexina/panexina, potencializando o efeito citotóxico do ATP. Esse efeito parece estar relacionado à mudanças na composição e arquitetura da membrana celular, visto que o uso do agente fluidizador de membrana benzil álcool foi capaz de mimetizar o efeito do *heat shock* na potencialização do receptor P2X7 a 37ºC. Este estudo fornece evidências adicionais sobre o papel da sinalização purinérgica no contexto da biologia celular tumoral e abre novas perspectivas para o uso dos nucleotídeos de adenina associados a hipertermia como agentes adjuvantes na terapia do câncer.

Palavras-chaves: ATP, Adenosina, P2X7, Sistema Purinérgico, Câncer Cervical, Câncer de Cólon, Alvos Terapêuticos.

ABSTRACT

The tumor microenvironment is rich in ATP and adenosine, suggesting an involvement for purinergic signaling in cancer development and surveillance. The P2X7 receptor, among the P2 purinergic receptors, is broadly recognized as the “death receptor”, because it promotes cell apoptosis when exposed to high levels of extracellular ATP. Researches have been shown that P2X7 protein levels are decreased at the tumor site in comparison to adjacent healthy tissue, suggesting a mechanism of tumor escape to cell death. Thus, understanding purinergic signaling in a cancer context becomes urgent and opens a new field for therapeutic strategies. Here, we evaluated adenine nucleotides and nucleosides cytotoxicity, as well as P2X7 role in cell death induction using cervical cancer cell lines. Indeed, we investigated heat shock effect on P2X7 functionality through exposing colon cancer cell shortly to ATP at 40ºC. According to our data, adenosine uptake formed from ATP metabolism is the main responsible for the extracellular ATP cytotoxicity in cervical cancer cells. While inside of the cell, adenosine is converted to AMP, leading to AMPK activation, p53 increase and autophagy induction. ATP induced cell death *per se* through P2X7 in this context seems to be less important, since P2X7 blockage or knocking down reduced only 20% of cell death. In colon cancer cells, we found that heat shock stress was able to increase P2X7 pore formation independently of heat shock protein interaction or native pore-forming transporters association (e.g pannexin-or connexin-type channels), thus leading to an increase ATP cytotoxicity. The mechanism enrolled in this process seems to be related to changes in the lipid composition and architecture of membrane, as the membrane fluidizer benzyl alcohol could reproduce heat stress effect in potentiating P2X7 activation at 37ºC. In conclusion, our work provides further evidence for a purinergic signaling role in the cancer biology context and opens new perspectives for the utility of purine-based drugs associated to hypertermia as adjunctive agents in cancer therapy.

Keywords: ATP, Adenosine, Purinergic System, P2X7, Cervical Cancer, Colon Cancer, Therapeutical Target.

SUMÁRIO

I. Introdução

I.1. Sistema Purinérgico

O sistema purinérgico é composto por três elementos principais, os nucletídeos/nucleosídeos da adenina extracelulares, os receptores (purinoceptores) e as enzimas (ectonucleotidases), que interagem entre si modulando o processo de sinalização purinérgica (Feng *et al.*, 2014). Em muitos casos, essa sinalização se inicia pela liberação do nucleotídeo ATP do meio intracelular para o extracelular (Idzko *et al*., 2014). Uma vez presentes no meio extracelular, ATP e ADP podem atuar nos receptores purinérgicos P2, que são subdivididos em duas famílias: os receptores metabotrópicos P2Y e os receptores ionotrópicos P2X. Os receptores P2Y são acoplados a proteínas G e são subdivididos, por sua vez, nos tipos $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14.}$ Já os receptores P2X atuam como canais ionotrópicos ativados por ATP e são divididos em sete subtipos (P2X1-7) (Di Virgilio, 2012; Burnstock & Di Virgilio, 2013; Idzko *et al*., 2014; Roger *et al*., 2014).

A ativação dos receptores P2 é finalizada pela difusão e hidrólise do ATP a adenosina. Este processo ocorre em duas etapas e é controlado principalmente pela ação da enzima nucleosídeo trifosfato difosfohidrolase 1 (NTPDase1 ou CD39), que converte ATP/ADP em AMP, seguido pela ação da enzima ecto-5'-nucleotidase (CD73), que forma adenosina a partir do AMP. A adenosina formada no meio extracelular pode, por sua vez, atuar nos receptores purinérgicos P1, que são subdivididos em quatro tipos (A_1, A_{2A}, A_{2B} e A_3), ser captada para o interior da células por meio de transportadores (ENTs) ou ser degradada a inosina por meio da ação da enzima adenosina deaminase (ADA), que se encontra tanto na membrana citoplasmática quanto no citoplasma das células (Idzko *et al*., 2014; Roger *et al*., 2014).

Dependendo dos níveis de ATP/adenosina que se encontram no meio extracelular e da ativação dos receptores P1 e P2, processos como quimiotaxia, ativação de células imunes, fagocitose, liberação de citocinas, proliferação, diferenciação, maturação, migração, adesão e morte celular são controlados (Di Virgilio, 2012; Roger *et al*., 2014). Sendo assim, um desequilíbrio nessa sinalização pode ser um dos responsáveis pelo desenvolvimento de inúmeras patologias, inclusive o câncer (Feng *et al.*, 2014).

I.2. ATP e adenosina extracelular no microambiente tumoral

Em condições fisiológicas, o ATP encontra-se em altas concentrações no interior das células, na faixa de 3-10 mM, e em baixas concentrações no meio extracelular (em torno de 10 nM). Sendo assim, altas concentrações desse nucleotídeo no meio extracelular representa um sinal de dano tecidual e/ou stress celular, como uma molécula sinalizadora (Feng *et al.*, 2014; Roger *et al*., 2014). No microambiente tumoral, estudos tem demonstrado que a concentração de ATP é relativamente alta, na faixa de mM, quando comparado aos tecidos saudáveis, onde é quase indetectável (Pellegatti *et al*., 2008). Inicialmente, acreditava-se que esse ATP extracelular era secretado pelas células cancerígenas, mas estudos mais recentes indicam que a sua principal fonte é proveniente de células em processo de morte celular, principalmente aquelas localizadas em regiões de grande hipóxia nos tecidos tumorais sólidos (Wang *et al*., 2004b; Roger *&* Pelegrin, 2011).

O papel do ATP extracelular no microambiente tumoral, modulando o desenvolvimento do câncer, vai depender do mecanismo pelo qual as células fonte deste nucleotídeo estão morrendo (necrose, apoptose, autofagia), do tipo de liberação pelas células (liberação passiva ou secreção ativa), da concentração de ATP no meio, do tipo de receptor purinérgico e da sinalização ativada. Portanto, dependendo do conjunto envolvido, o ATP extracelular pode gerar uma ação anti-tumoral com propriedades imunogênicas ou pró-tumoral, promovendo proliferação e migração das células cancerígenas (Roger *et al*., 2014).

Assim como o ATP, a concentração de adenosina nos fluidos intersticiais em condições fisiológicas é extremamente baixa e o seu aumento no meio extracelular normalmente se deve a condições patológicas como hipóxia, isquemia, inflamação ou trauma, caracterizando um sinal de dano tecidual (Hasko *et al.*, 2008). Além desta liberação passiva, o aumento de adenosina no meio extracelular também pode ocorrer devido a uma alteração do metabolismo de componentes do sistema purinérgico, caracterizado pelo acelerado catabolismo de ATP extracelular e/ou da inibição da degradação de adenosina, encontrado em vários tipos de câncer (Linden, 2006) (Figura 1).

No microambiente neoplásico, a adenosina, quando acumulada, exerce um papel crucial na regulação autócrina e parácrina estimulando a

imunossupressão e a angiogênese e favorecendo desta forma o crescimento tumoral (Ohta *et al*., 2006). Além deste efeito no estroma tumoral, a adenosina extracelular pode atuar diretamente nas células cancerígenas regulando a proliferação, diferenciação e apoptose. Sendo assim, o papel desse nucleosídeo no desenvolvimento, progressão e metástase tumoral se deve tanto a uma ação indireta no microambiente tumoral como também a uma ação direta sobre as células cancerígenas (Antonioli *et al.*, 2013).

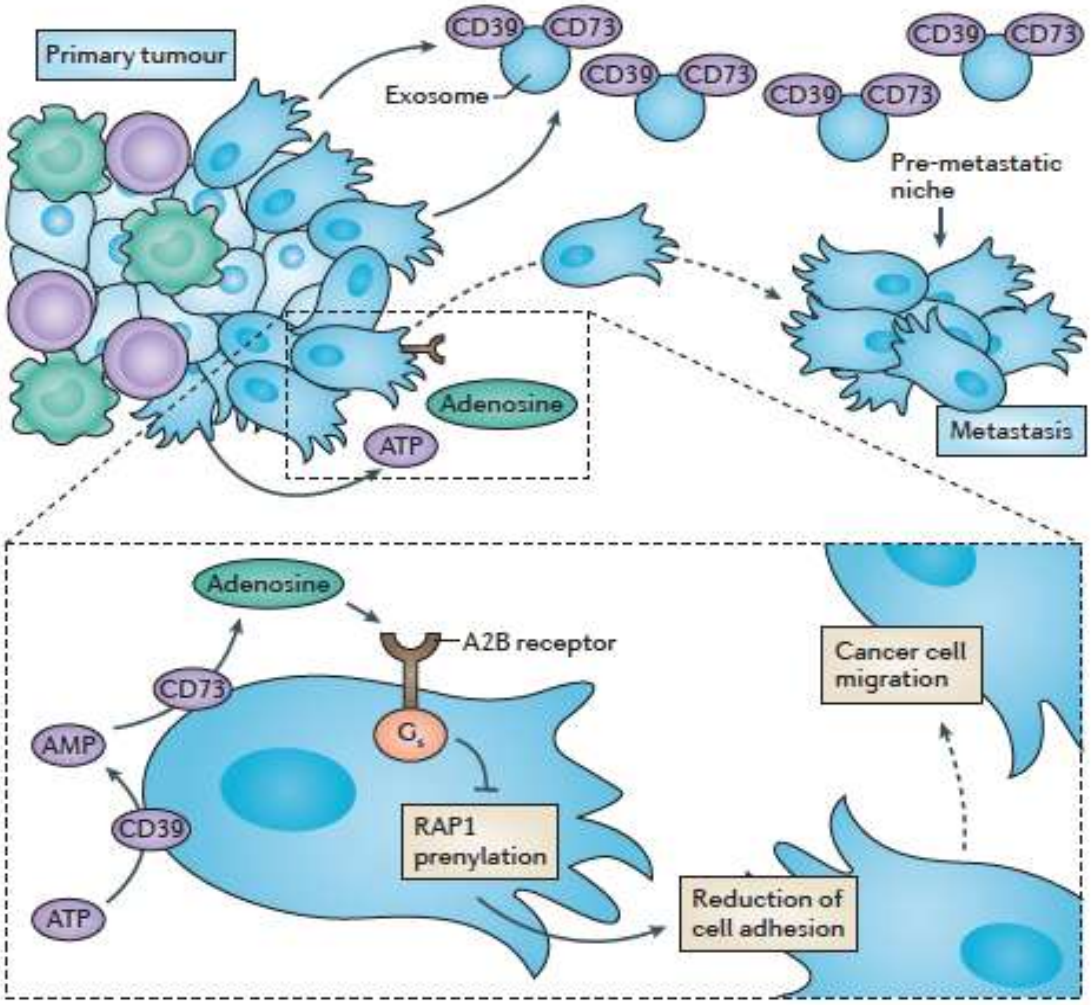


Figura 1. Adenosina formada pelo metabolismo do ATP no microambiente tumoral estimula a migração de células tumorais via receptores A2B. As células tumorais, bem como exosomos produzidos por elas, expressam CD39 e CD73, levando ao aumento dos níveis de adenosina no microambiente tumoral. Este nucleosídeo quando ligado aos receptores A2B inibe vias de sinalização importantes para o processo de adesão celular, estimulando a migração tumoral. Adaptado de Antonioli *et al.,* 2013. Cópia *autorizada por Nature Publishing Group*, número da licença 3733201443155.

I.3. Papel do ATP *vs* Adenosina extracelular no crescimento tumoral

Considerando a complexa via de sinalização purinérgica, a resposta final gerada pelos diferentes níveis de ATP *versus* adenosina no microambiente tumoral irá depender do painel de expressão e ativação dos diferentes subtipos de receptores P1 e P2 presentes no tecido, podendo ser tanto pró- ou anti-tumoral (Feng *et al*., 2014).

I.3.1. Atividade pró-tumoral

Atividades pró-tumorais já foram descritas para os receptores $P2Y_1$ em câncer de tireóide (Pines *et al*., 2005), P2Y2 em melanoma, câncer de células escamosas, mama, pulmão, tireóide e fígado (Dixon *et al.*, 1997; Greig *et al*., 2003; Schafer *et al*., 2003; White *et al*., 2005a; White & Burnstock, 2006; Shabbir *et al.*, 2008b), $P2Y_6$ e $P2Y_{12}$ em câncer de pulmão e astrocitoma (Schafer *et al*., 2003; Kim *et al*., 2003; Nishimaki *et al.*, 2012; Sodelar o Bilbao & Boland, 2013; Burnstock & Di Virgilio, 2013; Ide *et al*., 2014). Já os receptores A_{2A} e A_{2B} são amplamente conhecidos por seu papel na proliferação tumoral, tendo uma ação importante no estímulo da proliferação de células tumorais pulmonares, pancreáticas, intestinais e de próstata (Kalhan *et al*., 2012; Mediavilla-Varela *et al*., 2013; Wei *et al*., 2013).

I.3.2. Atividade anti-tumoral

Rapaport em 1983 foi o primeiro a descrever o efeito citotóxico do ATP ao verificar que esse composto bloqueava o crescimento celular quando adicionado ao meio de cultura de células cancerígenas (pâncreas e cólon) (Rapaport, 1983). Desde então, muitos estudos avaliando a atividade anti-neoplásica do ATP, tanto *in vitro* quanto *in vivo,* estão sendo publicados.

A redução na proliferação celular é resultado da ação conjunta dos processos de apoptose e diferenciação celular desencadeados pela ativação de vários subtipos de receptores purinérgicos pelo ATP (Feng *et al*., 2014). Funções tumoricidas já foram descritas para os receptores P2X5/P2X7 e/ou $P2Y_{11}$ em células de câncer de próstata (PC-3) e bexiga (HT-1376) (Fang *et al*, 1992; Calvert *et al*., 2004; Shabbir *et al.*, 2008b), bem como para os receptores

$P2Y_1$ e $P2Y_2$ em células de melanoma, de câncer de cólon e de esôfago (Hopfner *et al*., 1998; Maaser *et al*., 2002; White *et al*., 2005a; White *et al*., 2005b; Coutinho-Silva *et al*., 2005). Em linhagens celulares de neuroblastoma o efeito citotóxico do ATP extracelular é mediado basicamente pelos receptores P2X7, $P2Y_2$ e $P2Y_6$ (Feng *et al*., 2014).

Assim como o ATP, a adenosina também parece ter um efeito na redução do crescimento tumoral. A ativação do receptor A_{2A} está associada à indução de morte celular em linhagens de câncer de cólon (Caco-2), hepatoma (HepG2), mieloma múltiplo e linfoma (Yasuda *et al*., 2009; Serra *et al*., 2011; Rickles *et al*., 2012). Já o estímulo do receptor A_1 leva à indução de apoptose nas linhagens de câncer de cólon (CW2) (Saito *et al*., 2010) e glioblastoma (Synowitz *et al*., 2006). O receptor A_3, dentre todos os receptores P1, é o mais conhecido pelo seu efeito citotóxico, inibindo o crescimento de uma variedade de tipos tumorais, dentre eles o linfoma, melanoma, câncer de próstata, pulmão, cólon, câncer neural e carcinoma hepatocelular (Fishman *et al*., 2000; Bar-Yehuda *et al*., 2001; Merighi *et al*., 2002 Fishman *et al*., 2002; Madi *et al*., 2003; Fishman *et al*., 2004; Aghaei *et al*., 2011; Bar-Yehuda *et al*., 2008; Otsuki *et al*., 2012; Aghaei *et al*., 2012; Vincenzi *et al*., 2012). Além do seu efeito citotóxico direto via receptores P1, a adenosina pode induzir apoptose quando captada para o interior das células via transportadores de membrana (ENTs). Esse mecanismo de morte celular já foi descrito para as linhagens de câncer de mama (MCF-7) (Tsuchiya *et al.,* 2012), astrocitoma (RCR1) (Sai *et al*., 2006) e câncer gástrico (GT3-TKB) (Saitoh *et al*., 2004).

Essa ambigüidade da sinalização purinérgica com relação ao crescimento tumoral ainda não está totalmente elucidada. O que se sabe é que a presença nos tecidos tumorais de um ou vários tipos de receptores purinérgicos com diferentes sensibilidades aos níveis de ATP/adenosina no meio extracelular geram distintas vias de sinalização intracelular, que interagem/concorrem para produzir uma resposta celular final, que pode ser de crescimento, quiescência ou morte (White & Burnstock, 2006; Pellegatti *et al*., 2008; Weber *et al*., 2010; Wilhelm *et al*., 2010; Michaud *et al*., 2011).

I.4. O receptor P2X7 e suas peculiaridades

Dentre os subtipos de receptores P2X, o receptor P2X7 é peculiar e apresenta características únicas que vão desde a sua estrutura molecular até as suas propriedades biofísicas e farmacológicas (Jiang *et al*., 2013). Com relação a sua estrutura molecular, o receptor P2X7 difere dos demais por apresentar uma cauda carboxi-terminal. Já em relação as propriedades funcionais, ele é o único receptor capaz de responder a altas concentrações de ATP (acima de 1 mM) e formar poros permeáveis a grandes moléculas (até 900 Da). A formação do poro só ocorre após estímulo continuado por altas concentrações de ATP e é considerado o principal mecanismo envolvido na indução de apoptose celular (Adinolfi *et al*., 2005; Burnstock & Di Virgilio, 2013; Roger *et al.*, 2014).

Apesar de ser um canal iônico, o receptor P2X7 também poder interagir com 11 diferentes proteínas formando um complexo protéico responsável pela geração do sinal intracelular (Kim *et al*., 2001). Dentre essas 11 proteínas, três são moléculas da matrix extracelular: laminina α3, integrina ß2 e proteína tirosina fostase do tipo receptor ß (RPTPß); e oito são proteínas intracelulares: α-actinina 4, ß-actina, supervilina, três *heat shock proteins* (Hsp90, Hsc71 e HsP70), fosfatidilinositol 4-quinase 230 (PI4K) e guanitalo quinase P55 associada a membrana (MAGuK). Ainda não se sabe exatamente a função de cada proteína nesse contexto, mas estudos sugerem que elas possam controlar o funcionamento do canal iônico do receptor P2X7. Por exemplo, o bloqueio da atividade da enzima RPTPß leva ao aumento da fosforilação da tirosina do receptor P2X7, culminando em maior atividade do mesmo (Kim *et al.*, 2001). Outro caso, é o controle exercido pela *heat shock protein* 90 (Hsp90), cuja fosforilação da tirosina parece levar à inibição da função do receptor P2X7 (Adinolfi *et al*., 2003). Desta forma, a ativação do receptor P2X7 parece ser muito mais complexa do que uma simples abertura de poro na membrana celular e mais estudos para elucidar essas interações são necessários.

O P2X7 é amplamente distribuído em células do sistema imune como monócitos, macrófagos, células dendríticas, linfócitos B e T, mastócitos e

células de Langerhans, onde participa fortemente da resposta imune levando a ativação do inflamassoma e da caspase-1, com conseqüente estímulo da liberação de IL-1ß pró-inflamatória pelos macrófagos e microglia (Ferrari *et al*, 1997; Di Virgilio, 2007). Também pode ser encontrado em células ósseas como osteoblastos (Gartland *et al*., 2001; Gartland *et al*., 2012) e osteoclastos (Gartland *et al*., 2013); em células epiteliais (Garcia-Marcos *et al.*, 2003; Pochet *et al*., 2007) e renais (Hillman *et al*., 2002), sendo portanto importante para a resposta ao ATP extracelular principalmente em células não-excitáveis.

I.5. P2X7 e sua relevância no câncer

Conhecido como “o receptor da morte”, o P2X7 tem sido apontado como o principal receptor envolvido na indução de morte celular pelo ATP extracelular em vários tipos de câncer, como o melanoma (Feng *et al*., 2011; Shabbir & Burnstock, 2009; Bian *et al*., 2013), carcinomas de células escamosas (Deli & Csernoch, 2008), glioma (Gehring *et al*., 2012), câncer cervical (Wang *et al*., 2004a) e câncer endotelial (Li *et al*., 2006). A formação do poro na membrana plasmática, característica desse receptor quando exposto à altas concentrações de ATP ou benzoil-ATP (BzATP), leva ao aumento da concentração intracelular de cálcio ($[Ca^{2+}]_i$), à reorganização do citoesqueleto, à externalização da fosfatidil serina na membrana plasmática, à formação de corpos apoptóticos e conseqüentemente indução de apoptose celular (Feng *et al.*, 2014). Ainda, baixos níveis de expressão ou defeito no seu funcionamento estão sendo associados ao desenvolvimento do câncer, visto que esse seria um mecanismo de escape das células tumorais ao efeito pró-apoptótico deste receptor (Huang *et al*., 2013).

Sendo assim, durante a última década, muitos estudos tem avaliado a expressão do receptor P2X7 em diferentes tecidos tumorais e em linhagens celulares de câncer com a finalidade de utilizá-lo como um marcador diagnóstico e/ou como um alvo terapêutico no tratamento do câncer (Feng *et al.*, 2014, Roger *et al.*, 2014). No entanto, a expressão do receptor P2X7 e a sua funcionalidade parecem variar de acordo com o tipo celular e com o método utilizado para a sua detecção. Alguns estudos tem demonstrado uma redução na sua expressão, diferentemente de outros que reportam um aumento nos níveis de RNAm, de proteína ou de ambos em comparação ao

tecido normal (Roger *et al.*, 2014). Ainda, nos tumores que expressam P2X7 a sua ativação pode levar à indução de morte celular (efeito anti-cancerígeno) ou ao estímulo da proliferação celular (efeito pró-cancerígeno), dependendo do tipo celular estudado e dos níveis de ATP no meio extracelular (Roger *et al.*, 2014).

I.5.1 Atividade pró-cancerígena

O receptor P2X7 pode contribuir de forma direta e/ou indireta na proliferação celular. Indiretamente, sua ação se dá através da liberação de baixas quantidades de ATP no meio extracelular (Ohshima *et al*., 2010; Hattori *et al*., 2012) e diretamente através da sua atividade trófica, basal, quando não estimulado por grandes quantidades de ATP exógeno (Di Virgilio *et al.*, 2009).

O seu papel no crescimento celular já foi descrito em vários tipos tumorais. Em células de melanoma (B16/F10), o bloqueio farmacológico do receptor P2X7 com o antagonista A438079 ou a redução da sua expressão gênica com RNA de interferência (RNAi), levou a uma redução significativa da proliferação celular *in vitro*. Ainda, camundongos inoculados com células B16/F10 que foram tratados com o antagonista do P2X7, ATP oxidado (oATP), apresentaram uma redução significativa no crescimento tumoral quando comparados com o grupo controle (Hattori *et al*., 2012). Em células de neuroblastoma (Neuro-2), o tratamento das células com apirase (enzima que hidrolisa ATP) ou oATP ou BBG (antagonista inespecífico do P2X7) levou a uma diminuição significativa da viabilidade celular (Wu *et al*., 2009). Em células de carcinoma de ovário (SKOV-3 e CAOV-3) o estímulo do P2X7 com o seu agonista seletivo BzATP gerou um aumento nos níveis de $[Ca^{2+}]_i$ e da fosforilação da AKT (pAKT) e ERK (pERK), duas principais vias de sinalização da proliferação celular. Ainda, esse efeito foi completamente bloqueado pelo uso do antagonista do P2X7, A438079. De acordo com os autores, a atividade basal do receptor P2X7, induzida pela auto-liberação de ATP, gerou o aumento dos níveis de pAKT e pERK, o que culminou na manutenção da viabilidade celular (Vazquez-Cuevas *et al*., 2014).

A idéia de que o estímulo basal do receptor P2X7 seja crucial para a manutenção da proliferação celular vem sendo defendida por Di Virgilio e

colaboradores (Di Virgilio *et al.*, 2009). Segundo estes autores, a atividade basal do P2X7 poderia suportar diferentes aspectos celulares que são críticos para os processos de transformação oncogência, proliferação celular e crescimento tumoral. Para comprovar esta hipótese, células humanas embriônicas de rim (HEK293) foram transfectadas com o receptor P2X7 humano *full-lenght*. Diferentemente das células controle, as células transfectadas apresentaram uma alta capacidade de permeabilização, um alto potencial mitocondrial de repouso, uma maior concentração basal de Ca^{2+} mitocondrial e uma maior concentração de ATP intracelular, o que resulta numa maior atividade mitocondrial. Esta última, por sua vez, leva ao aumento da proliferação celular e confere as células resistência para crescer em meio desprovido de soro. Interessantemente, todos esses efeitos foram abolidos na presença de apirase, demonstrando a dependência do estímulo tônico do P2X7 pelo ATP liberado de forma autócrina/parácrina no meio extracelular. Como o esperado, o estímulo contínuo do P2X7 à altas concentrações de ATP exógeno resultaram em morte celular (Adinolfi *et al*., 2005). Os mesmos autores demonstraram ainda o crescimento tumoral dessas células transfectadas com P2X7 *in vivo.* De acordo com esse estudo, as células HEK293 expressando P2X7 apresentaram um fenótipo mais tumorigênico e anaplástico, secretaram altas quantidades de VEGF e possuíram um alto potencial angiogênico quando comparado com as células controle. Além disso, o crescimento tumoral foi reduzido pela injeção intratumoral de oATP e de um anticorpo anti-VEGF (Avastin) (Adinolfi *et al.*, 2012).

Além do efeito na proliferação celular, o receptor P2X7 também parece ter um papel importante na indução da migração e invasão tumoral. Estudos realizados com células de glioma (C6) mostram que o estímulo com BzATP produz um aumento na expressão gênica (RNAm) e protéica do P2X7. Ainda, esse tratamento com BzATP leva a um aumento significativo na migração celular (Wei *et al*., 2008). Estudos com linhagens de células de câncer de mama altamente invasivas como MDA-MB-435 mostram que a ativação do receptor P2X7 pelo ATP leva à liberação de cisteíno-catepsinas responsáveis pela degradação da matriz extracelular, facilitando desta forma a invasão tumoral. E mais, o bloqueio farmacológico do P2X7 tanto por antagonistas

competitivos quanto não-competitivos, assim como a redução da sua expressão pelo uso de siRNA, inibem significativamente a capacidade de invasão basal das células, ou seja, na ausência de estímulo exógeno pelo ATP (Jelassi *et al*., 2011; Jelassi *et al*., 2013). Estes dados reforçam a hipótese de que a ativação basal do receptor P2X7 pelo ATP endógeno liberado de forma autócrina/parácrina pelas células tumorais locais é importante para o efeito pró-tumoral do P2X7 (Roger *et al.*, 2014). Em contrapartida, estudos com linhagens celulares de câncer de pulmão, A549, PC-9 e H292, demonstram que tanto a ativação do P2X7 pelo ATP e BzATP exógenos, quanto pelo ATP endógeno, levam ao aumento da invasão celular. Ainda, esse efeito é bloqueado tanto pelo antagonista sintético (A438079) quanto pelo antagonista natural (emodin) do receptor P2X7 (Jelassi *et al*., 2013; Takai *et al.*, 2012. Takai *et al*., 2014).

Um último ponto a ser considerado é o efeito do receptor P2X7 na adaptação celular metabólica ao microambiente tumoral. Em algumas regiões tumorais de intensa taxa de proliferação, ocorrem pontos de hipóxia com conseqüente liberação e ativação do fator induzível por hipóxia 1 humano (HIF-1α). Em linhagens de câncer de mama MCF-7 e MDA-MB-231 a produção de HIF-1α leva ao aumento da expressão do receptor P2X7, que por sua vez ativa as vias ERK1/2 e AKT e aumenta a atividade das metaloproteinases MMP-2 e 9 (Tafani *et al*., 2013). Sendo assim, o receptor P2X7 parece ser não apenas responsável pela manutenção da viabilidade celular como também pela invasão tumoral.

I.5.2 Atividade anti-cancerígena

Diferentemente, quando estimulado por altas concentrações de ATP, por um longo período de tempo, o receptor P2X7 passa a ter uma atividade anti-tumoral, induzindo apoptose em muitos tipos de células cancerígenas. Desta forma, acredita-se que os níveis de ATP encontrados no meio extracelular e o tempo de ativação do receptor P2X7 é que irão ditar o efeito final sobre o tumor (Figura 2) (Roger *et al*, 2014).

Vários estudos utilizando linhagens celulares de câncer de mama (MCF-7, MDA-MB-231) (Huang *et al*., 2013), carcinoma de células escamosas (A431) (Greig *et al*., 2003), adenocarcinoma de cólon (HCT8, Caco-2, MCA38) (Coutinho-Silva *et al*., 2005; Bian *et al*., 2013), melanoma (A375, B16) (White et

al., 2005b; Bian *et al*., 2013) e glioma (GL261) (Tamajusuku *et al*., 2010) relatam a indução de apoptose mediada pelo receptor P2X7 após o estímulo exógeno de altas concentrações de ATP ou de BzATP. Nestes estudos a formação do poro com conseqüente aumento da permeabilização da membrana e do $[Ca^{2+}]_i$ são os fatores que precedem a ativação do processo de apoptose.

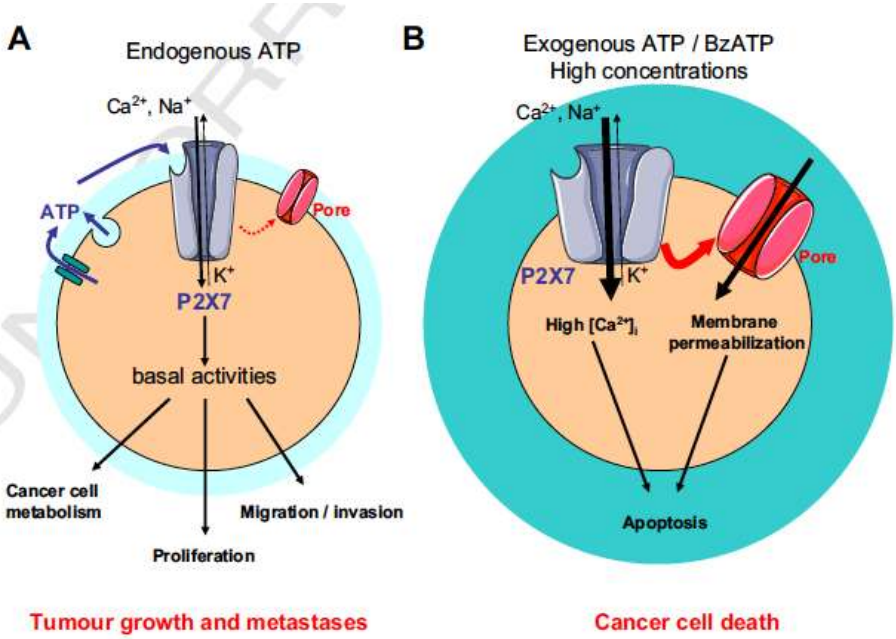


Figura 2. Representação esquemática do papel do receptor P2X7 na biologia celular do câncer epitelial. (A) Em células de câncer epitelial, a liberação autócrina/parácrina de ATP, por difusão transmembranar ou por liberação de vesículas, leva à formação de um halo pericelular que ativa o receptor P2X7 num nível basal, com fraca ou sem permeabilização da membrana e que culmina no aumento do metabolismo, proliferação, migração e invasão celular. (B) A estimulação exógena do P2X7 com altas concentrações de ATP ou BzATP promove uma hiper-ativação do receptor, com conseqüente aumento da concentração do Ca^{2+} intracelular e da permeabilização da membrana. Nesse nível de ativação, o receptor P2X7 induz morte celular via apoptose. Adaptado de Roger *et al.,* 2014. Copia autoriza por *Elsevier,* número da licença 1982084.

O efeito do ATP e BzATP, combinados ou não com a irradiação (2Gy), na morte de linhagens celulares humanas de glioma radioresistentes (U-138MG e U-251MG) e radiosensíveis (M059J) também foram relatadas (Gehring *et al.,* 2012). De acordo com os autores, as células sensíveis à radioterapia apresentaram maiores níveis de expressão protéica do receptor P2X7 e foram também sensíveis aos efeitos citotóxicos de agonistas do receptor. Em contrapartida, as células resistentes à radiação apresentaram

menores níveis de expressão do receptor e foram também resistentes à morte celular induzida tanto pelo ATP quanto pelo BzATP. Neste estudo, os autores enfatizaram a relevância da ativação do receptor P2X7 no aumento da sensibilidade dos gliomas à radioterapia, mas ressaltam a necessidade de mais estudos para provar essa hipótese.

O efeito anti-tumoral do P2X7 também foi demonstrado em estudos *in vivo.* Camundongos atímicos inoculados com células humanas de melanoma A375 e tratados diariamente com 50 mM de ATP por 39 dias apresentaram uma redução no volume tumoral de 50% e nenhuma perda no peso corporal quando comparados com os animais controle (White *et al.,* 2009). Em um modelo animal de câncer de pele induzido pela administração cutânea do agente carcinogênico DMBA (7,12-dimetilbenzantraceno) e do promotor carcinogênico TPA (acetato de tetradecanoilforbol), a aplicação tópica de 100 μM de BzATP levou a uma redução no tamanho da lesão tumoral e a um aumento na sobrevida dos animais (Fu *et al*., 2009). Em ambos os estudos os autores propõem o uso de agonistas do receptor P2X7 como uma nova forma de tratamento do câncer.

Recentemente Dr. Robson e seu grupo de pesquisa descreveram uma nova via de sinalização envolvida no processo de indução de morte celular após ativação do receptor P2X7 (Bian *et al*., 2013). De acordo com os autores, após o estímulo agudo das células de adenocarcinoma de cólon (MCA38) e de melanoma (B16/F10) com 5 mM de ATP e 2 mM de BzATP, a via de sinalização PI3K/AKT é inibida e a via AMPK-PRAS40-mTOR é estimulada de forma integrada e dependente do receptor P2X7, resultando em morte celular. Nesse trabalho, os autores demonstraram pela primeira vez o papel da autofagia na indução de morte celular via ativação do receptor P2X7. A autofagia é um processo celular fisiológico que leva a degradação e reciclagem de componentes do citosol e organelas celulares danificadas a fim de manter a homeostase celular em condições adversas, como privação de nutrientes, presença de patógenos e toxinas (Filippi-Chiela *et al*., 2011). Quando hiper estimulada a autofagia induz à morte celular ao invés de auxiliar na sobrevivência e adaptação celular (He and Klionsky, 2009; Yang and Klionsky, 2010). O receptor P2X7 parece induzir o processo de autofagia pelo fato de

causar um desequilíbrio dos níveis dos nucleotídeos de adenina intracelulares, promovendo um aumento da razão AMP/ATP, que representa o sinal intracelular de gasto metabólico.

Mais que um efeito na indução da morte celular, a ativação do receptor P2X7 por agonistas exógenos também parece ser importante para a inibição da migração celular. Um estudo recente utilizando linhagens celulares de câncer de mama (MDA-MB-231, Py8119) mostra que o tratamento das células com o análogo não-hidrolizável do ATP, ATPγS, reduz o crescimento e a migração celular *in vitro* e inibe o crescimento tumoral *in vivo* (Zhou *et al*., 2014).

Considerando todos esses estudos, pode-se dizer que o efeito anti-proliferativo ou pró-apoptótico do receptor P2X7, após o estímulo exógeno, está intimamente associado com a sua capacidade de indução da permeabilização da membrana plasmática. Acredita-se que em alguns tipos de câncer, apesar do receptor P2X7 ser totalmente funcional como um canal iônico, ele não é capaz de induzir a permeabilização da membrana, devido a presença de uma variante truncada na região C-terminal do receptor ou a sua incapacidade de se ligar a canais formadores de poros como a panexina-1. Sendo assim, nesses casos, o receptor P2X7 pode estar envolvido com o controle de outros processos celulares como a invasão celular, por exemplo. Se esta hipótese for verdadeira, a indução seletiva da permeabilização da membrana plasmática em células tumorais e não a manipulação do receptor P2X7 *per se* pode representar uma nova estratégia no tratamento do câncer (Roger *et al*., 2014).

I.6. Expressão e funcionalidade do receptor P2X7 nos tecidos tumorais sólidos

1.6.1 Câncer cervical

O câncer da cérvice uterina é a quarta neoplasia mais frequente entre as mulheres no mundo, sendo que para o ano de 2012 foram esperados cerca de 528.000 novos casos (GLOBOCAN, 2012). No Brasil, esta patologia detém o terceiro lugar em incidência e o quarto em mortalidade e no ano passado as estatísticas apontavam cerca de 17.540 novos casos (INCA, 2014). Essa

diferença na incidência se deve à falta de acesso da população ao sistema de screening e prevenção, como o exame do Papanicolaou e atualmente à vacina contra o HPV (vírus do papiloma humano) nas regiões menos desenvolvidas.

Embora a maioria dos casos de câncer cervical sejam relacionados com a infecção pelo HPV, apenas a presença do vírus não é suficiente para desencadear o processo de carcinogênese. Para que a ocorra a progressão tumoral é necessário que alterações nos processos de crescimento e diferenciação das células epiteliais também estejam presentes (Schiffman *et al.*, 2011). É nesse contexto que o estudo do receptor P2X7 se tornou um alvo interessante no entendimento da biologia celular do câncer cervical.

Várias publicações tem relacionado a redução da expressão do receptor P2X7 com o desenvolvimento do câncer cervical. Gorodeski e colaboradores foram os primeiros a verificar que os níveis de RNAm e proteína do receptor P2X7 são menores em células escamosas de carcinoma escamoso e em adenocarcinomas endocervical e endometrial quando comparados com células epiteliais normais provenientes da região do endométrio, da endocérvice e da ectocércive (Li *et al*., 2006; Li *et al*., 2007; Li *et al*., 2009). Ainda, lesões hiperplásicas pré-cancerígenas do endométrio já apresentavam uma expressão reduzida do receptor quando comparado ao tecido normal.

Essa redução da expressão do receptor P2X7 pode ser justificada pela presença dos micro RNAs miR-186 e miR-150 que estão aumentados nas células de câncer cervical (Zhou *et al*., 2008). Ou ainda, pela presença de uma variante truncada do receptor P2X7 (P2X7j) que parece interferir na ativação do receptor *full leght* (P2X7A). Essa variante é desprovida da região C-terminal e portanto é incapaz de formar poros e induzir apoptose. A expressão de P2X7j é similar nas células normais e de câncer cervical, mas a expressão do receptor P2X7A é maior nas células normais. Sendo assim, acredita-se que a variante P2X7j apresente um efeito inibitório sobre o receptor P2X7A, que se torna incapaz de induzir apoptose mesmo sob o estímulo do BzATP (Feng *et al*., 2006a; Feng *et al*., 2006b).

Esses estudos indicam que o receptor P2X7, bem como a sua variante truncada (P2X7j) podem ser usados como novos biomarcadores no estudo do câncer cervical.

1.6.2 Câncer de cólon e reto

O câncer de cólon e reto é o terceiro tipo de câncer mais frequente entre os homens (746.000 casos) e o segundo entre as mulheres (614.000 casos) em todo mundo (GLOBOCAN, 2012). No Brasil, as estatísticas apontam as mesmas frequencias encontradas para o mundo, sendo que para o ano de 2014 foram esperados cerca de 15.070 novos casos para homens e 17.530 novos casos para mulheres (INCA, 2014). Diferentemente do câncer cervical, o câncer de cólon e reto é mais frequente em países mais desenvolvidos e está intimamente relacionado com os hábitos de alimentares e de vida.

Estudos sobre a expressão tecidual do receptor P2X7 em tumores de cólon e reto são limitados e não conclusivos. Um estudo realizado com biópsias de tecido humano mostrou que o receptor P2X7A encontra-se na região basolateral do epitélio intestinal normal, enquanto que em células de adenocarcinoma de cólon o receptor encontra-se amplamente distribuído nas células. Ainda, em todos os casos estudados não foram encontrados diferenças nos níveis de receptor P2X7 nas células normais e de adenocarcinoma (Li *et al*., 2009).

Considerando a alta incidência do câncer de cólon e reto no mundo e no Brasil, a busca de novos marcadores moleculares que possam ajudar no seu diagnóstico, prognóstico e tratamento se torna de suma importância. Com relação ao receptor P2X7, mais estudos são necessários a fim de avaliar o seu papel no desenvolvimento desse tipo tumoral.

I.7 Sinalização Purinérgica como alvo terapêutico no câncer

Duas abordagens terapêuticas podem ser consideradas no uso da sinalização purinérgica no tratamento do câncer, o sistema imune do hospedeiro e/ou o próprio tecido tumoral (Di Virgílio, 2012). Com relação ao sistema imune do hospedeiro, sabe-se que a adenosina apresenta um papel importante na imunossupressão enquanto o ATP exerce um papel crucial na ativação da via P2X7/inflamassoma, levando ao recrutamento das células imunes à região tumoral (Ghiringhelli *et al*., 2009; Michaud *et al*., 2011).

Vários modelos experimentais de tumores mostram que uma redução nos níveis de adenosina está claramente relacionada à uma parada da progressão tumoral e à prevenção de metástases (Stagg *et al*., 2011). Essa redução da concentração de adenosina no interstício tumoral pode ser alcançada por meio da inibição da atividade das enzimas CD39 e/ou da CD73, ou alternativamente por meio do aumento da concentração da enzima ADA na região tumoral através do uso de um conjugado PEG-ADA direcionado ao tumor (Sauer *et al*., 2012). O bloqueio da enzima CD39, pelo uso de inibidores ou anticorpos monoclonais parece ser a melhor estratégia terapêutica visto que a sua inibição resulta tanto no bloqueio da formação de adenosina, quanto no acúmulo do ATP (Di Virgilio, 2012; Feng *et al*., 2014).

Com relação a abordagem terapêutica no tecido tumoral, a manipulação da sinalização purinérgica é mais complicada e controversa. Levando-se em conta o efeito pró-apoptótico do receptor P2X7 quando exposto a altas concentrações de ATP ou BzATP (Wang *et al*., 2004a), o uso de agonistas seletivos com a finalidade de induzir a morte do tecido tumoral pode ser apontada com uma opção terapêutica (White & Burnstock, 2006). No entanto, considerando-se a atividade pró-tumoral do receptor P2X7, a estratégia oposta também é valida e o uso de antagonistas do receptor pode ajudar a prevenir o crescimento tumoral e a formação de metástases (Roger & Pelegrin, 2011). Experimentos *in vivo* mostram que as duas opções são válidas, mas o resultado final varia de acordo com o tipo de tumoral (Di Virgilio, 2012).

O uso da adenosina também pode ser considerado controverso, levando-se em conta o seu efeito anti ou pró-tumoral. De qualquer forma, o efeito final da administração de agonistas ou antagonistas dos receptores P1 vai depender dos diferentes subtipos de receptores presentes no tecido tumoral, podendo resultar tanto em estímulo da proliferação, liberação de VEGF ou apoptose (Di Virgilio, 2012). Dentre todos os receptores P1, o receptor A3 parece ser o alvo terapêutico mais promissor no combate ao câncer (Varani *et al*., 2011).

Finalmente, alguns estudos clínicos avaliaram a aplicação terapêutica do ATP intravenoso em pacientes com câncer (Agteresch *et al*., 2000; Beijer *et al*., 2009). Os resultados mostram que apesar de ser bem tolerado e melhorar o

estado nutricional e a qualidade de vida dos pacientes, o ATP não teve efeito significativo na redução do tamanho tumoral.

Apesar de ambíguo, o sistema purinérgico, com os seus nucleotídeos de adenina, receptores e enzimas está emergindo com um novo e importante alvo terapêutico no tratamento do câncer. No entanto, mais estudos são necessários para que o real papel desse sistema seja compreendido e possa ser implementado na prática clínica.

Considerando essa necessidade, a presente tese busca trazer novas informações sobre o papel dos nucleotídeos de adenina e do receptor P2X7 no desenvolvimento e indução de morte celular do câncer cervical e de cólon. Esse trabalho se apóia na hipótese de que o receptor P2X7 possa estar envolvido com o mecanismo de morte e resistência celular, podendo ser um novo alvo para a pesquisa terapêutica do câncer e também um possível marcador de agressividade tumoral.

II. Objetivos

Considerando o papel do receptor P2X7 na indução de morte celular em alguns tipos tumorais e a busca urgente por novos alvos terapêuticos no tratamento do câncer esta tese tem como objetivo geral estudar o envolvimento do receptor P2X7 no mecanismo de morte e desenvolvimento tumoral em linhagens celulares de carcinoma cervical e adenocarcinoma de cólon.

Assim, os seguintes objetivos específicos foram propostos:

1. Investigar o mecanismo pelo qual o ATP induz apoptose na linhagem de carcinoma cervical SiHa, via ativação dos receptores P2X7;

2. Investigar o efeito do ATP na proliferação e indução de apoptose, na linhagem celular SiHa silenciada para o gene do receptor P2X7;

3. Avaliar o efeito dos nucleotídeos da adenina (ATP, ADP e AMP) e do nucleosídeo adenosina, na proliferação e morte celular, em células de carcinoma cervical HeLa e C33A;

4. Avaliar o efeito do *Heat Shock* e do ATP na indução de morte celular pelo receptor P2X7, utilizando linhagens celulares de adenocarcinoma de cólon (MCA38), silenciadas ou não para o gene do receptor P2X7.

5. Investigar o mecanismo envolvido nesse efeito citotóxico do *Heat Shock* + ATP.

III. Artigos Científicos

III. 1. CAPÍTULO 1 - Paola de Andrade Mello, Eduardo C. Filippi-Chiela, Jéssica Nascimento, Aline Beckenkamp, Danielle Santana Bertodo, Fraciele Kipper, Emerson André Casali, Alessandra Nejar Bruno, Juliano Paccez, Luiz Fernando Zerbini, Márcia Rosângela Wink, Guido Lenz and Andréia Buffon. **Adenosine uptake is the major effector of extracellular ATP toxicity in human cervical cancer cells.**

Artigo publicado na Revista *Molecular Biology of the Cell* no mês de Outubro de 2014. *Mol Biol Cell. 2014 Oct 1;25(19):2905-18. doi: 10.1091/mbc.E14-01-0042. Epub 2014 Aug 7*

MBoC | ARTICLE

Adenosine uptake is the major effector of extracellular ATP toxicity in human cervical cancer cells

Paola de Andrade Mello[a], Eduardo Cremonese Filippi-Chiela[b], Jéssica Nascimento[a], Aline Beckenkamp[a], Danielle Bertodo Santana[a], Franciele Kipper[b], Emerson André Casali[c], Alessandra Nejar Bruno[d], Juliano Domiraci Paccez[e], Luiz Fernando Zerbini[e], Marcia Rosângela Wink[f], Guido Lenz[b], and Andréia Buffon[a]

[a]Laboratory of Biochemical and Cytological Analysis, Faculty of Pharmacy, Federal University of Rio Grande do Sul, Porto Alegre, RS 90610-000, Brazil; [b]Department of Biophysics and Center of Biotechnology, Federal University of Rio Grande do Sul, Porto Alegre, RS 91501-970, Brazil; [c]Department of Morphological Science and Department of Biochemistry, Institute of Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS 90000-000, Brazil; [d]Federal Institute of Education, Science and Technology, Porto Alegre, RS 90035-007, Brazil; [e]International Center for Genetic Engineering and Biotechnology, Cancer Genomics Group, Cape Town 7925, South Africa; [f]Laboratory of Cell Biology, Federal University of Health Sciences of Porto Alegre, Porto Alegre, RS 90050-170, Brazil

ABSTRACT **In cervical cancer, HPV infection and disruption of mechanisms involving cell growth, differentiation, and apoptosis are strictly linked with tumor progression and invasion. Tumor microenvironment is ATP and adenosine rich, suggesting a role for purinergic signaling in cancer cell growth and death. Here we investigate the effect of extracellular ATP on human cervical cancer cells. We find that extracellular ATP itself has a small cytotoxic effect, whereas adenosine formed from ATP degradation by ectonucleotidases is the main factor responsible for apoptosis induction. The level of P2×7 receptor seemed to define the main cytotoxic mechanism triggered by ATP, since ATP itself eliminated a small subpopulation of cells that express high P2×7 levels, probably through its activation. Corroborating these data, blockage or knockdown of P2×7 only slightly reduced ATP cytotoxicity. On the other hand, cell viability was almost totally recovered with dipyridamole, an adenosine transporter inhibitor. Moreover, ATP-induced apoptosis and signaling—p53 increase, AMPK activation, and PARP cleavage—as well as autophagy induction were also inhibited by dipyridamole. In addition, inhibition of adenosine conversion into AMP also blocked cell death, indicating that metabolization of intracellular adenosine originating from extracellular ATP is responsible for the main effects of the latter in human cervical cancer cells.**

Monitoring Editor
Carl-Henrik Heldin
Ludwig Institute for Cancer Research

Received: Feb 6, 2014
Revised: Jun 23, 2014
Accepted: Jul 28, 2014

INTRODUCTION

Cervical cancer, although easily preventable by Papanicolaou screenings, is still high in the rank of cancers affecting women, with the third-highest incidence and fourth-highest fatality rate among females worldwide (Jemal *et al.*, 2011). Although almost all cases of cervical cancer are linked with human papillomavirus (HPV) infection, virus presence per se is not sufficient to trigger carcinogenesis. Acute infection with carcinogenic-type HPV is the first step, followed by viral persistence (rather than clearance) and subsequent precancer development and invasion (Schiffman *et al.*, 2011). This dynamic process suggests that alterations in growth and differentiation of epithelial cells are involved in cancer progression. Thus, understanding cervical cell biology and the mechanisms by which cells become altered during cancer development is extremely necessary.

Among the extracellular purines, ATP is a key extracellular signaling molecule and participates in several physiological processes, including immune response, neurotransmission, vascular tonus,

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E14-01-0042) on August 7, 2014.

Address correspondence to: Andréia Buffon (andreia.buffon@ufrgs.br).

Abbreviations used: ATPγS, adenosine 5′-*O*-(3 thiotriphosphate); BzATP, 2′,3′-*O*(4-benzoylbenzoyl)ATP; DIP, dipyridamole; EGTA, ethylene-bis(oxyethylenenitrilo) tetraacetic acid; LDH, lactate dehydrogenase; oATP, oxidized ATP; zAsp, zAsp-CH2-DCB.

Supplemental Material can be found at:
http://www.molbiolcell.org/content/suppl/2014/08/02/mbc.E14-01-0042.DC1.html

pain sensation, cell proliferation, differentiation, development, and death (Lee *et al.*, 2006; Tamajusuku *et al.*, 2010). To execute these functions, ATP can act through two types of purine receptors: P2X receptors, which are ligand-gated ion channels; and P2Y receptors, which are G protein–coupled receptors (Burnstock *et al.*, 2012).

Among the P2X receptors, only the P2×7 subtype is able to form a pore permeable to hydrophilic molecules up to 900 Da in size when activated by high ATP concentration over relatively long periods. This pore formation leads to an increase in intracellular cytosolic free calcium ions and the induction of cell death (Burnstock *et al.*, 2012). High levels of extracellular ATP and 2′,3′-*O*(4-benzoylbenzoyl)ATP (BzATP, a P2×7 receptor agonist) induce a significant reduction in keratinocyte number in primary human keratinocyte cultures, indicating the role of P2×7 in the control of epithelial growth (Burnstock *et al.*, 2012). In the same way, P2×7 was found to control baseline apoptosis in normal human ectocervical epithelial cells (hECEs) through an autocrine/paracrine mechanism involving ATP secretion by cells, P2×7 activation, cytosolic calcium influx, and mitochondrial apoptotic pathway induction (Wang *et al.*, 2004). Finally, Bian *et al.* (2013) described a role for P2×7 in ATP-induced autophagy in melanoma and colon cancer cells through the modulation of two important intracellular pathways involved in cell growth and death, phosphoinositide 3-kinase (PI3K)/Akt and AMP-activated protein kinase (AMPK)/PRAS40/mTOR. However, the role of autophagy in this context was not assessed.

Autophagy is a physiological mechanism involved in the degradation of old and/or injured cell components. It is triggered by metabolic alterations, such as nutrient deprivation or hypoxia, toxins, cytotoxic drugs, or other stressful conditions, and interferes with cell fate in a dual manner: it contributes to cell survival and adaptation in an adverse context but can contribute to cell death if triggered in high levels or for a long time (He and Klionsky, 2009; Yang and Klionsky, 2010). Two important components in this process are the proteins LC3 and p62. LC3 (microtubule-associated protein 1 light chain 3 α) is cytosolic (LC3 I) and, after proautophagic stimulus, is lipidated to form LC3 II (Kabeya *et al.*, 2004). P62 (sequestosome 1), on the other hand, marks cell components to be targeted to autophagosomal degradation and directly interacts with LC3 II in the autophagosomal membrane, being reduced when autophagic flux is increased (Pankiv *et al.*, 2007; Matsumoto *et al.*, 2011).

In addition to the direct cytotoxic effect described by extracellular ATP through P2×7 activation, other adenine nucleotides and adenosine can act as cell death inducers. Among them, adenosine, historically recognized as a key modulator of tumor progression, has emerged as an important factor in cell death and differentiation (Saitoh *et al.*, 2004; Sai *et al.*, 2006). Extracellular adenosine at high concentration can induce apoptosis in a variety of cancer cells via an intrinsic and/or an extrinsic pathway. The former pathway was described in human epithelial cancer cells originating from breast, colon, and ovary and is marked by adenosine uptake into cells via specific transporters, conversion to AMP by adenosine kinase, and AMPK activation (Saitoh *et al.*, 2004). The latter pathway was found in glioma cells, myeloid leukemia cells, mammary carcinoma cells, embryonic epithelial cells, granulose cells, thymocytes, and B lymphocytes and neutrophils and is characterized by adenosine as activation of the A_1, A_{2a}, A_{2b}, and A_3 receptors (Saitoh *et al.*, 2004; Sai *et al.*, 2006; Tsuchiya *et al.*, 2012). Depending on cell type, intracellular adenosine can induce apoptosis by a caspase-dependent or -independent mechanism (Tsuchiya *et al.*, 2012).

Tumor microenvironment is rich in extracellular ATP (Pellegatti *et al.*, 2008) and adenosine (Ohta *et al.*, 2006; Ghiringhelli *et al.*, 2012), and the effect of these molecules depends on both ATP concentration and the rate of ATP degradation to adenosine by ectonucleotidases present at the extracellular membrane (Beckenkamp *et al.*, 2014), as well as on the panel of P2 receptor expressed by the tumor (Di Virgilio, 2012; Burnstock and Di Virgilio, 2013). Therefore, to understand this complex network in the context of cancer development, here we studied the effect of extracellular ATP and its metabolite adenosine on cervical cancer cells. Therefore we investigated the importance of P2×7 and the mechanism underling ATP toxicity on cervical cancer, searching for a possible new target for diagnosis, prognosis, or treatment of this neoplasia.

RESULTS

SiHa cell line expresses high mRNA levels of the P2×7 receptor

Because lower levels of P2×7 were described in epithelial cancer cells than in normal tissue (Li *et al.*, 2009), we investigated the levels of P2×7 mRNA in different cervical cancer cell lines and in an immortalized human epithelial cell line (HaCaT) used as nontumorigenic control cells (Figure 1A). Cervical cancer cell lines (SiHa, HeLa, and C33A) and HaCaT exhibited different amounts of P2×7 mRNA. Among them, the SiHa cell line presented the highest levels of P2×7 mRNA, and therefore we chose it to investigate the role of P2×7 in the response of cervical cancer to extracellular ATP.

Extracellular ATP promotes cell death in a dose- and time-dependent way

To initially assess the cytotoxic effect of extracellular ATP, we treated cervical cancer cells with increasing doses of ATP for 24 h, with a maximum cytotoxic effect of 30% with 5 mM (Figure 1B). After 72 h, 5 mM ATP reduced the number of cells by 80% in relation to control (Figure 1C, bottom). Surviving cells had reduced long-term viability, since clonogenic survival of cells that survived 72 h was only 31%, indicating a slow mechanism of cell death (Figure 1C, top).

Extracellular ATP–induced cell death shows features of apoptosis but not necrosis

ATP, 5 mM, led to cell shrinkage in a time-dependent manner, as observed by forward scatter, suggesting apoptotic cell death (Figures 2A and Supplemental Figure S1). Indeed, treatment with extracellular ATP induced only a slight increase of lactate dehydrogenase (LDH) levels in the culture medium after 72 h (Figure 2B) and no increase of propidium iodide (PI) staining (Figure 2C), showing that necrosis was not the primary mechanism of ATP toxicity in SiHa cells. On the other hand, cells presented some phenotypic alterations that resemble apoptosis, including membrane blebbing, cell shrinkage, and chromatin condensation after 48 and 72 h. In agreement, ATP treatment highly increased annexin V staining (Figure 2C), confirming that ATP exerts a cytotoxic effect in SiHa cancer cells mainly through induction of apoptotic cell death.

ATP-induced apoptosis is not prevented by caspases inhibitors or calcium chelators

The main mechanism of P2×7-mediated cell death in normal human ectocervical epithelial cells (hECEs) involves pore formation, calcium influx, and apoptosis induction through caspase activation (Wang *et al.*, 2004). Surprisingly, neither ethylene-bis(oxyethylenenitrilo) tetraacetic acid (EGTA) nor the pancaspase inhibitor zAsp-CH(2)-DCB (zAsp) was able to reduced ATP toxicity (Figure 3A). Of interest, BzATP, a P2×7 agonist, showed a cytotoxic effect in a dose-dependent manner, with $EC_{50} \approx 100$ μM (Figure 3B), and this cytotoxicity was apoptotic and completely blocked by EGTA and zAsp (Figure 3A), suggesting that P2×7 is functional in inducing cell death but

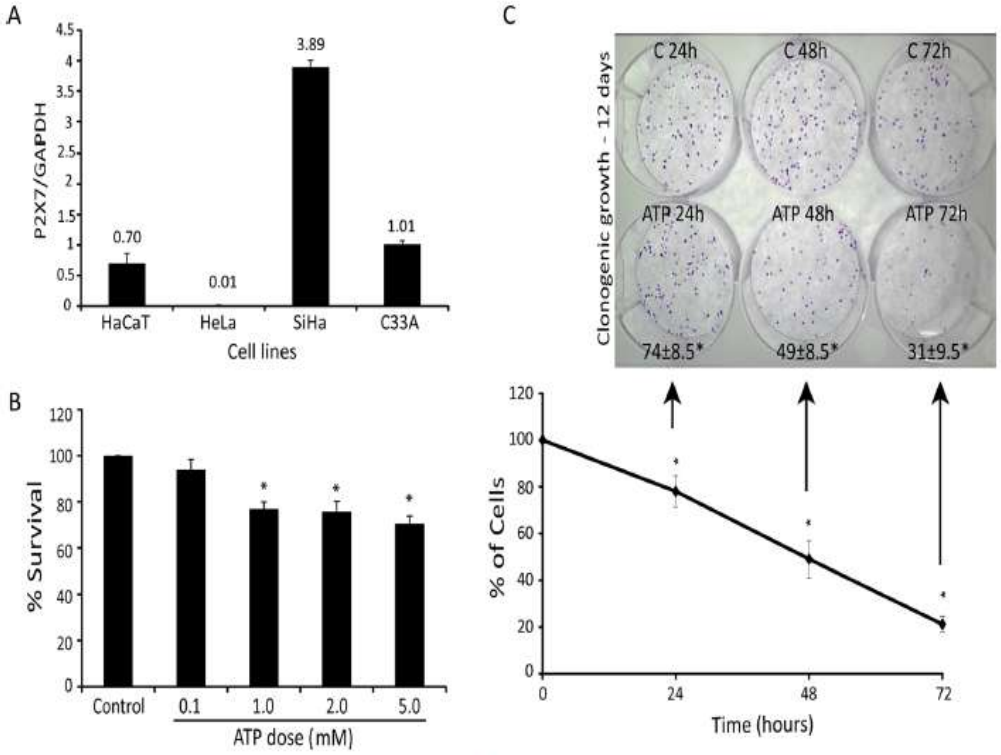


FIGURE 1: Extracellular ATP exerts acute and chronic toxicity in cervical cancer cells. (A) Comparison of P2×7 mRNA expression in SiHa, HeLa, C33A, and HaCaT cell lines by quantitative real-time PCR analysis. Results are presented as the ratio cDNA/GAPDH. (B) SiHa cell viability after exposure to increased ATP concentration for 24 h using MTT assay. (C) Bottom, time curve of SiHa cells treated with 5 mM ATP for 24, 48, and 72 h, determined by number of viable cells not marked by trypan blue. Top, 100 viable cells were seeded in clonogenic assay, and colony formation was evaluated. Numbers at bottom are survival fraction according to clonogenic assay. C, control; ATP, treatment with 5mM ATP. *$p < 0.05$ compared with control (one-way ANOVA, followed by Tukey's test).

does not respond to concentrations of ATP above the described concentration needed for activation of this receptor. In addition, when SiHa cells were treated with ATPγS, a nondegradable ATP form, only ~20% of cells died (Figure 3C), which was completely blocked by calcium chelation and caspase inhibition (Figure 3, C and D), indicating that a degradation product of ATP plays a role in its cytotoxicity.

This relatively small contribution of P2×7 to cell death was confirmed with the use of a P2×7 antagonist, oxidized ATP (oATP), which only partially prevented the effect of ATP (Figure 4A). To reinforce these findings, P2×7 knockdown (KD) SiHa cells (Figure 4B) were slightly more resistant to ATP than were wild-type (WT) or KD control cells (Figure 4C), confirming the partial role of this receptor in the cytotoxic effect of ATP.

ATP treatment preferentially eliminates cells expressing high P2×7 levels

To investigate which cells are killed by ATP directly, we determined the P2×7 protein levels in cells after treatment with ATP in two ways: 1) cells were treated with 5 mM ATP for 24, 48, and 72 h, and the remaining adherent cells were lysed and tested for P2×7 levels (Figure 5A); and 2) cells were treated with 5 mM ATP for 24, 48, and 72 h, followed by medium removal, two washes with 1× phosphate-buffered saline (PBS), and growth in ATP-free medium for an additional 4 d; adherent cells were then collected, and Western blot analysis was performed (Figure 5B). As shown in Figure 5, A and B, in both treatments, cells that remained adherent showed lower P2×7 protein levels than control, indicating that a subpopulation of cells with higher P2×7 levels was eliminated by ATP, as was also observed in glioma cells (Tamajusuku *et al.*, 2010). Together these results suggest that extracellular ATP per se is responsible for only a small part (~20%) of the toxicity of extracellular ATP through the P2×7 receptor and that the remaining cytotoxic effect might be through a metabolite derivative.

Adenosine uptake formed from ATP degradation is the major cytotoxic factor of extracellular ATP

Thus far our data suggest that P2×7 activation per se only eliminates cells with high expression levels of P2×7. To understand where the additional toxicity of ATP comes from, we turned our attention to adenine nucleotides and adenosine formed from ATP by the action of ectonucleotidases, which are expressed in human cervical cancer cells (Beckenkamp *et al.*, 2014). Most of the extracellular ATP was degraded to its metabolites over 72 h (Figure 6A; see also Supplemental Table S1). All of the main metabolites of ATP had toxic effects in cervical cancer cell lines (SiHa, HeLa, and C33A) and a human epithelial cell line (HaCaT), with C33A and HeLa cells presenting a more resistant profile. Of importance, only adenosine significantly reduced cell viability in all cell lines (Figures 6B and Supplemental Figure S3A). Because several studies

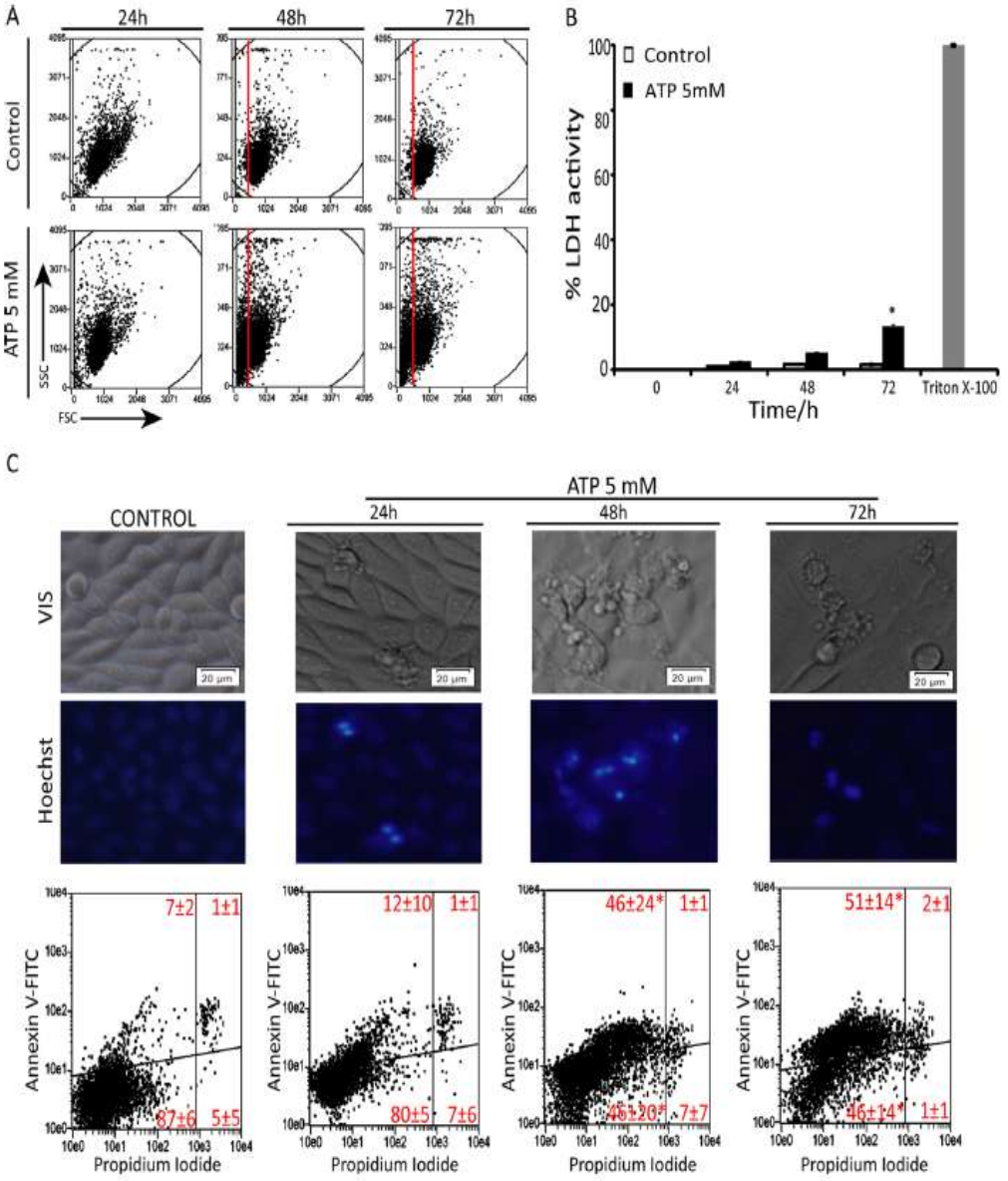


FIGURE 2: Extracellular ATP triggers apoptosis in SiHa cervical cancer cells. (A) Forward scatter analysis after treatment with 5 mM ATP for 24, 48, and 72 h. (B) Loss of membrane integrity measured by LDH release after treatment with 5 mM ATP for 24, 48 and 72 h. Triton X-100 was used as positive control for LDH release. (C) Top, images of SiHa cell treatment with 5 mM ATP for 24, 48, and 72 h. Cell nuclei was stained with Hoescht 35565665 according to manufacturer's instruction. Note apoptotic features such as cell shrinkage and blebbing and fragmented nuclei when cells were treated with ATP. Scale bars, 20 μm; magnification, 20×. Bottom, apoptosis and necrosis measured by annexin V– and PI-positive cells, quantified by flow cytometry in the same conditions as in C. Values refer to average of the percentage of cells in each gate of three independent experiments ± SD. *$p < 0.05$ compared with control (one-way ANOVA, followed by Tukey's test).

attributed a cytotoxic effect to adenosine in other cell types (Saitoh *et al.*, 2004; Sai *et al.*, 2006), we investigated whether uptake of extracellular adenosine formed by ATP degradation, which did not accumulate in extracellular medium (Supplemental Figure S5A), was responsible for apoptosis induction after ATP treatment. We treated SiHa cells with 10 μM dipyridamole (DIP), an inhibitor of adenosine transport, 30 min before ATP exposure (Figure 6C, left). DIP reduced cell shrinkage (Supplemental Figure S1), cell number reduction (Figure 6C, right), and annexin V staining (Figures 6D, right, and Supplemental Figure S1) induced by ATP treatment. Furthermore, phenotypic observation confirmed the reduction of apoptotic features such as cell shrinkage, membrane blebbing, and nuclear condensation and fragmentation (Figure 6D, left and middle), which were present after ATP treatment only (Figure 2C). After 72 h, there was a reduction of 20% in the cell number in DIP plus ATP treatment, which was not altered by DIP replacement each 24 h (unpublished data) but was by knockdown of the P2×7 receptor (Supplemental Figure S2), suggesting that

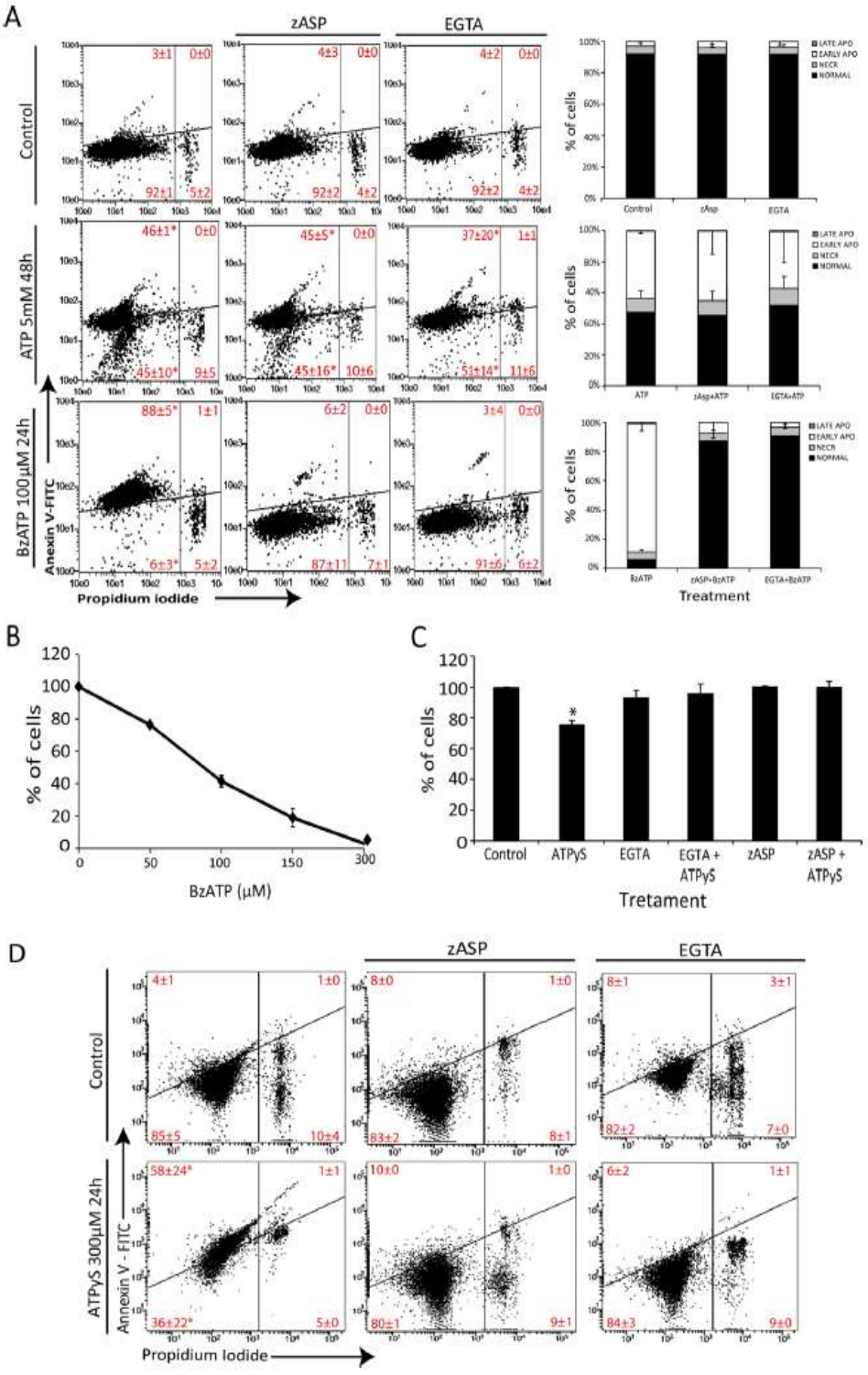


FIGURE 3: Extracellular ATP triggers apoptosis in a caspase- and calcium influx–independent way. (A) SiHa was exposed or not for 30 min to 0.6 mM EGTA or 50 µM zAsp, and then 5 mM ATP or 100 µM BzATP was added for 48 or 24 h, respectively, and apoptosis and necrosis was measured according to annexin V/PI binding (see *Materials and Methods*). Right, average values measured in each gate. NORMAL cells, annexin V–/IP–; NECR cells annexin, V–/IP+; EARLY APO cells, annexin V+/IP–; LATE APO cells, annexin V+/IP+. (B) Dose–response curve of BzATP treatment for 24 h measured with number of viable cells using trypan blue dye exclusion. (C) Number of viable cells not stained with trypan blue, after exposure of SiHa cells to 300 µM nondegradable ATP, ATPγS, for 24 h with or without previous exposure to 0.6 mM EGTA or 50 µM zAsp. (D) Apoptosis and necrosis induction at these same conditions. *$p < 0.05$ compared with other treatments (two-way ANOVA, followed by Bonferroni posttest).

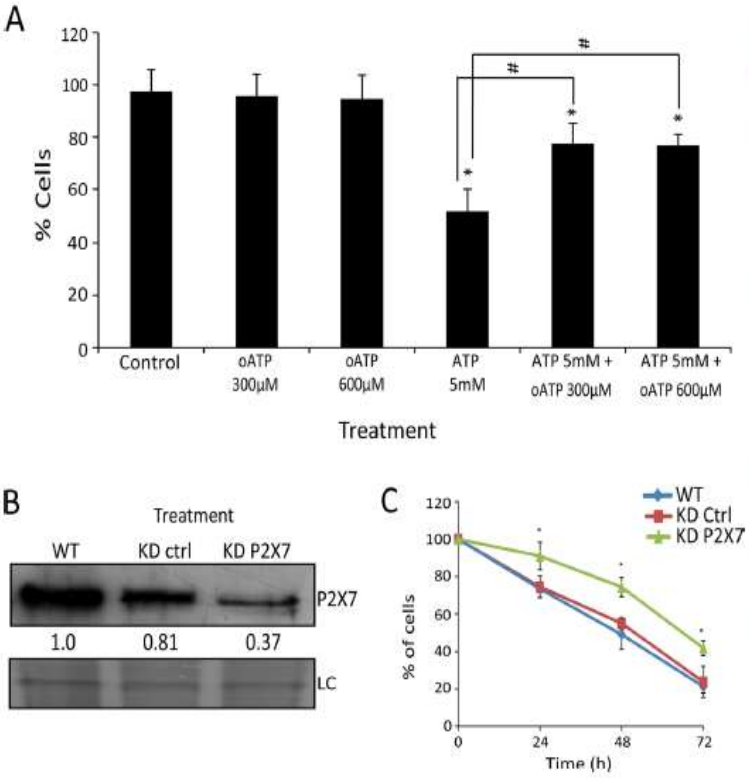


FIGURE 4: P2×7 receptor contributes little to the total cytotoxic effect of extracellular ATP in SiHa cells. (A) Blockage of 5 mM ATP induces cell death by the P2×7 antagonist oATP at two concentrations (300 and 600 μM). Control represents cells without treatment. *$p < 0.05$ for comparison vs. control and #$p < 0.05$ for comparison vs. respective group (two-way ANOVA, followed by Bonferroni posttest). (B) Knockdown for P2×7 confirmed by Western blot. SiHa WT, SiHa wild type; SiHa KD ctrl, SiHa cells transduced with nontarget sequence (knockdown control); SiHa KD P2×7, SiHa knockdown for P2×7. Loading control (LC) represents PVDF membrane stained with Coomassie blue. Numbers represent P2×7 protein amount in relation to SiHa WT. (C) Number of viable cells not marked by trypan blue after 5 mM ATP treatment for 24, 48, and 72 h. *$p < 0.05$ vs. SiHa KD Ctrl and WT at the respective times (one-way ANOVA, followed by Tukey's test).

this slight effect occurs through ATP-mediated P2×7 receptor activation and toxicity and is not due to the loss of action of DIP. Taken together, these results strongly suggest that adenosine uptake, formed by ATP degradation, is a central player in the cell death induced by extracellular ATP. In agreement, inhibition of adenosine kinase by ABT-702 completely reversed ATP-induced apoptosis (Figure 6E, bottom), indicating that intracellular adenosine phosphorylation and conversion to AMP is a key step in the toxicity of extracellular ATP. Indeed, as occurred with DIP plus ATP, there was a reduction of 20% in the number of cells after 72 h of treatment with ABT-702 plus ATP (Figure 6E, top), reinforcing the slight involvement of ATP-P2×7 in ATP-induced cell death and the importance of the metabolization of adenosine to AMP. Corroborating these data, sensitivity of cells to adenosine was strongly positively correlated ($r = 0.9$) with ATP cytotoxic effect in the four cell lines studied. On the other hand, ATP sensitivity at 24 h was not correlated with mRNA P2×7 levels (Supplemental Figure S3). Of interest, when cells were exposed to ATP for 48 or 72 h, the correlation between ATP sensitivity and mRNA P2×7 levels increased (unpublished data), suggesting that P2×7 activation could be important after a long exposure and thus could be involved with the cell death observed after DIP plus ATP at 72 h.

Adenosine uptake promotes dATP accumulation and intracellular nucleotide/nucleoside level imbalance, activates AMPK, increases p53, and induces autophagy

Measurement of intracellular nucleotides/nucleosides, as well as of deoxy-ATP (dATP), after ATP exposure pointed to an imbalance in the pool of nucleotides/nucleosides and an accumulation of dATP. Moreover, all of these intracellular effects were completely blocked by DIP (Supplemental Figure S5, B and C), suggesting that adenosine uptake alters the balance of intracellular nucleotide/nucleoside levels.

Extracellular ATP increased the levels of pAMPK(T172)—the active state of AMPK (Hardie *et al.*, 2006)—in a time-dependent manner, mainly after 48 and 72 h, accompanied by PARP cleavage (Figure 7A). p53 levels reached the highest levels at 48 h and these molecular alterations were suppressed almost completely by DIP pretreatment. Furthermore, DIP pretreatment increased the levels of Bcl2, a classical antiapoptotic protein, after 72 h of ATP treatment. Taken together, these data suggest that adenosine uptake is responsible for the main molecular alterations that underlie the toxicity of extracellular ATP in cervical cancer cells.

The induction of autophagy by extracellular ATP has been described (Bian *et al.*, 2013), but the role of autophagy in this context is poorly understood, despite its importance. Thus we examined the induction of autophagy after ATP treatment, with or without DIP, as well as the role of ATP-induced autophagy. As shown in Figure 7B, 5 mM ATP increased the LC3II/I ratio and decreased the amount of p62 after 48 and 72 h, suggesting autophagy induction. Corroborating data from molecular mechanisms triggered by ATP treatment, this proautophagic effect of ATP was reverted by DIP pretreatment, suggesting that adenosine uptake is responsible for autophagy triggering after ATP treatment.

Finally, the role of ATP-induced autophagy was assessed by pharmacological modulation of this process, followed by cell number evaluation and acridine orange (AO) staining, which is a marker of autophagolysosome formation. ATP alone significantly increased the percentage of AO-positive cells after 24 h. To inhibit autophagy, cells were pretreated with two autophagy inhibitors, 3-methyladenine (3MA) and bafilomycin A1 (Baf), whereas autophagy was activated by rapamycin (RAPA). 3MA and Baf inhibited ATP-induced autophagy only until 24 h, whereas RAPA potentiated the proautophagic effect of ATP for up to 48 h (Figure 7, C and D, and Supplemental Figure S4). After 24 h, the treatments that increased in autophagy (Figure 7D, top) induced a reduction in cell number (Figure 7D, bottom), with strong correlation ($r = 0.9$) between autophagy and cell death (Supplemental Figure S4B), suggesting a cytotoxic role for ATP-induced autophagy. On the other hand, after 48 h, all treatments presented the same index of autophagy, and cell number in the presence of autophagy modulators reached a plateau (Figure 7D).

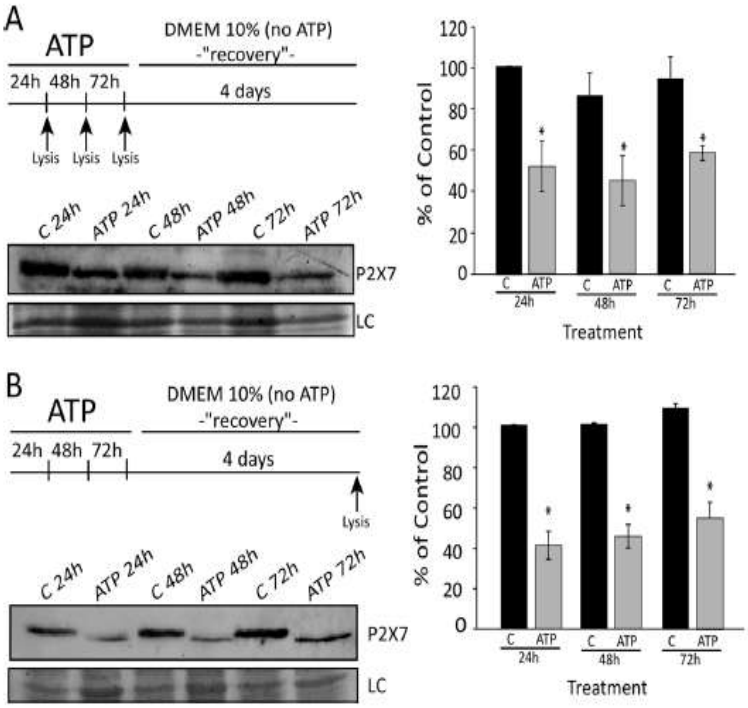


FIGURE 5: A subpopulation of cells with higher P2×7 levels was eliminated by ATP. (A) P2×7 protein expression analyzed by Western blot in SiHa cells that remain attached to the plate after treatment for 24, 48, and 72 h with 5 mM ATP and (B) after recovery with medium for 4 d. Note that expression of P2×7 is lower in an ATP-resistant subpopulation. C, control (no treatment); T, treatment with 5 mM ATP. *$p < 0.05$ vs. respective control (one-way ANOVA, followed by Tukey's test).

DISCUSSION

Among several receptors that comprise the purinergic system, the P2×7 subtype is implicated in terminal differentiation and apoptosis of stratified squamous epithelium and therefore has a special role in the control of cell death (Burnstock *et al.*, 2012). In human cervical epithelial cells, P2×7 activation by extracellular ATP culminates in pore opening, calcium influx, high cytosolic calcium, and apoptosis induction through the mitochondrial pathway (Wang *et al.*, 2004). When cervical cancer cells were exposed to high extracellular ATP, phenotypic features of apoptosis, such as cell shrinkage, membrane blebbing, and phosphatidylserine externalization, were found. However, in contrast to normal cervical epithelial cells, the induction of SiHa cell death by ATP, but not the nondegradable variants of ATP, did not occur by calcium influx and caspase activation. These data indicate that a caspase-independent mechanism could be involved in ATP-induced SiHa cell death. Recently Bian *et al.* (2013) described a new pathway for an antitumor effect of ATP on MCA38 colon cancer cells and on B16/F10 melanoma, which involves P2×7 activation and concurrent blockage of mTOR signaling through AMPK-PRAS40 and PI3K/AKT pathways, culminating in autophagy induction and cell death in a caspase- independent way. However, our data do not support such a major role for P2×7 in cervical cancer cell death. A pharmacological approach using oATP or P2×7silencing suppressed only 20% of the ATP-induced toxicity. Indeed, analyses of P2×7 levels in adherent cells that survived ATP cytotoxicity showed that only cells that expressed high levels of P2×7 were eliminated.

These data suggested that ATP could be acting also by producing a cytotoxic metabolite. Among ATP metabolites, extracellular adenosine exerted a cytotoxic effect in human epithelial cancer cells originating from breast, colon, and ovary. In these cells, adenosine leads to an increase in AMPK phosphorylation, probably through uptake and transformation into AMP by adenosine kinase, since this effect was blocked by the equilibrative nucleoside transporter (ENT) inhibitor dipyridamole (Saitoh *et al.*, 2004; Sai *et al.*, 2006). SiHa cell death was also marked by adenosine phosphorylation and transformation into AMP, which culminated in AMPK phosphorylation. In addition, p53 activation and PARP cleavage were also observed with ATP and blocked by DIP. In agreement with our results, treatment of human gastric cancer cells with adenosine-induced apoptosis independently of caspases and exclusively via an intrinsic pathway through AMPK activation (Saitoh *et al.*, 2004). In the same way, Nogi *et al.* (2012) found that AMP formed intracellularly from adenosine uptake induced an increase in p53 expression, resulting in malignant pleural mesothelioma cell death by caspase-independent apoptosis. Similar to our data, all of these effects were blocked by DIP, indicating that adenosine uptake was a key for apoptosis induction. Our work, however, also found a contribution of autophagy to SiHa cell death. This process seems to be important for ATP toxicity, since the increase of AO-positive cells strongly correlated with cell death after 24 h of treatment. However, the proautophagic effect of ATP was almost totally inhibited by DIP, indicating that this effect was mediated by adenosine uptake and not through P2×7 activation, as previously described in murine colon cancer cells and mouse melanoma cancer cells (Bian *et al.*, 2013).

Other evidence also suggests that adenosine is the main player responsible for ATP cytotoxicity, including the fact that extracellular AMP, which is not a ligand of any P2 receptors, also promotes cell death, indicating a role for adenosine formed from AMP degradation by 5 -nucleotidase on apoptosis induction (Wen and Knowles, 2003). Of importance, ATP and ADP can also be metabolized by ectonucleotidases at the cell surface of SiHa cells and generate adenosine (Beckenkamp *et al.*, 2014). Further evidence comes from our finding that after 72 h of treatment, DIP alone was able to inhibit ~80% of ATP-induced cell number reduction and block all ATP cytotoxicity in P2×7 KD cells. Additional evidence is provided by the fact that the adenosine metabolites inosine and hypoxanthine, although reaching high levels in extracellular medium and being transported into the cell by ENTs (Baldwin *et al.*, 2004), did not induce cell death, since preliminary data indicate a positive effect of inosine on proliferative but not cell death (unpublished data). Indeed, both inosine and hypoxanthine have been described as protective agents against cytotoxic compounds and hypoxic injury in normal and cancer cells (Obajimi and Melera, 2008; Ma *et al.*, 2011).

Increased intracellular levels of inosine and dATP after ATP exposure, which were blocked by DIP pretreatment, further support the entrance of adenosine into the cell. Accumulated dATP has been associated with accumulation of DNA strand breaks, activation of p53, and induction of apoptosis in lymphocytes (Joachims *et al.*, 2008; Johnston, 2011). Adenosine may promote cell death by

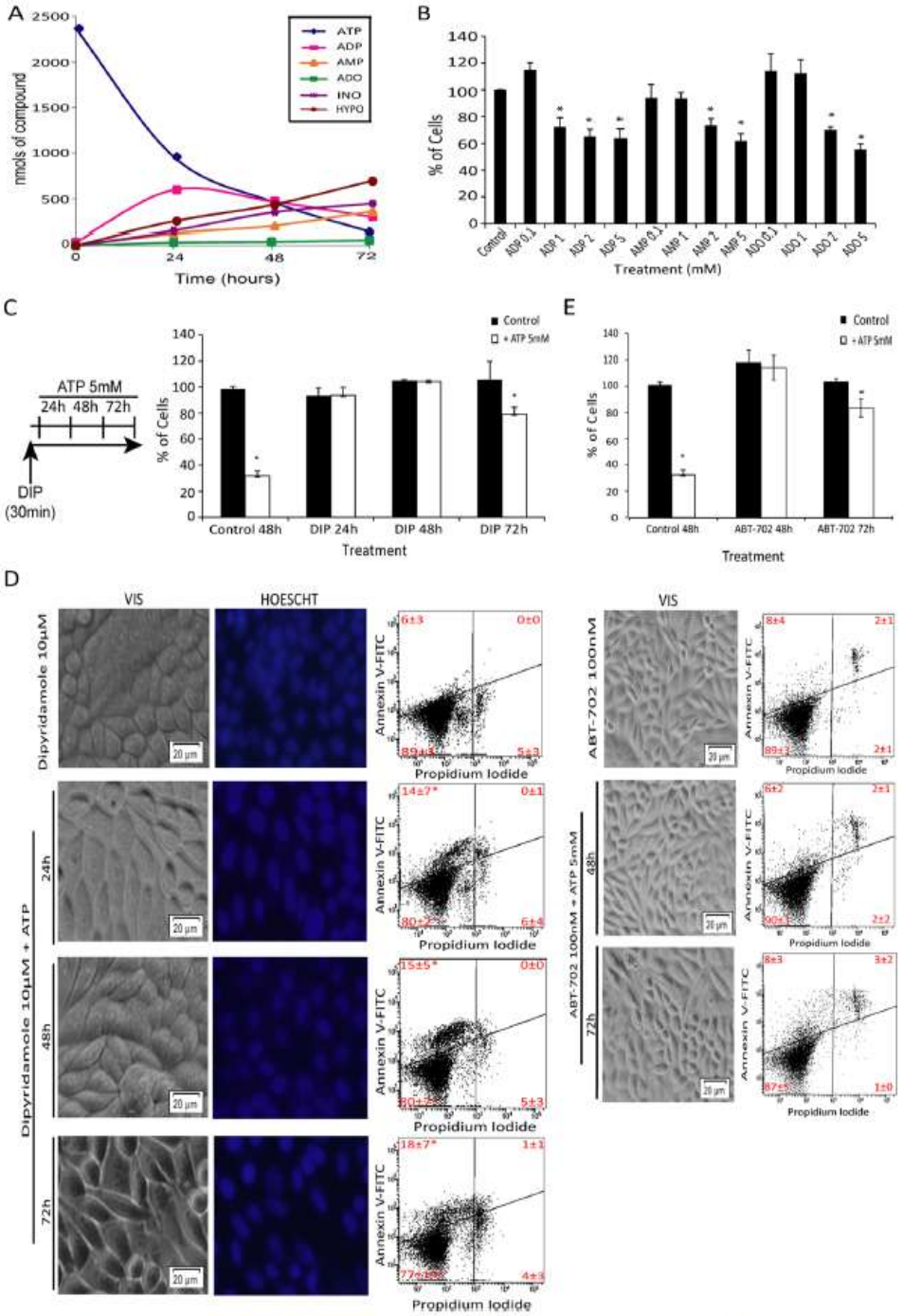


FIGURE 6: Adenosine uptake and conversion to AMP by adenosine kinase is the major mechanism of toxicity triggered by extracellular ATP in SiHa cells. (A) Extracellular ATP hydrolysis and product formation in SiHa cell line. Cells were incubated with 5 mM ATP, and levels of nucleotides in cell medium were analyzed by HPLC after treatment times of 0, 24, 48, and 72 h. A control without ATP was done for basal determination of nucleotides released by cells (Supplemental Table S1). ATP, ADP, AMP, adenosine (ADO), inosine (INO), and hypoxanthine (HYPO) contents in reaction medium were represented by exogenous (added) plus endogenous (secreted) purinergic compound as mean (nanomoles) ± SD. (B) Effect of ATP metabolites on SiHa cell death. Cells were incubated with different concentrations of ADP, AMP, and adenosine for 24 h, and the number of viable cells was determined as described in *Materials and Methods*. *$p < 0,05$ compared with control (one-way ANOVA, followed by Tukey's test). (C, D) Dipyridamole blockage of 5 mM ATP induces cell death by inhibiting extracellular adenosine uptake. SiHa cells were exposed to 10 μM dipyridamole alone or for 30 min, and then 5 mM ATP was added for 24, 48, and 72 h. (C) Number of viable cells after treatment. *$p < 0.05$ compared with control (two-way ANOVA, followed by Bonferroni posttest). (D) Apoptosis and necrosis index according

inducing an imbalance in deoxynucleotide triphosphate pools. Furthermore, we did not observe a correlation between P2×7 mRNA levels and ATP sensitivity, whereas adenosine and ATP sensitivity were highly correlated, indicating a preponderant role of the latter in relation to the former. Taken together, the evidence strongly suggests that adenosine formed from ATP metabolism, and not ATP itself, is the main agent responsible for SiHa cell death, contrary to what others found in other cell types (Wang *et al.*, 2004; Feng *et al.*, 2011; Di Virgilio, 2012; Bian *et al.*, 2013). Moreover, it seems that chronic exposure to micromolar levels of adenosine (100–200 µM) is a requirement for significant induction of cell death, in contrast to acute exposure, which requires millimolar levels. In both treatments, cells are exposed to a much higher concentration of adenosine than the amount found in the intracellular space (~10 nM; Kloor *et al.*, 2000). Wen and Knowles (2003) had already described a role for extracellular adenosine formed from ATP in a human hepatoma cell line death. However, in contrast to our result, they found that extracellular adenosine induced cell apoptosis via the A3 adenosine receptor. In our case, activation of the A3 receptor in cell death is unlikely, since DIP inhibited almost all cell death, and the SiHa cell line showed very low levels of A3 mRNA (unpublished data).

We propose that human cervical cancer cells comprise a heterogeneous population that responds differently to extracellular ATP toxicity according to the level of P2×7 receptor present in the cell membrane. Our hypothesis is that ATP per se is responsible for the elimination of a small subpopulation of cells (~20%) that express a high level of P2×7 and are killed through P2×7 activation, whereas adenosine acts in the remaining subpopulation, which is ATP resistant, expresses low levels of P2×7, and dies through adenosine uptake, AMPK phosphorylation, dATP accumulation, p53 activation, and autophagy induction (Figure 8). Thus cooperation among ATP and its metabolites seems to be important for cytotoxicity, with adenosine being necessary, but not sufficient, to induce cell death in the whole population of cells, which is of fundamental importance in cancer therapeutics. In conclusion, here we shed light on how cervical cancer cells respond to high extracellular ATP, which is a context commonly present in solid tumors and can be exploited to improve our understanding of tumor biology, as well as to increase therapy efficiency and overcome cell resistance.

MATERIALS AND METHODS

Reagents

Reagents, ATP, ADP, AMP, adenosine, BzATP, oATP, ATPγS, DIP, 3MA, BAF, RAPA, and AO were purchased from Sigma-Aldrich (St. Louis, MO). ABT-702, annexin V, and propidium iodide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The pan caspase inhibitor zAsp-CH2-DCB (zAsp; Peptide Institute, Tokyo, Japan) was kindly provided by Fabiana Horn (UFRGS) and dissolved in dimethyl sulfoxide (DMSO; Acros Organics).

Cell culture

Three cervical carcinoma cell lines, SiHa, HeLa, and C33A (American Type Culture Collection, Rockville, MD), were used in this study. SiHa cells contain integrated HPV 16; HeLa cells carry integrated HPV 18; and C33A cells are HPV negative and contain mutant p53. All culture materials were purchased from Gibco Laboratories (Grand Island, NY). Cervical carcinoma cell lines were maintained in culture flask in low-glucose DMEM supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere at 100% humidity. The spontaneously immortalized human epithelial cell line HaCaT was maintained at the same conditions but cultured with high-glucose DMEM.

Cell viability

Cell viability was assessed using the methylthiazolyltetrazolium bromide (MTT) assay. Cell lines (3500 cells/well) were seeded on 96-well multiwell plates, grown for 72 h, and treated with different concentration of ATP, ADP, AMP, and adenosine for 24 h at 37°C. After treatment, cells were incubated for 3 h at 37°C in MTT solution (0.5 mg/ml MTT dissolved in Ca^{2+}- and Mg^{2+}-free buffer). Formazan crystals formed by tetrazolium cleavage were dissolved with dimethyl sulfoxide (DMSO) and quantified at 570 and 630 nm using an EnVision Multilabel Plate Reader (PerkinElmer, Waltham, MA). Negative controls were made with DMEM supplemented with 10% fetal bovine serum (FBS). Results are expressed as percentage of control.

Cell counting

Cell lines (20,000 cells/well) were seeded on 24-well multiwell plates and treated with 5 mM ATP for 24, 48, and 72 h or with different concentrations of ADP, AMP, and adenosine for 24 h. At the end of treatment, the medium was removed, cells were washed with 1× PBS, and 200 µl of 0.25% trypsin/EDTA was added to detach the cells, which were counted in a hemocytometer (number of viable cells not marked by trypan blue). Negative controls were made with DMEM supplemented with 10% FBS. Results are expressed as percentage of control.

Clonogenic survival assays

Cells were assayed for the cytotoxic effect of ATP after cell survival according to established methods of performing the clonogenic assay (Franken *et al.*, 2006). Subconfluent cultures were exposed to 5 mM ATP for 24, 48, and 72 h. Then the surviving adherent cells were washed with PBS preheated to 37°C, trypsinized, counted, and replated in six-well plates (100 cells/well). After 12 d of incubation in complete culture medium, the colonies formed from each cell plated were stained with crystal violet after fixation with methanol and counted manually. In each case results are expressed as survival fraction, which was obtained by dividing the number of colonies that arise after treatment of cells by the number of cells seeded and plate efficiency (PE: number of colonies formed by untreated cells/number of cells seeded), multiplied by 100.

LDH activity measurement

Loss of membrane integrity was measured through LDH release. SiHa cells (20,000 cells/well) were seeded on 24-well multiwell plates and treated with 5 mM ATP for 24, 48, and 72 h. After cell medium

to annexin V/IP stain. Images were taken from SiHa cell line after the foregoing treatments. Cell nuclei were stained with Hoescht 35565665 according to manufacturer's instruction. Note the extinction of apoptotic features such as cell shrinkage and blebbing and fragmented nuclei when cells were treated with ATP only (Figure 2C). *$p < 0.05$ compared with dipyridamole alone (two-way ANOVA, followed by Bonferroni posttest). (E) Adenosine kinase inhibitor (ABT-702) blockage of 5 mM ATP induces cell death by inhibiting intracellularly transported adenosine phosphorylation and conversion to AMP. SiHa cells were exposed to 100 nM ABT-702 for 30 min and followed or not by 5 mM ATP for 48 and 72 h. ABT-702, 100 nM, was replaced each 24 h. Top, number of viable cells after treatment. *$p < 0.05$ compared with control (two-way ANOVA, followed by Bonferroni posttest). Bottom, apoptosis and necrosis index according to annexin V/IP stain and representative images of SiHa cells after foregoing treatments. Scale bars, 20 µm; magnification, 20×.

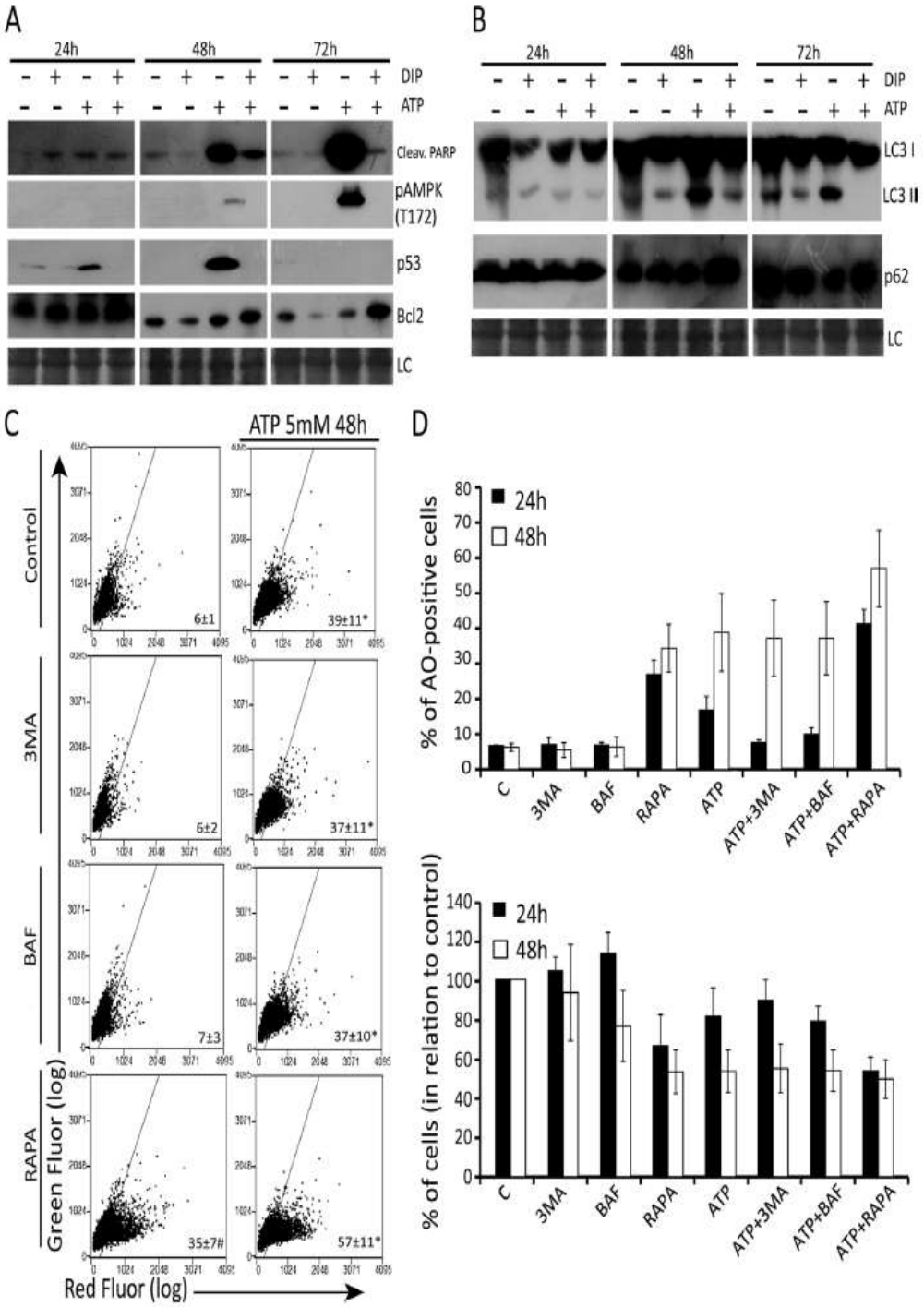


FIGURE 7: Adenosine uptake triggers AMPK phosphoactivation, p53 increase, and cytotoxic autophagy in SiHa cells. Expression of (A) p-AMPK, cleaved PARP, p53, and Bcl-2 and (B) p62 and LC3 II after 5 mM ATP exposure for 24, 48, and 72 h with or without 10 μM DIP pretreatment, determined by Western blot analysis as described in *Materials and Methods*. (C) Effect of the class III autophagy inhibitors 3MA and BAF and autophagy stimulator RAPA on AO staining after 48 h of ATP exposure. SiHa cells were pretreated with 2 mM 3MA, 100 nM BAF, or 200 nM RAPA before ATP treatment, and autophagy index was determined by AO staining as described in *Materials and Methods*. (D) Top, percentage of AO-positive cells after ATP exposure before or not to autophagy inhibitors or stimulator. Bottom, number of viable cells after treatment. Note that treatments that increased autophagy induced a reduction in cell number (see also Supplemental Figure S4). *p <0.05 compared with treatments without ATP; #p < 0.05 compared with control (two-way ANOVA, followed by Bonferroni posttest).

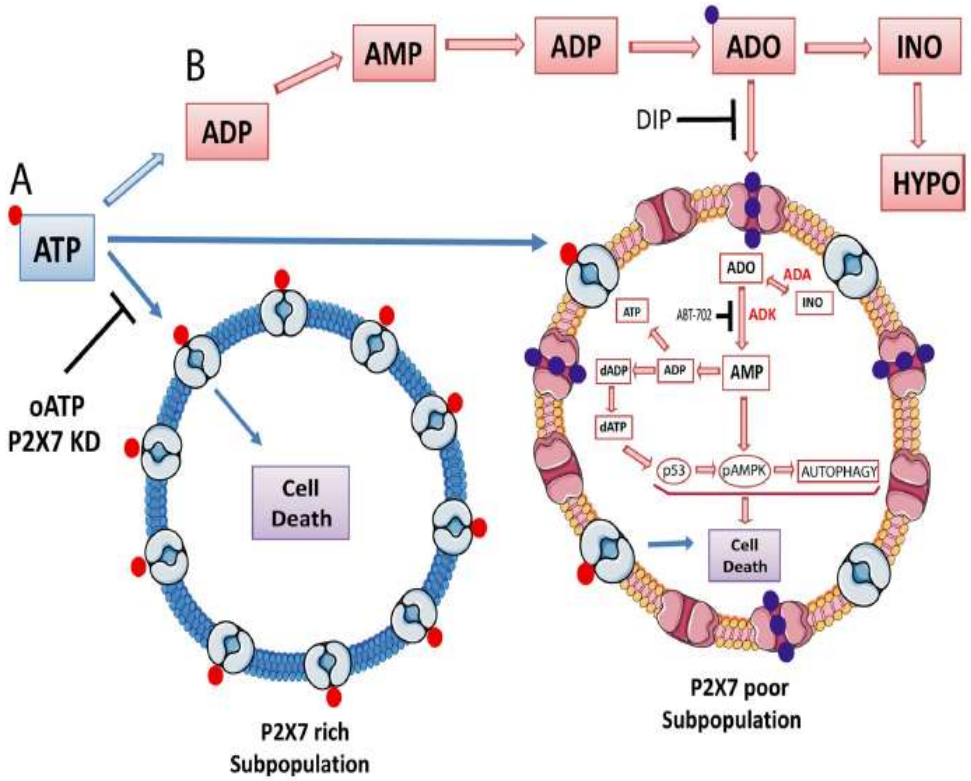


FIGURE 8: Schematic illustration of how adenosine uptake induces SiHa cervical tumor cell death in response to high levels of extracellular ATP. (A) A small subpopulation that expresses high levels of P2×7 died after being exposed to high levels of extracellular ATP via P2×7 activation by an unknown intracellular mechanism. The P2×7 antagonist oATP and receptor knockdown partially blocked ATP-induced cell death. (B) A major subpopulation of cells that express low levels of P2×7 died after being exposed to ATP metabolites such as ADP, AMP, adenosine, inosine, and hypoxanthine. In these cells, adenosine is taken up and partially converted to inosine through adenosine deaminase (ADA) activity. Concomitantly, adenosine is also phosphorylated to AMP by adenosine kinase (ADK), which leads to dATP accumulation, AMPK phosphorylation, p53 activation, autophagy induction, and finally cell death through apoptosis. Dipyridamole (DIP), an adenosine transporter inhibitor, and ABT-702, an adenosine kinase inhibitor, completely blocked cell death induction by these pathways. →, stimulation; –|, inhibition. ADO, adenosine; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; dADP, deoxyadenosine 5′-diphosphate; dATP, deoxyadenosine 5 -triphospate; HYPO, hypoxanthine; INO, inosine.

was withdrawn, transferred to a microtube on ice, and centrifuged for 1 min at 10,000 rpm, an aliquot of the supernatant was used for enzymatic assay using an LDH kit from Labtest Diagnostica (Minas Gerais, Brazil). Results are expressed as percentage of 0.5% Triton X-100–induced LDH release.

Annexin V and propidium iodide staining

Phosphatidylserine externalization was determined by the annexin fluorescence signal of an annexin V–fluorescein isothiocyanate conjugate (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) according to the manufacturer's protocol. Cell cultures were treated, trypsinized, and centrifuged for 6 min at 1600 rpm, and the supernatant was discarded. The pellet was suspended with 150 µl of annexin binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), incubated with annexin V at 0.75 µl/sample and PI at 15 µl/sample for 15 min at room temperature in the dark, and analyzed on a Guava EasyCyte flow cytometer, using Guava EasyCyte software for analysis (Millipore, Billerica, MA). Cisplatin, 40 µM, was used as positive control for apoptosis, and 0.5% Triton X-100 was used as a positive control for necrosis.

Analysis of intracellular and extracellular ATP metabolism by high-performance liquid chromatography

For extracellular ATP metabolism analysis, SiHa cells (20,000 cells/well) were seeded on 24-well multiwell plates and treated with 5 mM ATP for 24, 48, and 72 h after or not with10 µM DIP pretreatment. After the incubation time, the reaction medium was withdrawn and transferred to a microtube on ice, followed by centrifugation for 1 min at 10,000 rpm, supernatant dilution, and high-performance liquid chromatography (HPLC) analysis. The results are expressed as total amount of the different compounds (nanomoles), represented by the exogenous (added and extracellularly metabolized) plus endogenous (secreted) purinergic compounds, in the respective incubation time. The control for cellular purine secretion was done by growing cells in complete medium (DMEM plus 10% FBS) for 24, 48, and 72 h; very low levels of purinergic compounds were found in the extracellular medium (Supplemental Table S1).

For analysis of intracellular nucleoside triphosphate levels, SiHa cells (96,000 cells/well) were seeded on six-well multiwell plates and treated as described. All extraction steps were performed on ice, as described by Huang *et al.* (2003). The resulting supernatant was stored at –80°C until HPLC analyses. The intracellular concentration

of each nucleotide was normalized by the number of viable cells in the sample and is expressed as nanomoles/10^6 cells.

For HPLC analysis, 40-µl aliquots were applied to a reversed-phase HPLC system (Shimadzu, Japan) using a 15-cm C_{18} Resteck column at 260 nm with a mobile phase containing 60 mM KH_2PO_4 (Sigma-Aldrich) and 5 mM tetrabutylammonium chloride (Sigma-Aldrich), pH 6.0, in 30% methanol according to a previously described method (Casali *et al.*, 2003). All peaks were identified by retention time and comparison with standards. All incubations were carried out in triplicate, and controls to correct for nonenzymatic hydrolysis of nucleotides were done by measuring the peaks present in the same reaction medium incubated without cells.

Pharmacological profile assays

To test whether ATP-induced cell death involves P2×7 activation, we evaluated the effect of agonists and antagonists of this receptor. We seeded SiHa cells (20,000 cells/well) on 24-well multiwell plates and treated them as indicated. For the BzATP dose–response curve, we treated SiHa cells with 50, 100, 150, and 300 mM BzATP for 24 h. The effect of adenosine 5′-*O*-(3 thiotriphosphate) (ATPγS), a nonhydrolyzed P2 agonist, was determined by treating cells with 300 µM ATPγS for 24 h. For blockage of ATP-induced cell death, cells were previously treated with 300 and 600 mM oATP, a nonspecific P2×7 antagonist, for 2 h. After this time of incubation, cells were treated with 5 mM ATP for 24 h. At the end of all treatments, the viable cells were counted as described.

To test whether ATP was degraded and extracellular adenosine uptake was responsible for apoptosis induction, SiHa cells were pretreated with 10 µM DIP for 30 min before addition of 5 mM ATP for 24, 48, and 72 h. At the end of treatment, the viable cells were counted, and apoptosis status was determined by annexin V/IP staining as described. In addition, we investigated the effect of adenosine kinase inhibition on the induction of apoptosis. For this, SiHa cells were pretreated with 100 nM 4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl)pyrido[2,3-d]pyrimidine (ABT-702), a cell-permeable, adenosine-competitive and reversible adenosine kinase inhibitor, for 30 min, followed by the addition of 5 mM ATP for 48 and 72 h. ABT-702 was replaced every 24 h, and the number of viable cells and apoptosis status were determined after 48 and 72 h as described.

To verify that the mechanism that triggered apoptosis in SiHa cells after ATP exposure was P2×7 pore formation followed by increase of intracellular free calcium ions and caspase activation, we exposed subconfluent cultures to 0.6 mM EGTA, a calcium-chelating agent, or 50 µM zAsp, a pancaspase inhibitor, for 30 min. Then we treated cells with 5 mM ATP for 48 h, 100 µM BzATP for 24 h, or 300 µM ATPγS for 24 h, followed by cell number determination, phenotype observation, and annexin V/IP assay, as described.

Real-time PCR

Total RNA from SiHa, HeLa, C33A, and HaCaT cell lines was isolated with the RNA Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The cDNA species were synthesized with Super-Script II (Life Technologies, Carlsbad, CA) from 5 µg of total RNA in a total volume of 20 µl with both oligo (dT) primer and random hexamers in accordance with the manufacturer's instructions. SYRB Green I–based real-time PCR was carried out on a MJ Research DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research, Waltham, MA), as described (Zerbini *et al.*, 2003). All PCR mixtures contained PCR buffer (final concentration; 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 2.0 mM $MgCl_2$, and 0.1% Triton X-100), 250 µM deoxy-NTP (Roche Molecular Biochemicals, Penzberg, Upper Bavaria, Germany), 0.5 µM of each PCR primer indicated later, 0.5× SYBR Green I (Molecular Probes, Eugene, OR), 5% DMSO, and 1 U of taq DNA polymerase (Promega, Madison, WI) with 2 µl of cDNA in a 25-µl final volume reaction mix. The samples were loaded onto wells of Low Profile 96-well microplates. After an initial denaturation step for 1 min at 94°C, conditions for cycling were 35 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C. The fluorescence signal was measured right after incubation for 5 s at 79°C after the extension step, which eliminates possible primer dimer detection. At the end of the PCR cycles, a melting curve was generated to identify specificity for the PCR product. For each run, serial dilutions of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plasmids were used as standards for quantitative measurement of the amount of amplified DNA. In addition, for normalization of each sample, human GAPDH primers were used to measure the amount of GAPDH cDNA. All samples were run in triplicate, and the data are presented as the ratio cDNA/GAPDH. For human GAPDH, the primers used were as follows: sense primer, 5′-CAAAGTTGTCATGGATGACC-3′, and antisense primer, 5′-CCATGGAGAAGGCTGGGG-3′. For P2×7, the primers used were as follows: sense primer, 5′- AAA AGC CGG GGG CCT GCA TC-3′, and antisense primer, 5′-GCA GCT GGG CAG GAT GGC AA-3′. Oligonucleotides were obtained from Invitrogen.

Western blot analysis

Cell cultures were washed twice with cold PBS and homogenized in lysis buffer (4% SDS, 2.1 mM EDTA, and 50 mM Tris). Aliquots were taken for protein determination (Peterson, 1983), and β-mercaptoethanol was added to a final concentration of 5%. Thirty micrograms of protein was separated on 12% SDS–PAGE (Bio-Rad, Hercules, CA) and electrotransferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% M-TTBS (5% milk in Tween-20 in Tris-buffered saline [TTBS]) and further incubated with anti-P2×7 antibody (1:500; Santa Cruz Biotechnology), anti-Bcl2 (1:1000), anti-pAMPK (1:1000), anti–cleaved PARP (1:1000), anti-p62 (1:1000), anti-p53 (1:1000), and anti-LC3II (1:1000; Cell Signaling, Danvers, MA), diluted in TTBS, at room temperature. The membranes were then incubated with horseradish peroxidase–conjugated secondary antibody (1:2000) for 2 h at room temperature, and chemiluminescence was detected using x-ray films (X-Omat; Kodak, Rochester, NY). The films were scanned, and the percentage of band intensity was analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

P2×7 knockdown

Human P2RX7 (GeneID 18439) was knocked down by transduction of SiHa cells with lentivirus produced with the plasmid clone ID NM_002562.4-801s1c1 from the Mission RNAi library from Sigma-Aldrich. Nontarget (SHC001) sequence was used as a control. Lentiviruses were produced by cotransfecting the Mission RNAi plasmid with the helper plasmids pRSVREV, pVSV-G, and pMDLgRRE (Dull *et al.*, 1998) in subconfluent Hek293T cells with Superfect Reagent (Qiagen), according to the manufacture's protocol. Three days after transfection, supernatant was collected twice every day for 1 wk, filtered through a 0.22-mm membrane, and used immediately or stored at –80°C. We added 1 ml of virus-containing medium to target cells, also at subconfluent stage (12,000 cells/well) in 24-well plates, together with 8 µg/ml Polybrene overnight. Cells were allowed 48 h to express the selection marker and were then selected with 3 µg/ml puromycin for at least 10 d. Knockdown was confirmed by Western blotting.

Quantification of acidic vacuolar organelles by AO staining

The fluorescent dye AO is a marker of AVOs (acidic vacuolar organelles) that fluoresces green in the whole cell except in acidic compartments (mainly late autophagosomes), where it fluoresces red. AVO formation is a typical feature of autophagy, and its development indicates autophagosomes maturation and an efficient autophagic process, since only mature/late autophagosomes are acidic (Klionsky *et al.*, 2008). For AVO determination, cells (20,000 cells/well) were seeded on 24-well multiwell plates and exposed to autophagy inhibitors or inducers. Cells were treated with 2 mM 3MA, a blocker of autophagosome formation, for 1 h; 100 nM BAF, an inhibitor of the late phase of autophagy, for 24 h; or 200 nM RAPA, an autophagy inducer, for 24 h before ATP treatment. After the incubation time, medium was replaced and 5 mM ATP was added for 24 or 48 h. Then cells were trypsinized and incubated with AO (2.7 mM) for 15 min at room temperature, and fluorescence emission was analyzed by flow cytometry, as described previously (Jiang *et al.*, 2009), using a Guava flow cytometer and Guava Cytosoft.

Statistical analysis

Statistical analysis was performed with Prism 5 (GraphPad, La Jolla, CA). Data are expressed as percentage of control and presented as mean ± SD of at least three independent experiments. Statistical analyses for comparison among multiple groups were performed by one-way analysis of variance (ANOVA), followed by a Turkey post-hoc test. When more than one molecule was mixed to the same well at the same time, a two-way ANOVA was performed, followed by a Bonferroni posttest. Values were considered significant at $p < 0.05$. Correlation coefficients were calculated using the CORREL function of Excel 2013 (Microsoft, Redmond, WA).

ACKNOWLEDGMENTS

We thank the Brazilian agencies FAPERGS (Fundação de Amparo à Pesquisa do Rio Grande do Sul; Pronem 11/2072-2, Pesquisador Gaúcho 11/0916-1), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico; Universal 472512/2011-0), ICGEB BRA (International Centre for Genetic Engineering and Biotechnology, Brazil; 11/01), CAPES (Coordenação e Aperfeiçoamento de Pessoal de Nível Superior), and PROPESQ (Pró-Reitoria de Pesquisa da Universidade Federal do Rio Grande do Sul) for financial support.

REFERENCES

Baldwin SA, Beal PR, Yao SYM, King AE, Cass CE, Young JD (2004). The equilibrative nucleoside transporter family, SLC29. Pflugers Arch 447, 735–743.

Beckenkamp A, Santana DB, Bruno AN, Calil LN, Casali EA, Paccez JD, Zerbini LF, Lenz G, Wink MR, Buffon A (2014). Ectonucleotidase expression profile and activity in human cervical cancer cell lines. Biochem Cell Biol 92, 95–104.

Bian S, Sun X, Bai A, Zhang C, Li L, Enjyoji K, Junger WG, Robson SC, Wu Y (2013). P2Y integrates PI3K/AKT and AMPK-RAS40-mTOR signaling pathways to mediate tumor cell death. PLoS One 8, e60184.

Burnstock G, Di Virgilio F (2013). Purinergic signaling and cancer. Purinergic Signal 9, 491–540.

Burnstock G, Knight GE, Greig AVH (2012). Purinergic signaling in healthy and diseased skin. J Invest Dermatol 132, 526–546.

Casali EA, Souza LF, Gelain DP, Kaiser GR, Battastini AM, Sarkis JJ (2003). Changes in ectonucleotidase activities in rat Sertoli cells during sexual maturation. Mol Cell Biochem 247, 111–119.

Di Virgilio F (2012). Purines, purinergic receptors, and cancer. Cancer Res 72, 5441–5447.

Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L (1998). A third-generation lentivirus vector with a conditional packaging system. J Virol 72, 8463–8471.

Feng L, Sun X, Csizmadia E, Han L, Bian S, Murakami T, Wang X, Robson SC, Wu Y (2011). Vascular CD39/ENTPD1 directly promotes tumor cell growth by scavenging extracellular adenosine triphosphate. Neoplasia 13, 206–216.

Franken NAP, Rodermond HM, Stap J, Haveman J, Van Bree C (2006). Clonogenic assay of cells in vitro. Nat Protoc 1, 2315–2319.

Ghiringhelli F, Bruchard M, Chalmin F, Rébé C (2012). Production of adenosine by ectonucleotidases: a key factor in tumor immunoescape. J Biomed Biotechnol 2012, 473712.

Hardie DG, Hawley SA, Scott JW (2006). AMP-activated protein kinase-development of the energy sensor concept. J Physiol 574, 7–15.

He C, Klionsky DJ (2009). Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 43, 67–93.

Huang D, Zhang Y, Chen X (2003). Analysis of intracellular nucleoside triphosphate levels in normal and tumor cell lines by high-performance liquid chromatography. J Chromatrogr B Analyt Technol Biomed Life Sci 784, 101–109.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011). Global cancer statistics. CA Cancer J Clin 61, 69–90 [correction published in CA Cancer J Clin (2011). 61, 134].

Jiang H, White EJ, Conrad C, Gomez-Manzano C, Fueyo J (2009). Autophagy pathways in glioblastoma. Methods Enzymol 453, 273–286.

Joachims ML, Marble P, Knott-Craig C, Pastuszko P, Blackburn MR, Thompson LF (2008). Inhibition of deoxynucleoside kinases in human thymocytes prevents dATP accumulation and induction of apoptosis. Nucleosides Nucleotides Nucleic Acids 27, 816–820.

Johnston JB (2011). Mechanism of action of pentostatin and cladribine in hairy cell leukemia. Leuk Lymphoma 52(Suppl 2), 43–45.

Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T (2004). LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J Cell Sci 117, 2805–2812.

Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, *et al.* (2008). Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 4, 151–175.

Kloor D, Yao K, Delabar U, Osswald H (2000). Simple and sensitive binding assay for measurement of adenosine using reduced *S*-adenosylhomocysteine hydrolase. Clin Chem 46, 537–542.

Lee SG, Choi J K, Choi BH, Lim Y, Kim YH, Lee KH, Shin JC, Ahn WS (2006). The effect of adenosine 5-triphosphate on calcium mobilization and cell proliferation in cervical cancer cells. Eur J Obstet Gynecol Reprod Biol 127, 110–114.

Li X, Qi X, Zhou L, Fu W, Abdul-Karim FW, MacLennan G, Gorodeski GI (2009). P2Y receptor expression is decreased in epithelial cancer cells of ectodermal, uro-genital sinus, and distal paramesonephric duct origin. Purinergic Signal 5, 351–368.

Ma RQ, Yang H, Zhao XH, Zhang YK, Yao AH, Cheng P, Xie YB, Zhao HK, Ju G, Kuang F (2011). The protective effects of inosine against chemical hypoxia on cultured rat oligodendrocytes. Cell Mol Neurobiol 31, 1171–1186.

Matsumoto G, Wada K, Okuno M, Kurosawa M, Nukina N (2011). Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins. Mol Cell 44, 279–289.

Nogi Y, Kanno T, Nakano T, Fujita Y, Tabata C, Fukuoka K, Gotoh A, Nishizaki T (2012). AMP converted from intracellularly transported adenosine upregulates p53 expression to induce malignant pleural mesothelioma cell apoptosis. Cell Physiol Biochem 30, 61–74.

Obajimi O, Melera PW (2008). The depletion of cellular ATP by AG2034 mediates cell death or cytostasis in a hypoxanthine-dependent manner in human prostate cancer cells. Cancer Chemother. Pharmacol 62, 215–226.

Ohta A, Gorelik E, Prasad SJ, Ronchese F, Lukashev D, Wong MK, Huang X, Caldwell S, Liu K, Smith P, *et al.* (2006). A2A adenosine receptor protects tumors from antitumor T cells. Proc Natl Acad Sci USA 103, 13132–13137.

Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Øvervatn A, Bjørkøy G, Johansen T (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 282, 24131–24145.

Pellegatti P, Raffaghello L, Bianchi G, Piccardi F, Pistoia V, Di Virgilio F (2008). Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. PLoS One 3, e2599.

Peterson GL (1983). Determination of total protein. Methods Enzymol 91, 95–119.

Saitoh M, Nagai K, Nakagawa K, Yamamura T, Yamamoto S, Nishizaki T (2004). Adenosine induces apoptosis in the human gastric cancer cells

via an intrinsic pathway relevant to activation of AMP-activated protein kinase. Biochem Pharmacol 67, 2005–2011.
Sai K, Yang D, Yamamoto H, Fujikawa H, Yamamoto S, Nagata T, Saito M, Yamamura T, Nishizaki T (2006). A1 adenosine receptor signal and AMPK involving caspase-9/-3 activation are responsible for adenosine-induced RCR-1 astrocytoma cell death. Neurotoxicology 27, 458–467.
Schiffman M, Wentzensen N, Wacholder S, Kinney W, Gage JC, Castle PE (2011). Human papillomavirus testing in the prevention of cervical cancer. J Natl Cancer Inst 103, 368–383.
Tamajusuku ASK, Villodre ES, Paulus R, Coutinho-Silva R, Battasstini AMO, Wink MR, Lenz G (2010). Characterization of ATP-induced cell death in the GL261 mouse glioma. J Cell Biochem 109, 983–991.
Tsuchiya A, Kanno T, Saito M, Miyoshi Y, Gotoh A, Nakano T, Nishizaki T (2012). Intracellularly transported adenosine induces MCF-7 human breast cancer cells by accumulating AMID in the nucleus. Cancer Lett 321, 65–72.
Wang Q, Wang L, Feng YH, Li X, Zeng R, Gorodeski GI (2004). P2'7 receptor-mediated apoptosis of human cervical epithelial cells. Am J Physiol Cell Physiol 287, 1349–1358.
Wen LT, Knowles AF (2003). Extracellular ATP and adenosine induce cell apoptosis of human hepatoma Li-7A cells via the A3 adenosine receptor. Br J Pharmacol 140, 1009–1018.
Yang Z, Klionsky DJ (2010). Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol 22, 2124–131.
Zerbini LF, Wang Y, Cho JY, Libermann TA (2003). Constitutive activation of nuclear factor _B p50/p65 and Fra-1 and JunD is essential for deregulated interleukin 6 expression in prostate cancer. Cancer Res 63, 2206–2215.

Supplemental Materials

Molecular Biology of the Cell

Mello et al.

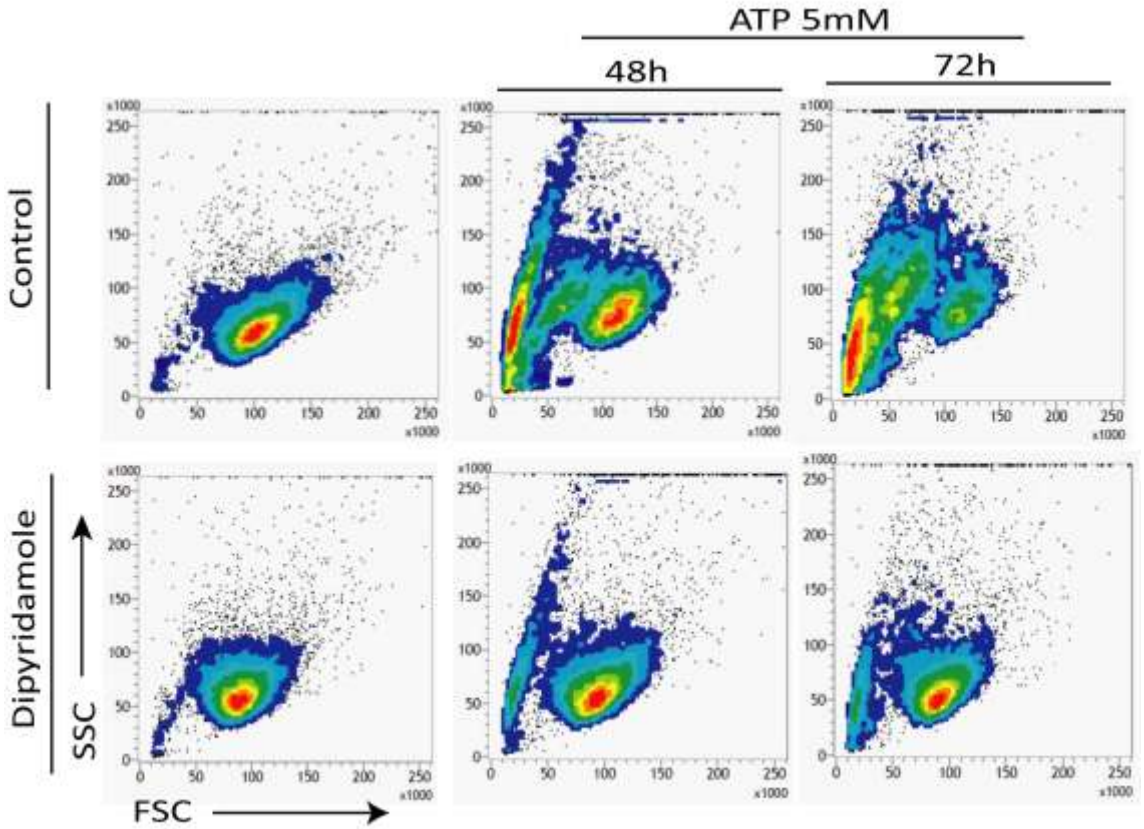
Supplementary Table S1 Mello et al, 2014

Table S1. Basal amount of adenine nucleotides and nucleoside released by SiHa.

Time (hours)	ATP (nmol) ± DP	ADP (nmol) ± DP	AMP (nmol)± DP	ADO (nmol) ± DP	INO (nmol) ± DP	HYPO (nmol) ± DP
24	56.18±0.00033	34.52±0.0008	3.46±0.00009	0±0	5.79±0.000008	4.51±0.000003
48	58.54±0.0004	28.75±0.00008	2.29±0.000018	0±0	9.53±0.000022	4,98±0.00001
72	11.17±0.00005	31.66±0.00003	1.19±0.00001	0±0	12.69±0.0009	4.27±0.000017

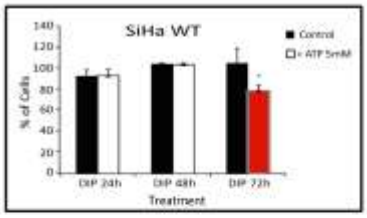
SiHa cells were grown in complete medium (DMEM + 10% SBF) for 24, 48 and 72h and reaction medium was withdrawn and analyzed by HPLC as described in Material and Methods. Results were represented by purinegic compound as mean (nmols) ± DP.

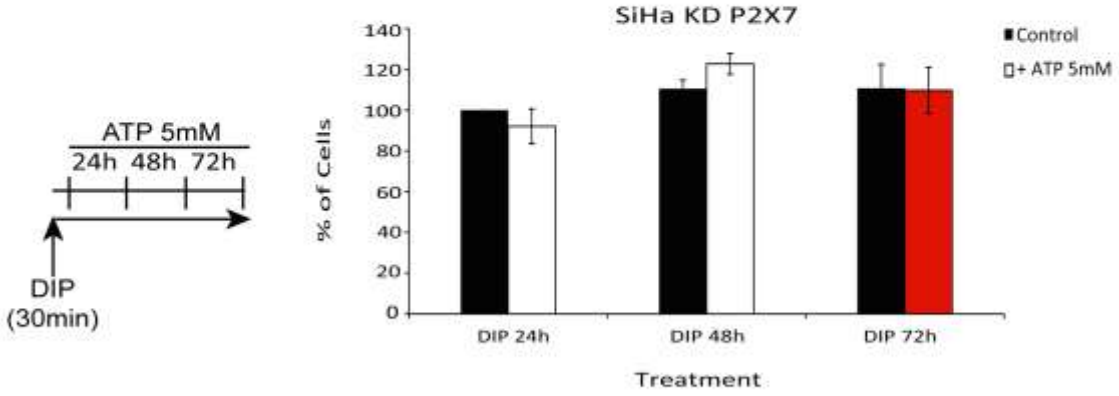
Mello et al, 2014



Supplementary Figure S1. Cellular distribution (FSC x SSC) and density after treatment with ATP 5mM alone or Dipyridamole 10 µM previous to ATP for 48 and 72h. Note the reduction on left subpopulation (apoptotic subpopulation) when dipyridamole was pre-added compared to ATP treatment alone.

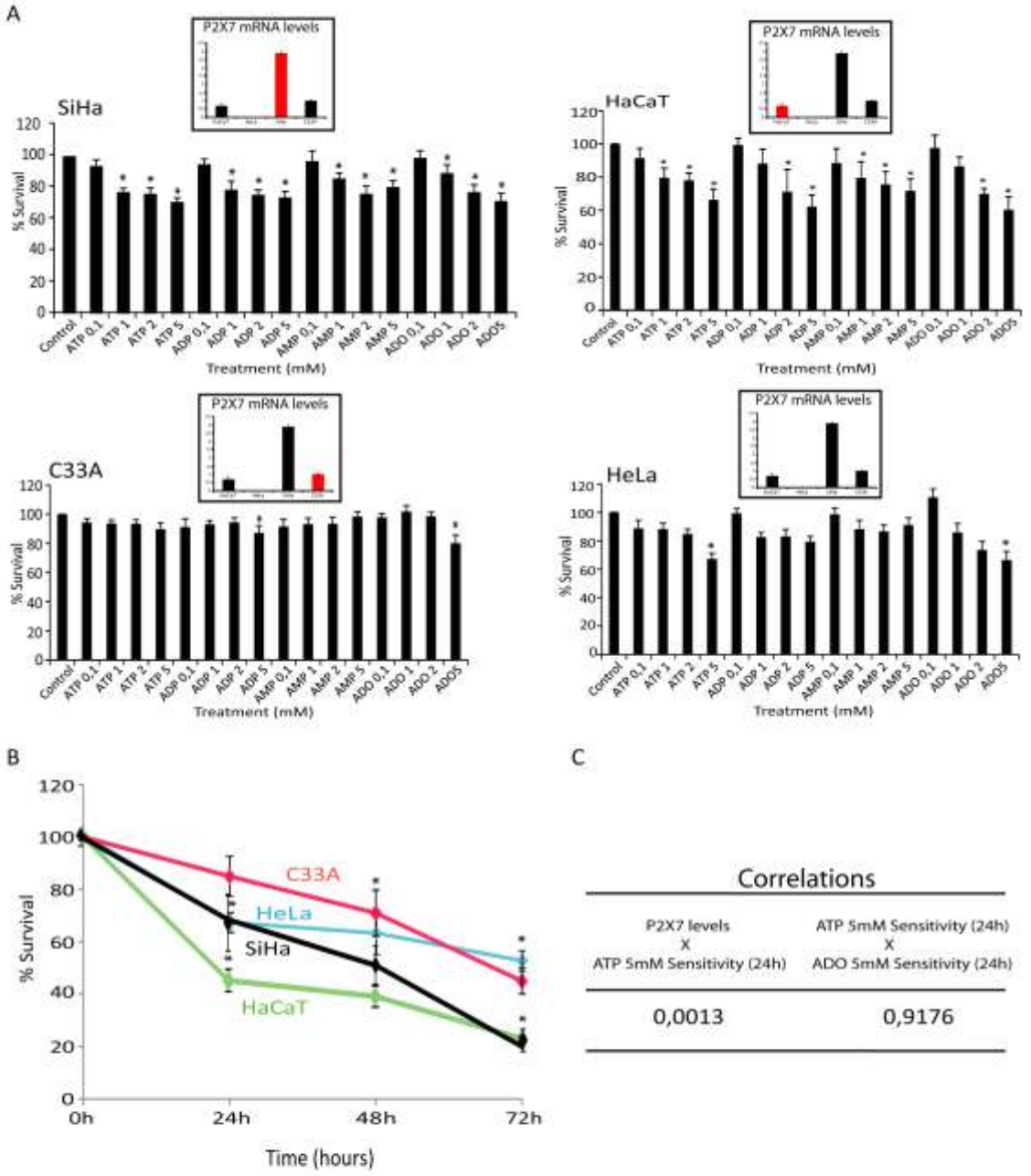
Mello et al, 2014



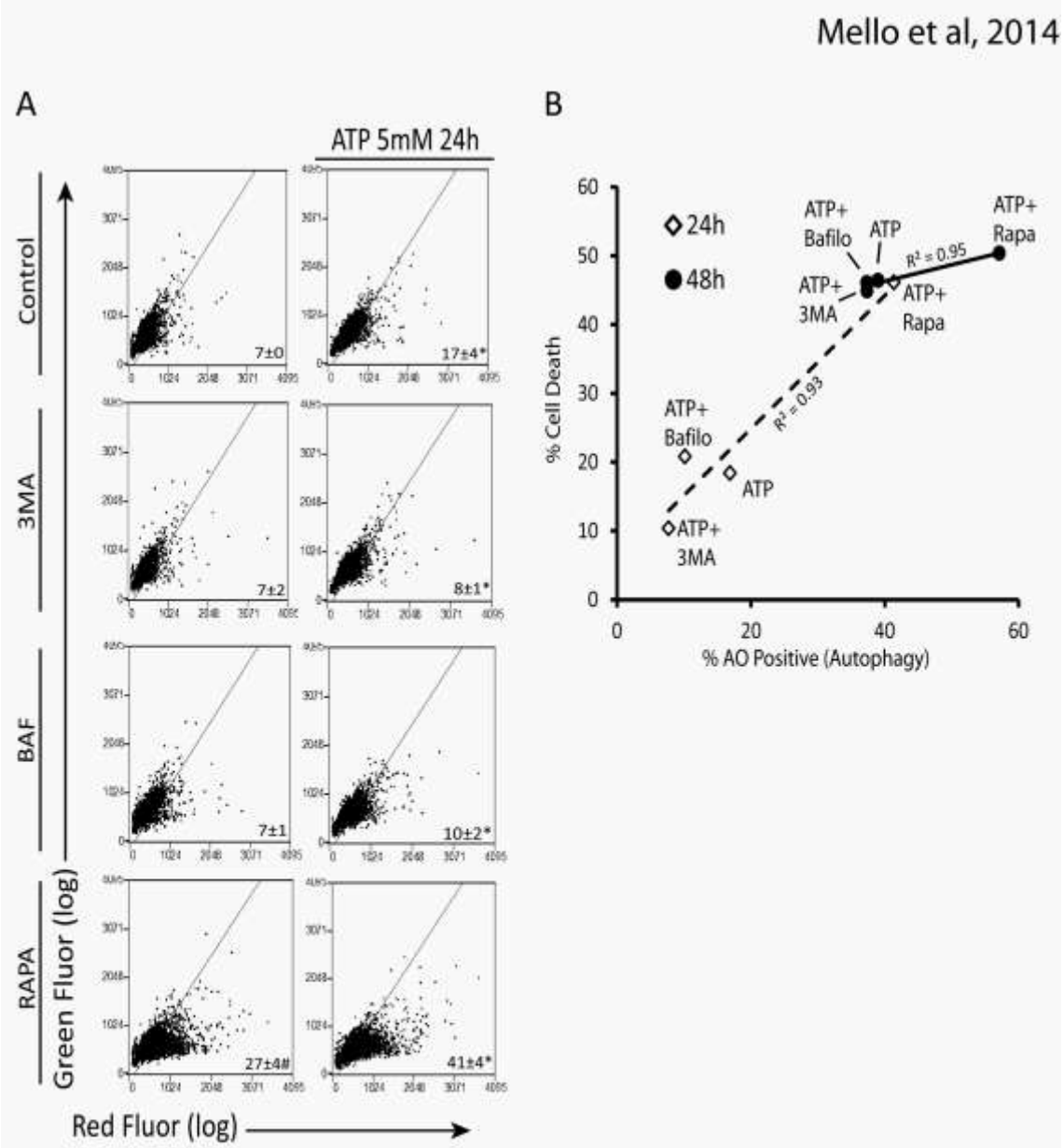


Supplementary Figure S2. P2X7 knockdown contributes with dipyridamole on cell death prevention after ATP exposure. SiHa cells KD for P2X7 were exposure to dipyridamole 10μM (DIP) alone or for 30min and then ATP 5mM was added for 24, 48 and 72h. Number of viable cells not stained by trypan blue was determined. Note the extinction of cell death after 72h of treatment with ATP + DIP when compared to the same experiment using SiHa WT (upper and small graph highlighted in red, as showed in Figure 6C).

Mello et al, 2014

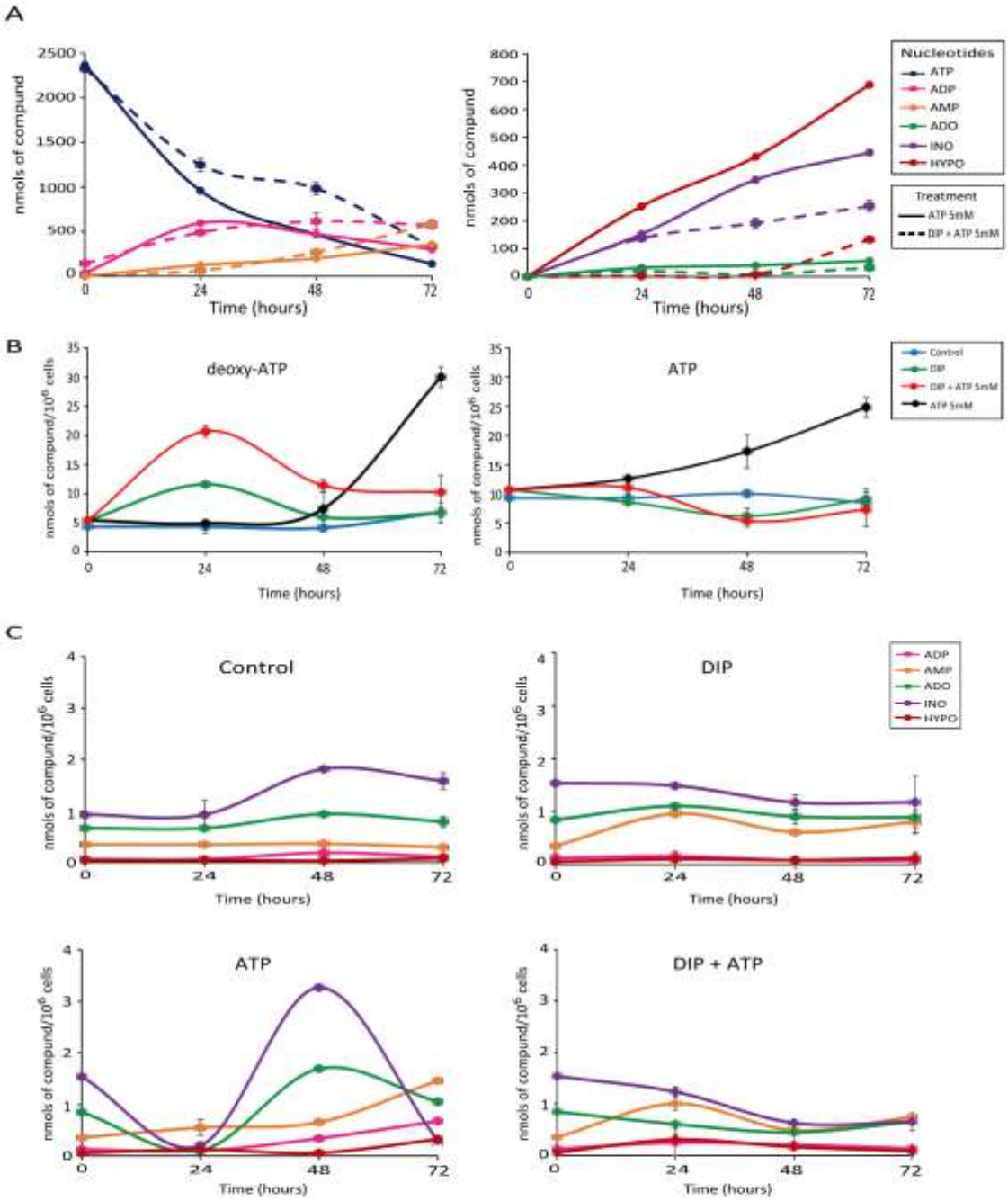


Supplementary Figure S3. Susceptibility of cervical cancer cells death to ATP is correlated with adenosine sensitivity and not with mRNA P2X7 levels. (A) Cervical cancer cell lines (SiHa, HeLa and C33A) and human epithelial cell line (HaCaT) were exposed to different concentrations of ATP, ADP, AMP and adenosine for 24h and cell viability was accessed by MTT assay. Upper and small graph in red represents the mRNA P2X7 amount for respective cell line, determined by Real Time PCR as described in Figure 1A. (B) Cells were exposed to ATP 5mM for 24, 48 and 72h and cell viability was determined by MTT assay. Note the different sensitivity to ATP (HaCaT>SiHa>HeLa>C33A), which is not correlated to mRNA P2X7 amount (SiHa>C33A>HaCaT>HeLa), but is correlated to adenosine (ADO) sensitivity, as demonstrated by numbers in (C). * $p < 0.05$ when compared to control (one-way ANOVA followed by Turkey´s test).



Supplementary Figure S4. (A) Effect of autophagy inhibitors (3MA and Baf) and autophagy stimulator RAPA on Acridine Orange (AO) staining after 24h of ATP exposure. SiHa cells were pretreated with 2mM of 3-MA, 100nM of BAF or 200nM of RAPA prior to ATP treatment and authopagy index were determined by AO staining as described in Material and Methods. (B) Graphic representation showing the strong positive correlation between AO positive cells and % of cell death. * $p < 0.05$ when compared with treatments without ATP, #$p<0,05$ when compared to control (two-way ANOVA followed by Bonferroni post-test).

Mello et al, 2014



Supplementary Figure S5. Levels of extracellular and intracellular nucleotides/nucleosides post extracellular ATP or DIP + ATP treatment. (A) Extracellular ATP metabolism when SiHa cells were pretreated with dipyridamole 10μM (DIP) followed by ATP 5mM for 24, 48 and 72h (dotted line) compared to the same experiment exposing cells to ATP 5mM only (bold line, as showed in Figure 6A). Note the accumulation of ATP, ADP and AMP and the reduction of inosine and hypoxantine levels when DIP was added to reaction medium. (B) Levels of intracellular deoxy-ATP and ATP post extracellular treatment with ATP 5mM, DIP pretreatment followed by ATP 5mM (DIP+ATP), DIP alone or culture medium (Control). Note the intracellular accumulation of deoxy-ATP and ATP at 72h after ATP exposure, which was completely blocked by DIP pretreatment (DIP + ATP). (C) Levels of other intracellular nucleotides/nucleosides after exposing cells to the same treatments described above. Note the disturbance on nucleotide/nucleosides levels at 48h after ATP treatment alone, which was reverted by DIP pretreatment in DIP + ATP.

III.2. CAPíTULO 2- Paola de Andrade Mello, Shu Bian, Luiz Eduardo Baggio Savio, Jingping Zhang, Wolfgang Junger , Márcia Wink, Guido Lenz, Yan Wu, Andréia Buffon and Simon C. Robson. **Heat shock increases ATP-mediated cancer cell death by inducing P2X7 hyperactivation.**

Essa parte da tese foi desenvolvida durante a realização do doutorado sanduíche no *Department of Medicine, Gatroenterology & Transplatation of Beth Israel Deaconess Medical Center, Harvard Medical School*, na cidade de Boston (USA), do mês de Setembro de 2014 ao mês de Agosto de 2015. E representa uma prévia do manuscrito a ser submetido a um periódico da área nos próximos meses.

HEAT SHOCK INCREASES ATP-MEDIATED CANCER CELL DEATH BY INDUCING P2X7 HYPERACTIVATION

Paola de Andrade Mello[1,2], Shu Bian[2], Luiz Eduardo Baggio Savio[2,3], Jingping Zhang[4], Wolfgang Junger[4], Márcia Wink[5], Guido Lenz[6], Yan Wu[2], Andréia Buffon[1] and Simon C. Robson[2*]

[1] Laboratório de Análise Bioquímica e Citológica, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil;
[2] Department of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, Harvard University, Boston, MA, USA;
[3]Programa de Imunobiologia, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil;
[4]Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Harvard University, Boston, MA, USA;
[5] Laboratório de Biologia Celular, Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Porto Alegre, RS, Brazil.
[6] Departamento de Biofísica e Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

*Corresponding Author: Simon C. Robson
Department of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School
3 Blackfan Circle, Rm612,
Boston, MA 02215, USA
Tel: + 617-735-2924; Fax. 617-735-2930
E-mail: srobson@bidmc.harvard.edu

ABSTRACT

Background: Extracellular ATP directly promotes P2X7-mediated cancer cell death. We have previously reported that two intracellular signaling axes are involved in this tumor killing process: the known P2X7-PI3K/AKT axis and a novel P2X7-AMPK-PRAS40-mTOR axis, that acts independently and synergistically to disrupt the balance between cell growth and autophagy. Although these mechanisms efficiently induce cancer cell death, high levels of exogenous ATP were required. Therefore, finding a way to increase P2X7 sensitization to a purine-related drug could maximize tumor cell death with no side effects.

Aims: In this study, we investigated the use of heat shock as a new strategy to increase P2X7 functionality, thus increasing tumor cell death in the presence of extracellular ATP.

Methodology/principal findings: MCA38 colon cancer cells expressing or knockdown for P2X7 receptor were shortly exposed to ATP 1mM either at 37ºC or at 40ºC (mild heat stress). According to our data heat shock stress was capable to increase ATP-P2X7-AKT/PRAS40/mTOR elicited tumor cell death, by altering P2X7 functionality independently of heat shock protein interaction or native pore-forming transporters association (e.g pannexin-or connexin-type channels). The mechanism by which heat stress increase P2X7 pore dilatation seems to be related to changes in the lipid composition and architecture of membranes, as the membrane fluidizer benzyl alcohol could reproduce heat stress effect in potentiating P2X7 activation at 37ºC.

Conclusion: Our work provides further evidence for a purinergic signaling role in the cancer biology context and opens new perspectives for the utility of purine-based drugs associated with hypertermia as adjunctive agents in cancer therapy.

KEY WORDS: ATP, P2X7 receptor, heat shock, cancer cell death

Introduction

Adenosine triphosphate (ATP) is normally present at low concentration in the extracellular space. Thus, any increase at this molecule levels represents a signal of danger (Roger *et al*., 2014). In the extracellular space, ATP acts through type 2 purinergic (P2) receptors to participate in many pathophysiological processes, such as cell proliferation, differentiation, apoptosis, inflammation and metabolism (White & Burnstock, 2006; Deaglio & Robson, 2011; Eltzschig *et al*., 2012). Two P2 receptor families that can be activated by ATP and other nucleoside triphosphates and diphosphates have already been described: the P2X receptors (P2X1-7), which are ATP-gated ion channels and the P2Y receptors ($P2Y_{1,2,4,6,11\text{-}14}$), that belong to G-protein-coupled family. These different P2 receptors have distinct agonist affinity/specificity hence controlling different cellular functions (Volonte *et al*., 2006; Khakh & North, 2006).

High concentrations of ATP in the extracellular tumor microenvironment have already been described (Mathew *et al*., 2007; Pellegati *et al*., 2008). Although the role for this extracellular ATP in tumor development is not completely understood, the final response, either pro-tumoral or tumoricidal, is largely determined by the specific expression/activation of P2R subtypes present at tumor tissue (Feng *et al*., 2014). To exert antitumor activity, pericellular ATP is thought to act through two main mechanisms, by stimulating antitumor immune responses and/or by directly inducing tumor cell death (Bian *et al*, 2014). The later effect has already been demonstrated on many types of tumor cells such as prostate cancer, melanoma, glioma and colon cancer cells (White & Burnstock, 2006; Souza *et al*., 2012). Among all P2 receptors, P2X5, P2X7, $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ seem to be involved in this tumor ATP-killing activity, besides the precise molecular mechanism remain unclear (White & Burnstock, 2006).

Although ATP-induced cell death may occur through different P2 receptors activation, P2X7 is far the most studied ATP-cytotoxic-related receptor. P2X7 has been associated with ATP-evoked tumor cell death in many

types of cancer, such as colon cancer, melanoma (Shabbir & Burnstock, 2009; Feng *et al*., 2011; Bian *et al*., 2013), squamous cell carcinomas (Deli & Csernoch, 2008), glioma (Gehring *et al.*, 2012), cervical cancer (Wang *et al*., 2004), and endometrial cancer (Li *et al*., 2006). While in the presence of high extracellular ATP, P2X7 is the only P2X receptor capable to form a membrane pore permeable to large molecules (up to 900 Da) and thus lead to tumor cell death through apoptosis (Adinolfi *et al.*, 2005). In this regard, low levels or defective P2X7 expression have been linked to cancer development by allowing tumor cells to escape from the P2X7 controlled pro-apoptotic mechanism (Huang *et al*., 2013). Thus, finding a mechanism that increases P2X7 activation and avoids cancer cell escape can be a new strategy to increase tumor cell death in association to traditional cancer treatments.

Recently, our group showed that short treatment of MCA38 colon cancer cells with high levels of ATP was able to induce cancer cell death in a P2X7 dependent way (Bian *et al*., 2013). But differently from another studies, we delineated a new downstream pathway involved in ATP-P2X7 signaling. Accordingly, two independent signaling axes, PI3K/AKT e AMPK-PRAS40-mTOR, appear to act synergistically in response to tumoricidal ATP-P2X7 signals to thereby elicit maximal tumor cell death by disrupting the balance between cell growth and autophagy. Considering these results, we proposed the use of purine-related drugs as adjunctive agents in cancer therapy.

An ideal strategy to maximized tumor cell death could be reached by using a substance that increases P2X7 sensitization in combination to a purine-related drug. Manipulation of plasma membrane cholesterol in order to increase P2X7 pore formation has already been described (Robinson *et al*., 2014). Accordingly, cholesterol depletion by using methyl-ß-cyclodextrin (MCD) caused a substantial increase in the rate of agonist-evoked pore formation, while cholesterol loading inhibited this process. It seems that P2X7 receptors localized in lipid rafts exhibit a more resistant mode of gating and addressing this receptor to non-rafts regions may be a way to increase P2X7 activation.

Besides being an ion channel, P2X7 can also form a receptor protein-complex comprised by at least 11 distinct proteins (Kim *et al*., 2001). Therefore, manipulating one of those intracellular interacting proteins may be a different

strategy to increase P2X7 activation. A role for heat shock protein 90 (HSP90) in this context has already been described (Adinolfi *et al.* 2003). In HEK 293 cells, HSP90 is normally tyrosine-phosphorylated in association with P2X7 receptor complex, but a reduction in its phosphorylation lead to an increase in P2X7 sensitivity to the agonist, suggesting that HSP90 may act as a negative regulator of P2X7 complex formation and function. In this study, geldanamycin, a HSP90 inhibitor, was used to decreased tyrosine phosphorylation of HSP90, but heat shock stress can also be applied to alter HSP90 activity *in vivo* (Yamada *et al.,* 2007).

In this regard, here we are proposing the use of heat shock stress to enhance the agonist efficacy in stimulate P2X7 pore opening and dilatation in MCA38 colon cancer cells. According to our data, heat shock stress is capable to increase P2X7 sensitization to ATP cytotoxicity independently of HSP interaction. The mechanism by which heat stress increases P2X7 pore dilatation seems to be related to alterations in the plasma microdomain structure, as the membrane fluidizer benzyl alcohol could reproduce heat stress effect in potentiating P2X7 activation at 37ºC. Our results point up the use of hyperthermia as a new strategy to modulate P2X7 activation and thus increase cancer cell death in condition where ATP is found in high concentrations, such as the tumor microenvironment.

Material and Methods

Reagents/Antibodies

Carbenoxolone (CBX), BAPTA-AM and thapsigargin (TG) were from Tocris Bioscience (Ellisville, MO); all other chemicals and cell culture media were from Sigma-Aldrich (St. Louis, MO). All other culture reagents were from Invitrogen (Carlsbad, CA). Rabbit anti-P2X7 antibody (#APR-004) was obtained from Alomone labs (Jerusalem, Israel); b-actin (AC-15, #ab6276) from Abcam (Cambridge, MA); HSP90α/β (N-17, #sc-1055), HSP70 (K-20, #sc-1060), Flotillin-2 (B6, #sc-28320) from Santa Cruz Biotechnology, Inc. (Dallas, TX), Caveolin-1 (7C8, #NB100-615) from Novus Biologicals (Littleton, CO), phospho-AKT (S473) (#9271), phospho- PRAS40 (T246) (#2997), phospho-S6K (T389) (#9205), phospho-S6 (S235/236) (#2211), HSP40 (C64B4) (#4871), HSP60

(D307) (#4870), from Cell Signaling Technology (Danvers, MA); HRP-conjugated goat anti-mouse (#31438), donkey anti-rabbit (#31458) and mouse anti-goat IgG (#31400) and the SuperSignal West Femto Maximum Sensitivity Substrate reagents (#PI-34096) were from Thermo Scientific (Rockford, IL).

Tumor Cell Lines

Syngeneic C57BL/6 murine MCA38 colon cancer cells (a gift of Dr. Nicholas P. Restifo, National Cancer Institute) were provided by Dr. Alan B. Frey at New York University School of Medicine (Koneru *et al*., 2005; Monu & Frey, 2007; Vazquez-Cintron *et al*., 2012). Cells were also tested for Mycoplasma and other infections by mouse IMPACT III PCR Profile via RADIL (Columbia, MO) and were maintained in culture flask in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin at 37ºC in a 5% CO2 atmosphere at 100% humidity. The generation of P2X7 knockdown cell line was already published and described by Bian *et al*., 2013. Puromicyn (3μg/mL) was added at the culture medium to keep cell lines selected.

Heat Shock treatment

In all experiments cells were pulse-treated with medium or ATP 1 mM for 20 min at 37ºC (incubator) or 40ºC (water bath). This mild temperature was chosen considering the cytotoxicity caused by higher temperatures.

Cell Viability

Cells ($7.5X10^3$) were seeded into 96-well plates and cultured for 24 hr. Cells were then treated as described above, replaced with fresh culture media, and grown for additional 24 hr. Cells viability was evaluated using Cell Counting Kit-8 (CCK-8, Dojindo Molecular Tech. Inc., Rockville, MD) that measures the activity of cellular dehydrogenases (correlating with cell proliferation), as previously established (Feng *et al*., 2011; Sun *et al*., 2013).

Annexin V and propidium iodide (PI) staining

Phosphatidylserine externalization was determined by the annexin

fluorescence signal of an annexin V–fluorescein isothiocyanate conjugate (BioLegend Inc, San Diego, CA) according to the manufacturer's protocol. Cell cultures were treated, trypsinized, and centrifuged for 5 min at 1200 rpm, and the supernatant was discarded. Cells were resuspend in Annexin V Binding Buffer at a concentration of 0.25- 1.0X10^7 cells/ml and then an aliquot of 100µl was taken and incubated with 5uL of FITC Annexin V and 10µl of PI for 15 min at room temperature in the dark. Samples were analyzed on BD FACS LSR II cytometer (BD Bioscience, San Diego, CA) using FlowJo V.10 software for analysis (Tree Star Inc, Ashland, OR).

Antagonist/Plasma membrane disturbance-treatment Experiments

Cells were pre-incubated with antagonists carbenoxolone 100 μM, BAPTA-AM 10 μM, thapsigargin 100 nM for 30 min and Geldanamycin (GAD) 0.5 μM or 1 μM for 6h before being exposed to pulse treatment with ATP or control medium. The cholesterol depleting agents, Methyl-β-cyclodextrin (MCD) 10 mM or Filipin 10 μM, were incubated for 20 min and removed before ATP treatment. Cholesterol loading at the plasma membrane was obtained by adding water-soluble cholesterol at 100 μg/mL for 30 min before and during ATP exposure. The membrane fluidizer benzyl alcohol (BA), a documented nondenaturant, was added together with ATP for 20 min in order to mimic the heat shock effect *per se*.

Analysis of intracellular and extracellular ATP levels by high-performance liquid chromatography

Intracellular and extracellular levels of ATP, ADP, and AMP were determined by highperformance liquid chromatography (HPLC) analysis as described (Chen *et al*., 2006; Corriden *et al*., 2008; Sun *et al*., 2013) with slight modifications. Cells were seeded into 35 mm X 10 mm dishes in culture media and kept for 24hr until reach 70% of confluence. Cells were then treated with ATP 1 mM or medium for 20 min, at 37ºC or 40ºC. The supernatant was removed, transferred to a new tube and centrifuged at 2,000 rpm for 10 min at 0ºC. A new supernatant aliquot was taken, centrifuged at 5,000rpm for 5 min, at 0ºC, precipitated by 5% 8 M perchloric acid (PCA) and stored at -80ºC for

subsequent HPLC analyses. The remaining cells were washed with ice-cold HBSS five times to remove excess extracellular ATP. Some wells were lysed with 130μL of protein lysis buffer for protein concentration measurement; and some wells were harvested with 600μL of HBSS containing 5% 8 M perchloric acid and then subjected to three frozen-thaw cycles. Cells were then scraped, transferred to a 1.5 ml Eppendorf tube, pulse sonicated on ice, and then stored at -80ºC for subsequent HPLC analysis using a Waters 484 system (Waters Corporation, Milford, MA) as previously described (Chen *et al*., 2006; Corriden *et al*., 2008; Sun *et al*., 2013).

Ethidium Bromide Uptake Assay

This assay was performed as previously published (Casas-Pruneda *et al.*, 2009) with slight modifications. Briefly, cells ($1.5X10^4$) were seeded into 96-black well plates. 24 hr later, cells were washed once with HBSS containing Ca^{2+}/Mg^{2+} and then incubated with 1.27 μM ethidium bromide (a cell impermeable organic dye) in the absence or presence of ATP 1 mM, for 20 min at 37ºC or 40ºC, followed by whole cell fluorescence measurement (in arbitrary units of fluorescence, AUF) at 544/610nm excitation/emission using the SoftMax Pro software on a SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA). AUF was normalized by number of cells and results are expressed as AUF/10^4 cells.

Western Blotting

Cells were seeded into 35 mm X 10 mm dishes in culture media and kept for 24hr until reach 70% of confluence. The next day, cells were treated with freshly made compounds and the reaction was stopped immediately by washing the cells with ice-cold PBS three times. Cells were then lysed in ice-cold modified-RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl) supplemented with Protease and Phosphatase Inhibitor Cocktails (Thermo Scientific). The lysates were kept on ice for at least 30 min and then centrifuged at 14,000 rpm for 20 minutes at 4ºC. The measurement of protein concentrations and detailed procedures of immunoblotting were described previously (Wu *et al.,* 2006; Sun *et al*., 2013).

Immunoprecipitation

Cells were seeded into 100 mm X 20 mm dishes and kept in culture media until reach 80% of confluence. Cells were then treated and the reaction was stopped immediately by washing the cells with ice-cold PBS three times. Cells lysate was performed as described in Western Blotting section. 150µg of the total cellular protein was incubated with 2µL (P2X7 or Caveolin-1) or 10µL (HSP90, HSP70 or Flotillin-2) of antibody with a end-over-end rotation at 4ºC for 4h. Then, 20µL of Protein G-Sepharose fast flow (Sigma Aldrich) were added into each mixture and incubated at 4ºC on a rotating device overnight. Another day, the mixture were centrifuged at 2500 rpm, for 5mim at 4ºC, washed three times with lyses buffer, eluted with 2X SDS reducing sample buffer (Bio-Rad, Hercules, CA) with 2% of ß-2-mercaptoethanol and resolved on a 4-12% gradient SDS-page gel (Bio-Rad, Hercules, CA) according to western blot methods.

Immunofluorescence

Cells were seeded into 24-well plates in a low confluence and cultured over a cover slip for 3 days until reach 50% of confluence. Then, cells were treated with medium or ATP 1 mM, for 20 min, at 37ºC or 40ºC, the supernatant was removed and cells were fixed with 2% paraformaldeahyde (PFA) (Electron Microscopy Sciences, Hatfield, PA) or cold methanol (Flotillin-2 staining only) for 15min. After fixed, cells were washed three times with phosphate buffered saline (PBS), incubated with 0.1M Glycine for 15 min, washed two times with PBS, incubated with 0.5% Triton X-100 for 30 min, washed two times with PBS, blocked with 7% Horse Serum (Vector Laboratories, Inc., Burlingame, CA) for 30 min and then incubated with P2X7 (1:2000) and HSP90 (1:100) or HSP70 (1:75) or Caveolin-1 (1:400) or Flotillin-2 (1:50) antibody overnight in the dark at 4ºC. Another day, cells were washed four times with PBS, incubated with anti-rabbit (Alexa Fluor®594) and anti-goat (Alexa Fluor®488) or anti-mouse (Alexa Fluor®488) (Life Technologies, Eugene, OR) secondary antibody (1:300) for 1h at 37ºC in the dark, washed two times with PBS, stained with Hoescht 33258 nuclear dye (1:10,000) (Life Technologies, Eugene, OR) for 3 min at 37ºC in the dark, washed two times with PBS and two times with distilled water, dried and

finally mounted in a microscope slide to be analyzed in a Zeiss AxioVert 200M Inverted Fluorescent Microscope (Peabody, MA). Pictures were taken at 40X increase and negative controls with anti-rabbit, anti-goat and anti-mouse IgG were tested in parallel. For caveolin-1/P2X7 and flotillin-2/P2X7 membrane co-immunostaining Triton X-100 step wasn't performed.

For filipin staining, paraformadehyde-fixed cells were incubated with 0.5 mg/mL filipin in PBS for 60 min and then washed twice with PBS (Eskelinen *et al*., 2004), twice with distilled water, dried and mounted in a microscope slide to be analyzed according to describe above.

Statistical analysis

Statistical analysis was performed with Prism 5 (GraphPad, La Jolla, CA). Data are expressed as percentage of control and presented as mean ± SD of at least three independent experiments. Statistical analyses for comparison among multiple groups were performed by one-way analysis of variance (ANOVA), followed by a Turkey post-hoc test. When more than one molecule was mixed to the same well at the same time, a two-way ANOVA was performed, followed by a Bonferroni post-test. Values were considered significant at $p < 0.05$.

Results

Heat Shock (HS) increases ATP tumor-killing activity in a P2X7-dependent manner leading cells to death via P2X7-AKT/PRAS40/mTOR signaling.

MCA38 colon cancer cells expressing P2X7 receptor became sensitive to ATP cytotoxicity when exposed to heat shock stress (HS=40ºC) (Fig. 1). Moreover, this effect seems to be completely dependent on P2X7 activation, since P2X7 knockdown cells were still resistant to ATP treatment in that condition (Fig. 1A and C). Pictures taken from cells and forward scatter analysis showed that ATP pulse treatment associated to HS led to cell shrinkage (Fig. 1B upper and middle) and FACS analysis using Annexin-V/PI staining pointed up a double stained in almost all cells (Fig. 1B, bottom right), suggesting apoptotic cell death. The intracellular pathway involved in this HS sensitization ATP-induced cell death seems to be via P2X7 dependent-AKT/PRAS40/mTOR

signaling (Fig.1C), as we can observe by a decreasing phosphorylation of pathway components of mTOR (p70 S6 kinase - S6K, S6 ribossomal protein – S6) and PI3/AKT (AKT and PRAS40) in response to eATP plus HS. According to previous data published by our group, these signaling perturb the balance between growth and autophagy, leading cells to death (Bian *et al*., 2013).

HS facilitates ATP cytotoxicity by increasing P2X7 functionality independently of calcium signaling and pannexin/connexin association.

Data from Figure 2 supports the heat shock increased P2X7 non-seletive pore formation activity in the presence of ATP. According to ethidium bromide uptake assay, which measure P2X7 channel function, ATP markedly stimulates ethidium bromide uptake only when cells were exposed to heat shock stress (Fig. 2A). Moreover, analysis of extracellular and intracellular adenine nucleotides by HPLC showed that in this condition a significant increase in the extracellular ATP levels occurs concomitantly with a decrease in the intracellular part in a P2X7 dependent way (Fig. 2B). This imbalance could be explained by the “porin” P2X7 channels function, which could facilitate adenine nucleotide efflux. However, we must consider that a large extracellular ATP amount may be coming from died cells as well as a certain intracellular amount may be consumed by the dying cells metabolism.

Taking account that P2X7 is a ligand-gated ion channel and it activation by high ATP levels normally leads to an increase Ca^{2+} influx into cells, we also investigated if this signaling is involved in HS-ATP-P2X7 mediated tumor killing activity. For this, two experimental approaches were employed using two chemical compounds that differently alter cytosolic Ca^{2+} levels: BAPTA-AM, a selective cell permeable calcium chelator to block intracellular Ca^{2+} stores, and thapsigargin (TG), a potent inhibitor of endoplasmic reticulum Ca^{2+}-ATPase causing an immediate increase in cytoplasmic Ca^{2+} levels. According to western blot analysis neither BAPTA-AM nor TG was able to blockage P2X7-AKT/PRAS40/mTOR signaling involved with cell death (Fig. 2C). Indeed, carbenoxolone (CBX), a pharmacological inhibitor of pannexin-or conexin type channels, was also unable to prevent the cell death induction, suggesting that the cytolytic pore formation is an intrinsic property of P2X7 receptor (Fig. 2C)

Heat shock proteins (HSP) seem to not contribute to HS stimulated P2X7 hyperactivation.

Besides being a ligand-gated ion channel, P2X7 was found to be associated with at least 11 distinct proteins to form a receptor-protein complex. Among these proteins, three heat shock proteins were identified, HSP90, HSP70 and HSC70 (Kim *et al*., 2001). Moreover, a tyrosine phosphorylation of HSP90 within the P2X7 receptor complex was found to negatively regulate P2X7 receptor activity in human embryonic kidney cells (Adinolfi *et al*., 2003). Based on this information, we next identify a possible heat shock proteins (HSP) role in P2X7 hyperactivation after HS+ATP treatment. According to figure 3, in MCA38 colon cancer cells P2X7 overactivation is no related to a HSP interaction. The HSP90 inhibitor, geldanamycin (GAD), acts by decreasing tyrosine phophorylation of HSP90 and it was capable to promote cell death by itself at 37ºC (Fig. 3A). Indeed, when GAD was associated with ATP an increase cell death independent of P2X7 was observed, suggesting another mechanism of cell death induction. No difference was found at the HSP protein levels (Fig. 3B) and localization (Fig. 3C) when cells were exposed to heat shock stress (with or without ATP). Moreover, analysis of immunofluorescence pointed up to a P2X7 profile change (from dots in control cells to spread in HS+ATP treated cells) at the plasma membrane. Finally, no association was established between P2X7 receptor and HSP90 or HSP70 in this cell line (Fig. 3C and D). As we expected, HSP90 and HSP70 interact to form a HSP complex (Fig. 3D).

HS increased P2X7 sensitivity to ATP is not associated to P2X7-lipid rafts perturbation at the plasma membrane.

Considering that P2X7 receptors can be associated with cholesterol-rich lipid rafts and this interaction may affects receptor channel properties (Robinson *et al*., 2014) we then evaluated a possible mechanism involving heat shock induction P2X7-lipid rafts perturbation at the plasma membrane. According to Figure 4, the interaction P2X7-lipid rafts seems not to be important to heat shock mediated P2X7 hyperactivation. A co-localization between P2X7 and the two types of lipid rafts, caveolae and planar lipid rafts were evaluated by

immunofluorescence (Fig. 4A and B, respectively). Representing caveolae, caveolin-1 immunofluorescence signal was detected concentrate in one side of the cell at the normal condition (37ºC). When cells were exposed to heat shock stress (with or without ATP), caveolin-1 becomes widely spread allover the cell (Fig 4A, right). No differences could be observed for P2X7 distribution or co-localization (Fig. 4A, left). A similar result was found when we evaluated flotillin-2, a planar lipid rafts marker. According to immunofluorescence signal, flotillin-2 seems to be normally concentrated in small dots at plasma membrane, but after heat shock disturb (associated with ATP), flotillin-2 becomes broadly spread at the plasma membrane and almost no dots can be observed (Fig. 4B, right). Again, no pronounced differences could be visualized for P2X7 receptor (Fig. 4B, left). These differences in caveolin-1 and flotillin-2 distribution may be related to alterations in the plasma membrane microdomain structure induced by heat shock stress.

In order to completely discard a lipid rafts role affecting P2X7 activity, we also performed an experiment using two different compounds that distinctly disrupt lipid rafts before exposing cells to heat shock and ATP treatment. According to Figure 4C, neither methyl β-cyclodextrin (MCD), which acts by depleting lipid rafts cholesterol (Fig. 4D) nor filipin, which acts by forming a multimeric globular complex with membrane cholesterol was capable to block P2X7 hyperactivation and cell death (Fig. 4C).

HS seems to induce profound reorganization of cholesterol-rich membrane domains thus leading to P2X7 increased activity.

Data from our immunofluorescence suggest that heat shock may induce alterations in the plasma membrane microdomain structure. Moreover, according to literature, there is a growing body of evidence linking the cellular response to heat stress to changes in the lipid composition and architecture of membranes (Nagy *et al*., 2007). In this regard, Nagy and co-workers showed that the membrane fluidizer benzyl alcohol (BA), a documented nondenaturant, acts similarly as heat stress *per se*, initiating profound reorganization of cholesterol-rich microdomains at the plasma membrane. In this way, we next tested the hypothesis that BA could increase P2X7 activation, as well as heat

shock does, but at 37ºC. As reported by figure 5, BA was capable to reproduce all heat shock effects in association with ATP, however at 37ºC. Our data reinforces the previous published data in relation to BA acts similarly as heat stress *per se*, but bring a new information about their abilities to alter P2X7 pore formation and thus increase tumor cell death upon ATP exposure.

At last, in order to confirm that there is no interaction between P2X7 and lipid rafts even after all this changes in the lipid composition and architecture of membranes, we performed P2X7, caveolin-1 or flotillin-2 immunoprecipitation (Fig. 5E). And, as we expected, no interaction between P2X7 and lipid rafts in this cell line seems to happen.

Even though we found that BA and heat shock facilitate P2X7 pore formation in the presence of ATP, we still don't know the mechanism involved in this process. To test the hypothesis that heat stress and BA induce a characteristic reorganization of cholesterol-rich membrane domains, we stained cells cholesterol with fillipin. According to figure 6A, both heat stress and BA alone appears to not be inducing alterations in the plasma membrane cholesterol, but the association with ATP makes cells present distinct cholesterol reorganization. Moreover, BA fluidizer property doesn't seems to be important to P2X7 hyperactivation, since loading cholesterol at the plasma membrane along the treatments do not prevent cancer cell death (Fig. 6B).

Altogether these findings suggest that a complex mechanism, involving more than changes in the physical state of membranes *per se,* is related to P2X7 hyperactivation after heat shock-ATP stimulation. The appearance of specific microdomains or changes in the composition of particular lipid involved directly in specific lipid-protein interactions are potentially and equally able to furnish stimuli for the activation or attenuation of certain protein receptors, such as P2X7 (Nagy *et al*., 2007).

Discussion

Tumor microenvironment is ATP rich in comparison to healthy tissues (Pellegatti *et al*., 2008). Moreover, after radiation and chemotherapy high levels of this nucleotide are released by dying tumor cells (Pellegatti *et al*., 2008; Aymeric *et al*., 2010). In this context, extracellular ATP has been identified as a

novel danger signal leading to immune system stimulation via P2X7-NLRP3 inflammasome activation (Ghiringhelli *et al*., 2009; Aymeric *et al*., 2010), thus increasing antitumor immune response. Indeed, extracellular ATP can also exert direct cytotoxicty on tumor cells and vascular endothelial cells, limiting tumor growth and angiogenesis, respectively (Feng *et al*., 2011). Therefore, it can be postulated that keeping ATP levels high in tumor tissues may be a good strategy to increase the efficacy of traditional anti-cancer therapy (Bian *et al*., 2013).

We recently reported that exposing tumor cells briefly to high levels of exogenous ATP was able to inhibit tumor growth, both in vitro and in vivo. Indeed, we described a new molecular mechanism that links extracellular ATP-P2X7 signals to the intracellular mTOR pathway (Bian *et al*., 2013). mTOR is a well-known sensor for extracellular nutrients and growth factors, being a central regulator of essential cellular functions such as proliferation/growth, survival, migration, adhesion, autophagy and metabolism (Bjornsti & Houghton, 2004). Two main upstream signal, Ras/mitogenactivated protein kinase (MAPK) and PI3K/AKT, converge to mTOR, inhibiting or stimulating it, respectively, thus controlling tumor cell growth (Shaw & Cantley, 2006). Accordingly to our previous published data, PI3K/AKT axes and AMPK/PRAS40/mTOR axes seem to act independently and synergistically in response to ATP-P2X7 activation, leading to tumor cell death by disrupting the balance between cell growth and autophagy (Bian *et al*., 2013).

In the present study, we further investigate a mechanism to increase P2X7 sensitivity to extracellular ATP in order to maximize cancer cell death in this context. We demonstrate for the first time that heat shock stress is capable to increase ATP-P2X7-mTOR elicited tumor cell death, by altering P2X7 functionality independently of heat shock protein interaction or native pore-forming transporters association (e.g pannexin-or connexin-type channels). The precise mechanism by which heat stress increase P2X7 pore dilatation is not completely understood, but it seems to be related to alterations in the plasma microdomain structure. There is a growing body of evidences linking the cellular response to heat stress to changes in the lipid composition and architecture of membranes (Nagy *et al*., 2007) and this effect may easily result in changes at

the plasma membrane protein distribution as well as in specific lipid-protein interaction.

Nagy *et al*. (2007) performed and elegant study using a fluorescent label probe that inserts into the cholesterol-rich membrane domains (fPEG-Chol) to evaluate plasma membrane changes caused by heat shock stress. According to this study, either heat stress or the membrane fluidizer, benzyl alcohol (BA), was able to produce profound alterations in the plasma membrane microdomains and therefore generate stress signal to activate *hsp* genes. As reported by the authors BA acts similarly as heat stress *per se* and thus can be used on its behalf to produce plasma membrane stress signaling. Here we demonstrated that BA was able to reproduce at 37ºC the heat shock effect in potentiate ATP-P2X7 induced cancer cell death, linking changes in the plasma membrane organization and/or composition to P2X7 increased functionality.

Taking account that heat shock initiates profound alterations in the plasma membrane microdomain structure we can presume that changes in temperature could also result in altered solubility, redistribution and activity of some proteins presents in the lipid rafts (Vigh *et al*., 2005 Vigh *et al*., 2007). Lipids rafts are microdomains rich in cholesterol and sphingolipids within the cell membrane that can have important signaling roles (Allsopp *et al*., 2010). Evidences for the involvement of P2X receptors in lipids rafts are emerging and the presence of P2X1 and P2X3 in rafts has also been described (Vacca *et al*., 2004; Vial & Evans, 2005). In both cases, their presence in the lipids rafts facilitated receptor function, suggesting that this association might be important for receptor stability and responsiveness. On the other hand, the association of P2X7 with lipids rafts seems to differ according to the cell type. But basically when present in non-rafts compartments P2X7 seems to have an increased ion channel activity in contrast to a more resistant mode of gating when it is localized in lipid rafts (Barth *et al*., 2007; Garcia-Marcos *et al*., 2006; Robinson *et al*., 2014). Here we couldn't find an association between P2X7 receptor and lipid rafts, moreover, cholesterol depletion or loading to the plasma membrane seems to not be important for P2X7 activation and sensitization, suggesting that changes in the order and arrangement of lipids around P2X7 may be the mechanism involved in heat shock inducing P2X7 hyperactivity.

Despite it has been shown that heat shock stress promotes changes in the levels of HSP (Nagy *et al*., 2007; Yamada *et al*., 2007) and HSP90 seems to negatively regulate P2X7 receptor complex (Adinolfi *et al*., 2003), in our work we could observe neither a change in HSP levels nor a role for HSP90 in modulating P2X7 activity. The later can be explained by the fact that HSP90 seems to not be interacting with P2X7 receptor at the MCA 38 plasma membrane cells neither in normal conditions nor after being stimulated by heat stress. The former can be justify by the fact that changes on the level of HSP are only observed when cells are exposed to a minimal temperature of 41ºC for at least 1h (Nagy *et al*., 2007), and in our work cells were exposed to a lower temperature (40ºC) and for a shorter time (20min).

The clinical use of hyperthermia for cancer therapy either singly or in combination with radiotherapy or chemotherapy has been employed by many researchers (Luk *et al*., 1984; Irish *et al*., 1986; Seegenschmiedt *et al*., 1993) with significant improvements in outcome for prostate (Van Den Berg *et al*., 2006; Hurwitz *et al.*, 2011), breast (Moros *et al*., 2010; Zagar *et al*., 2010), cervix (Vasanthan *et al*., 2005; Franckena & Van Der Zee, 2010) and head and neck cancer (Huilgol *et al*., 2010a; Huilgol *et al*., 2010b). Generally, associating hyperthermia to the conventional therapy has not resulted in increased toxicity, but has contributed to better control, cure and/or palliation. Our data support the use of hyperthermia as an adjunctive modality to treat cancer in association to traditional regimen and bring a new insight for this treatment modality regardless the modulation of P2X7 activation in the presence of high levels of ATP, such as tumor microenvironment.

In conclusion, we described a novel strategy to increase cancer cell death by modulating P2X7 activation using mild heat stress in association to high levels of ATP. Considering the importance of ATP-P2X7 activation in mediating antitumor immune responses, direct cytotoxicity of tumor and blockade of tumor angiogenesis, our work affirm the therapeutic potential use of hyperthermia associated with purine-related drugs to achieve maximal efficacy of cancer therapy.

Acknowledgments: Financial support from NIH/USA and doctoral scholarship from CAPES/Brazil.

References

ADINOLFI, E.; CALLEGARI, M. G.; FERRARI, D.; BOLOGNESI, C.; MINELLI, M.; WIECKOWSKI, M. R.; PINTON, P.; RIZZUTO, R.; DI VIRGILIO, F. Basal activation of the P2X7 ATP receptor elevates mitochondrial calcium and potential, increases cellular ATP levels, and promotes serum-independent growth. *Molecular Biology of the Cell*, v.16, p.3260–3272, 2005.

ADINOLFI, E.; KIM, M.; YOUNG, M. T.; DI VIRGILIO, F.; SURPRENANT A. tyrosine phosphorylation of HSP90 within the P2X7 receptor complex negatively regulates P2X7 receptors. *The journal of biological chemestry,* v.278, p.37344-51, 2003.

ALLSOPP, R. C.; LALO, U.; EVANS, R. J. Lipid raft association and cholesterol sensitivity of P2X1-4 receptors for ATP: chimeras and point mutants identify intracellular amino-terminal residues involved in lipid regulation of P2X1 receptors. *The Journal of Biologycal Chemistry*, v.285, p.32770-7, 2010.

AYMERIC, L.; APETOH, L.; GHIRINGHELLI, F.; TESNIERE, A.; MARTINS, I.; KROEMER, G.; SMYTH, M. J.; ZITVOGEL, L. Tumor cell death and ATP release prime dendritic cells and efficient anticancer immunity. *Cancer Research*, v.70, p.855–858, 2010.

BARTH, K.; WEINHOLD, K.; GUENTHER, A.; YOUNG, M. T.; SCHNITTLER, H.; KASPER, M. Caveolin-1 influences P2X7 receptor expression and localization in mouse lung alveolar epithelial cells. *The FEBS Journal*, v.274, n.12, p.3021-33, 2007.

BIAN, S.; SUN, X.; BAI, A.; ZHANG, C.; LI, L.; ENJYOJI, K.; JUNGER, W. G.; ROBSON, S. C.; WU, Y. P2X7 integrates PI3K/AKT and AMPK-PRAS40-mTOR signaling pathways to mediate tumor cell death. *PLoS One,* v.; 8, p.e60184, 2013.

BJORNSTI, M. A.; HOUGHTON, P. J. The TOR pathway: a target for cancer therapy. *Nature Reviews. Cancer*, v. 4, p.335–348, 2004.

CASAS-PRUNEDA, G.; REYES, J. P.; PEREZ-FLORES, G.; PEREZ-CORNEJO, P.; ARREOLA, J. Functional interactions between P2X4 and P2X7 receptors from mouse salivary epithelia. *The Journal of Physiology*, v.587, p.2887–2901, 2009.

CHEN, Y.; CORRIDEN, R.; INOUE, Y.; YIP, L.; HASHIGUCHI, N.; ZINKERNAGEL, A. NIZET, V.; INSEL, P. A.; JUNGER, W. G. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science*, v.314, p.1792–1795, 2006.

CORRIDEN, R.; CHEN, Y.; INOUE, Y.; BELDI, G.; ROBSON, S. C.; INSEL, P. A.; JUNGER, W. G. Ectonucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1/CD39) regulates neutrophil chemotaxis by hydrolyzing released ATP to adenosine. The Journal of Biologycal Chemistry, v.283, p.28480–28486, 2008.

DEAGLIO, S.; ROBSON, S. C. Ectonucleotidases as regulators of purinergic signaling in thrombosis, inflammation, and immunity. *Advances in pharmacology*, v.61, p.301-32, 2011.

DELI, T.; CSERNOCH, L. Extracellular ATP and cancer: an overview with special reference to P2 purinergic receptors. *Pathology Oncology Research*, v.14, p.219-31, 2008.

ELTZSCHIG, H. K.; SITKOVSKY, M. V.; ROBSON, S. C. Purinergic signaling during inflammation. *The New England Journal of Medicine*, v.367, p.2322-33, 2012.

ESKELINEN, E. L; SCHMIDT, C. K.; NEU, S.; WILLENBORG,M.; FUERTES, G.; SALVADOR, N.; TANAKA, Y.; LULLMANN-RAUCH, R; HARTMANN, D.; HEEREN, J.; VON FIGURA, K.; KNECHT, E.; SAFTIG, P. Disturbed Cholesterol Traffic but Normal Proteolytic Function in LAMP-1/LAMP-2 Double-deficient Fibroblasts. *Molecular Biology of the Cell*, v.15, p.3132-3145, 2004.

FENG, L.; SUN, X., CSIZMADIA, E.; HAN, L.; BIAN, S.; MURAKAMI, T.; WANG, X.; ROBSON, S. C.; WU, Y. Vascular CD39/ENTPD1 directly promotes tumor cell growth by scavenging extracellular adenosine triphosphate. *Neoplasia*, v.13, p.206-16, 2011.

FENG, L.; TAPPER, E. B.; SUN, X.; GEHRING, M.; ROBSON, S. C.; WU, Y. Purinegic Modulation and CD39/ENTPD1 in Cancer. *Frontiers in Anti-Cancer Drug Discovery*, Chapter 5, v.4, p.229-292, 2014.

FRANCKENA, M.; VAN DER ZEE, J. Use of combined radiation and hyperthermia for gynecological cancer. *Current Opinion in Obstetrics & Gynecology*, v. 22, p.9–14, 2010.

GARCIA-MARCOS, M.; POCHET, S.; MARINO, A.; DEHAYE, J. P. P2X7 and phospholipid signalling: the search of the “missing link” in epithelial cells. *Cellular Signalling*, v.18, p.2098–2104, 2006.

GEHRING, M. P.; PEREIRA, T. C.; ZANIN, R. F.; BORGES, M. C.; BRAGA FILHO, A.; BATTASTINI, A. M.; BOGO, M. R.; LENZ, G.; CAMPOS, M. M.; MORRONE, F. B. P2X7 receptor activation leads to increased cell death in a radiosensitive human glioma cell line. *Purinergic Signalling*, v.8, p.729-39, 2012.

GHIRINGHELLI, F.; APETOH, L.; TESNIERE, A.; AYMERIC, L.; MA, Y.; et al. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nature Medicine*, v.15, p.1170–1178, 2009.

HUANG, S.; CHEN, Y.; WU, W.; OUYANG, N.; CHEN, J.; LI, H.; LIU, X.; SU, F.; LIN, L.; YAO, Y. miR-150 promotes human breast cancer growth and malignant behavior by targeting the pro-apoptotic purinergic P2X7 receptor. *PLoS One*, v.8, p.e80707, 2013.

HUILGOL, N. G.; GUPTA, S.; SRIDHAR, C. R. Hyperthermia with radiation in the treatment of locally advanced head and neck cancer: a report of randomized trial. *Journal of Cancer Research and Therapeutics*, v.6, p.492–496, 2010a.

HUILGOL, N. G.; GUPTA, S.; DIXIT, R. Chemoradiation with hyperthermia in the treatment of head and neck cancer. *International Journal of Hyperthermia*, v.26, p.21–25, 2010b.

HURWITZ, M. D.; HANSEN, J. L.; PROKOPIOS-DAVOS, S.; MANOLA, J.; WANG, Q.; BORNSTEIN, B. A.; HYNYNEN, K.; KAPLAN, I. D. Hyperthermia combined with radiation for the treatment of locally advanced prostate cancer: long-term results from Dana-Farber Cancer Institute study 94–153. *Cancer,* v.117, p.510–516, 2011.

IRISH, C. E.; BROWN, J.; GALEN, W. P.; GALLUCCI, J. J.; HYMAN, M. D.; HOROWITZ, I. J.; SNEDECOR, P. A.; BAKER, H. W. Thermoradiotherapy for persistent cancer in previously irradiated fields. *Cancer*, v.57, p.2275–2279,1986.

KHAKH, B. S.; NORTH, R. A. P2X receptors as cell-surface ATP sensors in health and disease. Nature, V.442, p.527-32, 2006.

KIM, M.; JIANG, L.H.; WILSON, H. L.; NORTH, R. A.; SURPRENANT, A. Proteomic and functional evidence for a P2X7 receptor signalling complex. *The EMBO Journal,* v.20, p.6347-58, 2001.

KIM, N. H.; PARK, K. S.; SOHN, J. H.; YEH, B. I.; KO, C. M.; KONG, I. D. Functional expression of P2Y receptors in WERI-Rb1 retinoblastoma cells. Korean J Physiol Pharmacol, v.15, p.61–66, 2011.

KONERU, M.; SCHAER, D.; MONU, N.; AYALA, A.; FREY, A. B. Defective proximal TCR signaling inhibits CD8+ tumor-infiltrating lymphocyte lytic function. *Journal of Immunology*, v.174, p.1830–1840, 2005.

LI, X.; ZHOU, L.; FENG, Y. H.; ABDUL-KARIM, F. W.; GORODESKI, G. I. The P2X7 receptor: a novel biomarker of uterine epithelial cancers. *Cancer Epidemiology Biomarkers & Prevention*, v.15, p.1906-13, 2006.

LUK, K. H.; FRANCIS, M. E.; PEREZ, C. A.; JOHNSON, R. J. Combined radiation and hyperthermia: comparison of two treatment schedules based on data from a registry established by the Radiation Therapy Oncology Group (RTOG). *International Journal of Radiation Oncology, Biology, Physics*, v.10, p.801–809, 1984.

MATHEW, R.; KARANTZA-WADSWORTH, V.; WHITE, E. Role of autophagy in cancer. *Nature Reviews. Cancer,* v.7, p.961-7, 2007.

MONU, N., FREY, A. B. Suppression of proximal T cell receptor signaling and lytic function in CD8+ tumor-infiltrating T cells. Cancer Research, v.67, p.11447–11454, 2007.

MOROS, E. G.; PENAGARICANO, J.; NOVAK, P.; STRAUBE, W. L.; MYERSON, R. J. Present and future technology for simultaneous superficial thermoradiotherapy of breast cancer. *International Journal of Hyperthermia*, v.26, p.699–709, 2010.

NAGY, E.; BALOGI, Z.; GOMBOS, I.; AKERFELT, M.; BJORKBOM, A.; BALOGH, G.; TOROK, Z.; MASLYANKO, A.; FISZER-KIERZKOWSKA, A.; LISOWSKA, K.; SLOTTE, P. J.; SISTONEN, L.; HORVATH, I.; VIGH, L. Hyperfluidization-coupled membrane microdomain reorganization is linked to activation of the heat shock response in a murine melanoma cell line. *Proceedings of the National Academy of Sciences of the United of States of America*, v.104, p.7945-7950, 2007.

PELLEGATTI, P.; RAFFAGHELLO, L.; BIANCHI, G.; PICCARDI, F.; PISTOIA, V.; DI VIRGILIO, F. Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. *PLos One*, v.3, n.7, p.e2599, 2008.

ROBINSON, L. E.; SHRIDAR, M.; SMITH, P.; MURRELL-LAGNADO, R. D. Plasma Membrane Cholesterol as a Regulator of Human and Rodent P2X7 Receptor Activation and Sensitization. *The Journal of Biological Chemistry*, v.289, p.31983-94, 2014.

ROGER, S.; JELASSI, B.; COUILLIN, I.; PELEGRIN, P.; BRESSON, P.; JIANG, L.H. Understanding the roles of P2X7 receptor in solid tumor progression and therapeutic perspectives. *Biochimica et Biophysica Acta*, v.1848, n.10PtB, p.2584-602, 2014.

SEEGENSCHMIEDT, M. H.; SAUER, R.; MIYAMOTO, C.; CHALAL, J. A.; BRADY, L. W. Clinical experience with interstitial thermoradiotherapy for localized implantable pelvic tumors. *American Journal of Clinical Oncology*, v.16, p.210–222, 1993.

SHABBIR, M.; BURNSTOCK, G. Purinergic receptor-mediated effects of adenosine 5'-triphosphate in urological malignant diseases. *International Journal of Urology*, v.16, p.143-50, 2009.

SHAW, R. J.; CANTLEY, L. C. Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature*, v.441, p.424–430, 2006.

SOUZA, C. O.; SANTORO, G. F.; FIGLIUOLO, V. R.; NANINI, H. F.; DE SOUZA, H. S.; CASTELO-BRANCO, M. T.; ABALO, A. A.; PAIVA, M. M.; COUTINHO, C. M.; COUTINHO-SILVA, R. Extracellular ATP induces cell death in human intestinal epithelial cells. Biochimica at Biophysica Acta, v.1820, p.1867-78, 2012.

SUN, X.; HAN, L.; SETH, P.; BIAN, S.; LI, L.; CSIZMADIA, E.; JUNGER, W. G.; SCHMELZLE, M.; USHEVA, A.; TAPPER, E. B.; BAFFY, G.; SUKHATME, V. P. WU, Y.; ROBSON, S. C. Disordered purinergic signaling and abnormal cellular metabolism are associated with development of liver cancer in Cd39/Entpd1 null Mice. *Hepatology*, v.57, p.205–216, 2013.

VACCA, F.; AMADIO, S.; SANCESARIO, G.; BERNARDI, G.; VOLONTE, C. P2X3 receptor localizes into lipid rafts in neuronal cells. *Journal of Neuroscience Research*, v.76, p.653–661, 2004.

VAN DEN BERG, C. A.; VAN DE KAMER, J. B.; DE LEEUW, A. A.; JEUKENS, C. R.; RAAVMAKERS, B. W.; VAN VULPEN, M.; LAGENDIJK, J. J. Towards patient specific thermal modeling of the prostate. *Physics in Medicine and Biology*, v.51, p.809–825, 2006.

VASANTHAN, A.; MITSUMORI, M.; PARK, J. H.; ZHI-FAN, Z.; YU-BIN, Z.; OLIYNYCHENKO, P.; TATSUZAKI, H.; TANAKA, Y.; HIRAOKA, M. Regional hyperthermia combined with radiotherapy for uterine cervical cancers: a multi-institutional prospective randomized trial of the international atomic energy agency. International Journal of Radiation Oncology, Biology, Physics, v.61, p.145–153, 2005.

VAZQUEZ-CINTRON, E. J.; MONU, N. R.; BURNS, J. C.; BLUM, R.; CHEN, G.; LOPES, P.; MA, J.; RADOJA, S.; FREY, A. B. Protocadherin-18 is a novel differentiation marker and an inhibitory signaling receptor for CD8+ effector memory T cells. *PLoS One,* v.7, p.e36101, 2012.

VIAL, C.; EVANS, R. J. Disruption of lipid rafts inhibits P2X1 receptor-mediated currents and arterial vasoconstriction. The Journal of Biologycal Chemistry, v.280, p.30705–30711, 2005.

VIGH, L.; ESCRIBÁ, P.; SONNLEITNER, A.; SONNLEITNER, M.; PIOTTO, S.; MARESCA, B.; HORVÁTH, I.; HARWOOD, L. J. The significance of lipid composition for membrane activity: new concepts and ways of assessing function. Progress in Lipid Reseach, v.44, p.303–344, 2005.

VIGH, L.; TOROK, Z.; BALOGH, G.; GLATZ, A.; PIOTTO, S.; HORVATH, I. In Molecular Aspects of the Stress Response: Chaperones, Membranes and Networks, eds Csermely P, Vigh L (Springer, New York), p.114–131, 2007.

VOLONTE, C.; AMADIO, S.; D'AMBROSI, N.; COLPI, M.; BURNSTOCK, G. P2 receptor web: complexity and fine-tuning. *Pharmacology & Theraputics*, v.112, p.264-80, 2006.

WANG, Q.; WANG, L.; FENG, Y. H.; LI, X.; ZENG, R.; GORODESKI, G. I. P2X7 receptor-mediated apoptosis of human cervical epithelial cells. *American Journal of Physiology Cell Physiology*, v.287, p.C1349-58, 2004.

WHITE, N.; BURNSTOCK, G. P2 Receptors and cancer. *Trends in Pharmacological Science*, v.27, n.4, p.211-7, 2006.

WU, Y.; SUN, X.; KACZMAREK, E.; DWYER, K. M.; BIANCHI, E.; USHEVA A.; ROBSON, S. C. RanBPM associates with CD39 and modulates ecto-nucleotidase activity. The Biochemical Journal, v.396, p.23–30, 2006.

YAMADA, K.; FUKAO, Y.; HAYASHI, M.; FUKAZAWA, M.; SUZUKI, I.; NISHIMURA, M. Cytosolic HSP90 Regulates the Heat Shock Response That Is Responsible for Heat Acclimation in Arabidopsis thaliana. *The Journal of Biological Chemestry*, v. 282, p. 37794–37804, 2007.

ZAGAR, T. M.; OLESON, J. R.; VUJASKOVIC, Z.; DEWHIRST, M. W.; CRACIUNESCU, O. I.; BLACKWELL, K. L.; PROSNITZ, L. R.; JONES, E. L. Hyperthermia combined with radiation

therapy for superficial breast cancer and chest wall recurrence: a review of the randomised data. International Journal of Hyperthermia, v.26, p.612–617, 2010.

Figure captions

Figure 1. Heat Shock (HS) increases ATP cytotoxicity in a P2X7-dependent manner. A. MCA38 cells expressing (Ctrl KD) or knocking down (P2X7 KD) P2X7 receptor were exposed to ATP 1 mM for 20 min at 37ºC or 40ºC (heat stress) and cell viability was evaluated after 24h. Note that HS increased ATP cytotoxicity only in P2X7 expressing cells. **B.** Pictures taken from cells (upper), forward scatter analysis (middle) and Annexin-V/PI double staining (bottom) after respective treatments. **C.** Western Blot analysis showing the P2X7-AKT/PRAS40/mTOR signaling pathway involved in tumor cell death. *p<0.05 in relation to control (one-way ANOVA, followed by Tukey pos-test).

Figure 2. HS + ATP lead cells to decrease intracellular ATP and promote cell death through a Ca^{2+}/pannexin/connexin-independet pathway. A. P2X7 functionality measured by Etidium Bromide (EtBr) Uptake assay. Note that a significant increase in EtBr uptake occurred only when cells were exposed to HS+ATP, suggesting P2X7 pore formation in this condition. **B.** Extracellular (upper) and intracellular (bottom) adenine nucleotide levels measured by HPLC after ATP exposure. Results are expressed as nmols of compounds/mg of protein. Basal levels of compounds were determined without ATP treatment and no difference was found *(data not shown)*. **C.** Cells were pre-incubated with carbenoxolone 100μM, BAPTA-AM 10μM or thapsigargin 100nM previous ATP pulse treatment and then cells were lysed and western blot was performed. *p<0.05 in relation to control (one-way ANOVA, followed by Tukey pos-test).

Figure 3. Heat shock proteins (HSP) are not involved in P2X7 hyperactivation after HS + ATP treatment. A. Cells were pre-treated with geldanamycin (GAD), a HSP90 inhibitor, for 6h before being exposed to ATP 1mM for 20min at 37ºC and cell viability was verified 24h later. **B.** HSP levels were determined by western blot after cells being treated or not with ATP 1mM, for 20min, at 37ºC or 40ºC. **C.** Cells were immunostained with P2X7 (red) and HSP90 or HSP70 (green) antibody. Nucleus was stained with Hoescht 33258. Only merged images were shown. Observe that cells treated with HS+ATP present a slight orange color when compared to control. **D.** Total cellular protein

was immunoprecipitaded with P2X7 or HSP90 or HSP70 antibody and then western blot analysis was performed to detect the same proteins. *p<0.05 in relation to control (two-way ANOVA, followed by Bonferroni pos-test).

Figure 4. P2X7 translocation from rafts to non-rafts or *vice-verse* appears not to be the mechanism by which HS alters P2X7 sensitivity to ATP. To investigate P2X7 co-localization at the lipids rafts, cells were immunostained with P2X7 (red) and **A.** Caveolin-1 (green) or **B.** Flotilin-2 (green). Nuclei were stained with Hoescht 33258. Only merged images were shown. Note that HS+ATP promoted Caveolin-1 and Flotilin-2 spread at the plasma membrane and no differences in P2X7 localization was observed. **C.** Lipid rafts were disrupted by MCD (methyl β-cyclodextrin) (left) or Filipin (right) before cells being exposure to HS+ATP treatment. **D.** Cholesterol staining with filipin showing cholesterol depletion at the plasma membrane induced by 10mM of MCD. Observe the significant decrease at plasma cholesterol staining after MCD treatment when compared to control. *p<0.05 in relation to control (two-way ANOVA, followed by Bonferroni pos-test).

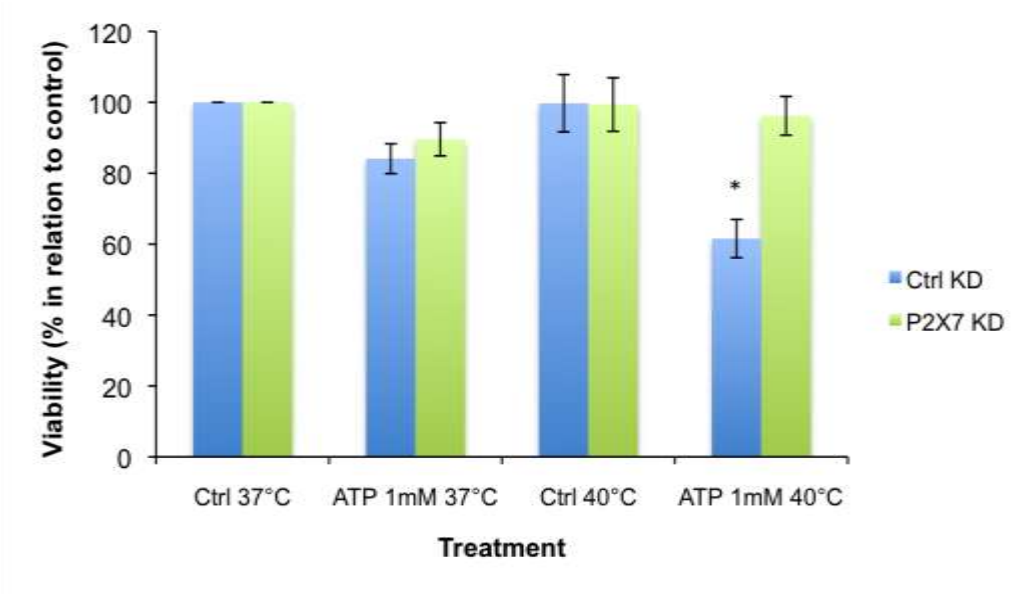
Figure 5. The membrane fluidizer benzyl alcohol (BA) acts similarly as heat stress *per se*, thus leading to P2X7 hyperactivation at 37ºC. A. Ctrl KD or P2X7 KD cells were exposed to BA 20 or 40mM alone or in combination with ATP 1mM for 20min at 37ºC (in order to mimic the heat shock effect *per se*) and then cell viability was evaluated after 24h. Note that, in both concentrations, BA promoted cell death in association with ATP at 37ºC (left), and this cytotoxic effect was completely blocked by P2X7 knockdown (right). Except for this experiment, all the following data was obtained using BA 40mM. **B.** Pictures taken from cells (upper), forward scatter analysis (middle) and Annexin-V/PI double staining (bottom) after respective treatments. Note that BA+ATP at 37ºC induces the same cell shrinkage effect and the double Annexin-V^+/PI^+ staining as HS+ATP. **C.** Western Blot analysis showing identical P2X7-dependent AKT/PRAS40/mTOR-induce cell death pathway either for HS+ATP or BA+ATP treatments. **D.** Immunofluorescence for P2X7 (red) and Caveolin-1 (green) or **E.** Flotilin-1 (green). Nuclei were stained with Hoescht 33258. Only merged images were shown. Observe the same Caveolin-1 and Flotilin-2 spread profile at the

plasma membrane when cells were exposure to ATP with HS or BA. **F.** P2X7 or Caveolin-1 or Flotilin-2 immunoprecipitation after respective treatments. Note the caveolin-1 and flotillin-2 were co-immunoprecipitated, which was expected considering lipid rafts composition. *p<0.05 in relation to control (two-way ANOVA, followed by Bonferroni pos-test).

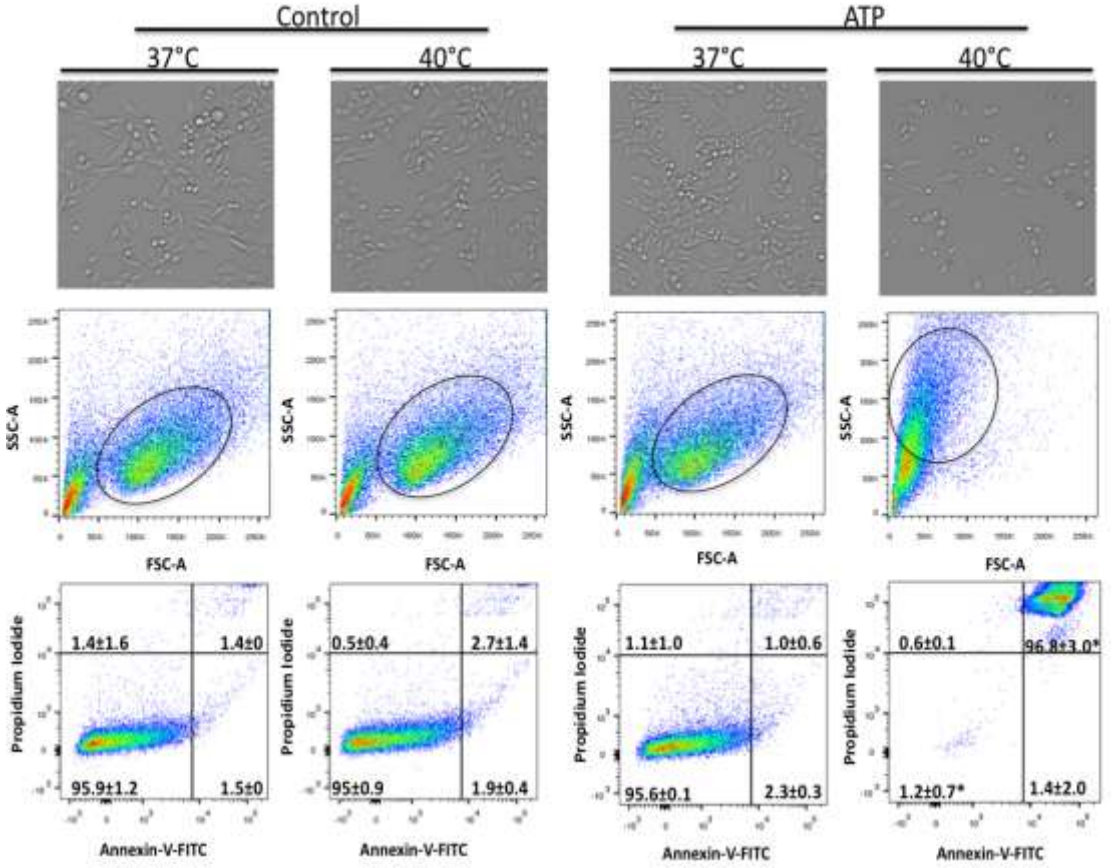
Figure 6. Both heat stress and BA treatment seems to induce a characteristic reorganization of cholesterol-rich membrane domains. A. Cholesterol staining with filipin after cells being treated with ATP at 40ºC or with BA + ATP at 37ºC. Controls without ATP were performed in parallel. Please pay attention to distinct reorganization of cholesterol-rich microdomains in cells exposed to ATP when compared to control. **B.** Cholesterol was loaded at the plasma membrane by adding water-soluble cholesterol at 100μg/mL for 30min before and during ATP exposure and then cell viability was evaluated after 24h. *p<0.05 in relation to control (two-way ANOVA, followed by Bonferroni pos-test).

Figure 1

A


Viability (% in relation to control)
120
100
80
60
40
20
0
*
Ctrl KD
P2X7 KD
Ctrl 37°C
ATP 1mM 37°C
Ctrl 40°C
ATP 1mM 40°C
Treatment

B


Control
ATP
37°C
40°C
37°C
40°C
SSC-A
FSC-A
Propidium Iodide
Annexin-V-FITC
1.4±1.6
1.4±0
95.9±1.2
1.5±0
0.5±0.4
2.7±1.4
95±0.9
1.9±0.4
1.1±1.0
1.0±0.6
95.6±0.1
2.3±0.3
0.6±0.1
96.8±3.0*
1.2±0.7*
1.4±2.0

C

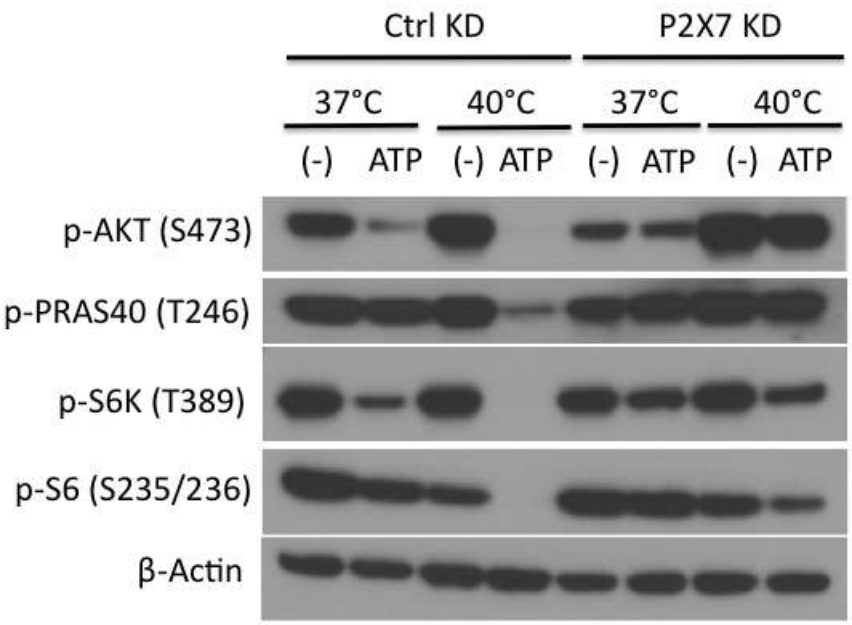
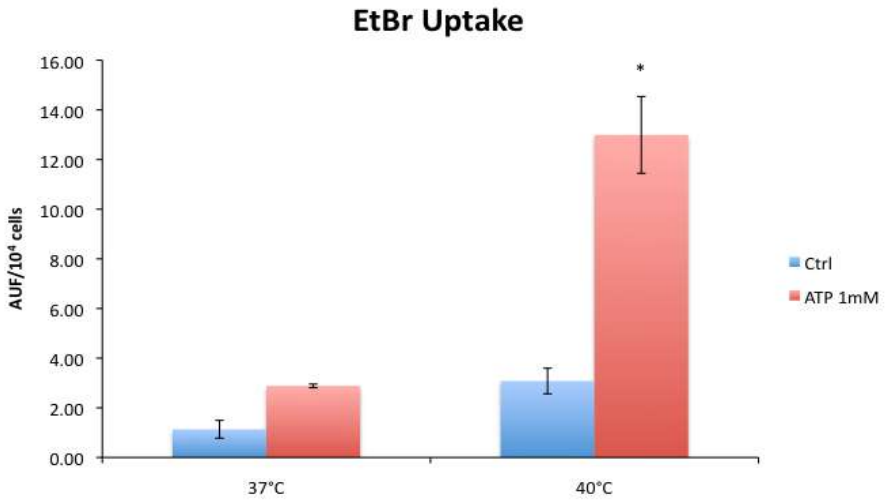
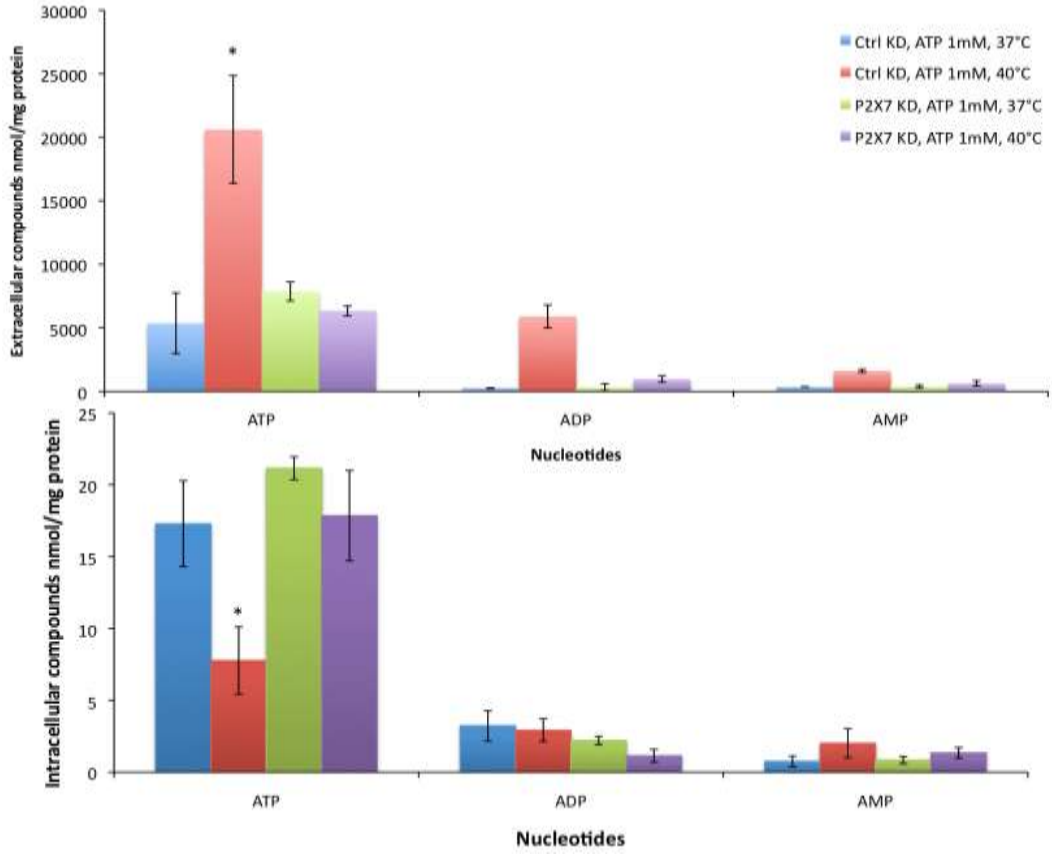

Ctrl KD
P2X7 KD
37°C
40°C
37°C
40°C
(-) ATP (-) ATP (-) ATP (-) ATP
p-AKT (S473)
p-PRAS40 (T246)
p-S6K (T389)
p-S6 (S235/236)
β-Actin

Figure 2

A


EtBr Uptake
AUF/10⁴ cells
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
37°C
40°C
*
Ctrl
ATP 1mM

B


Extracellular compounds nmol/mg protein
30000
25000
20000
15000
10000
5000
0
ATP
ADP
AMP
Nucleotides
Ctrl KD, ATP 1mM, 37°C
Ctrl KD, ATP 1mM, 40°C
P2X7 KD, ATP 1mM, 37°C
P2X7 KD, ATP 1mM, 40°C
Intracellular compounds nmol/mg protein
25
20
15
10
5
0
ATP
ADP
AMP
Nucleotides

C

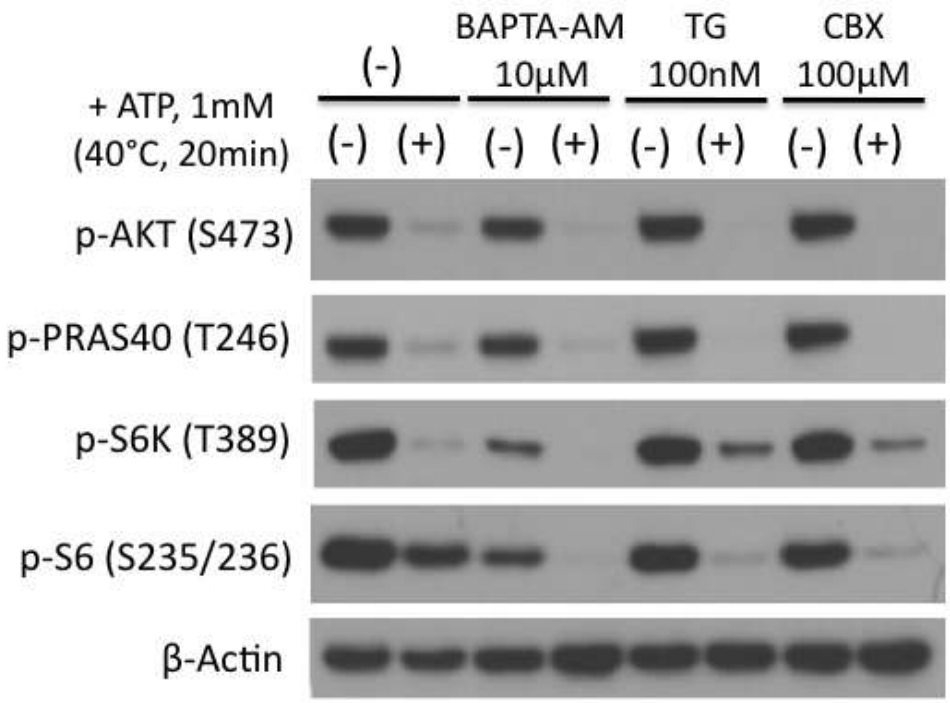
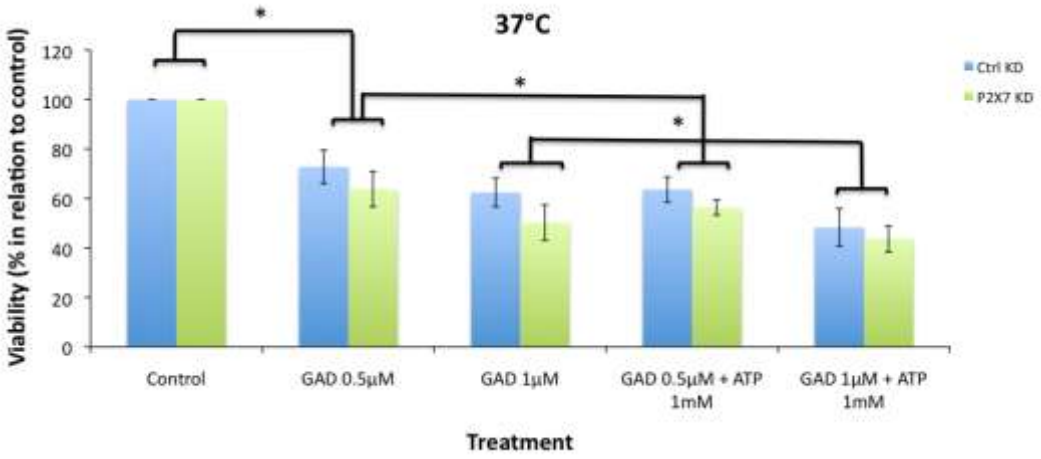
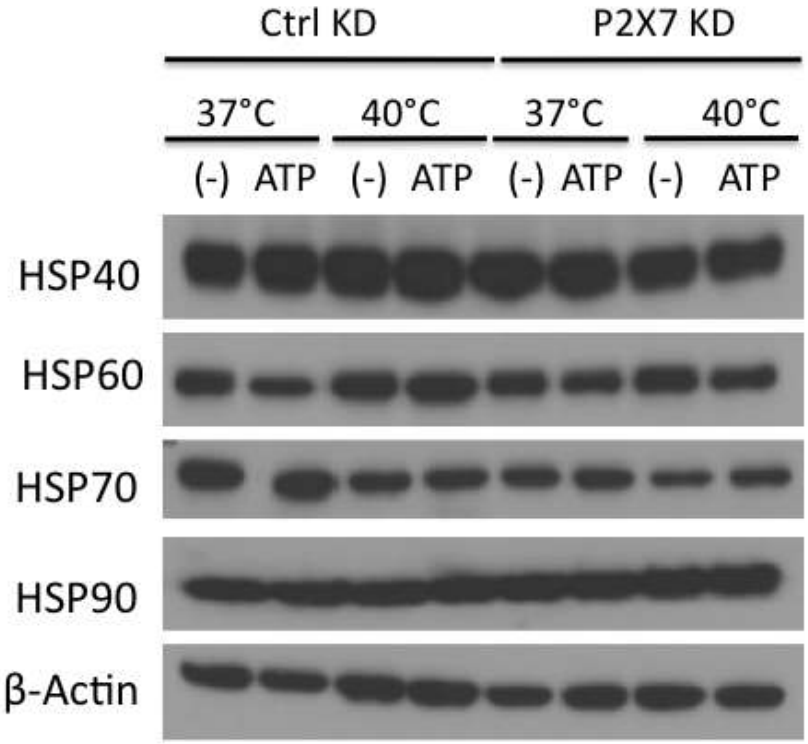

(-)
BAPTA-AM 10μM
TG 100nM
CBX 100μM
+ ATP, 1mM
(40°C, 20min)
(-) (+) (-) (+) (-) (+) (-) (+)
p-AKT (S473)
p-PRAS40 (T246)
p-S6K (T389)
p-S6 (S235/236)
β-Actin

Figure 3

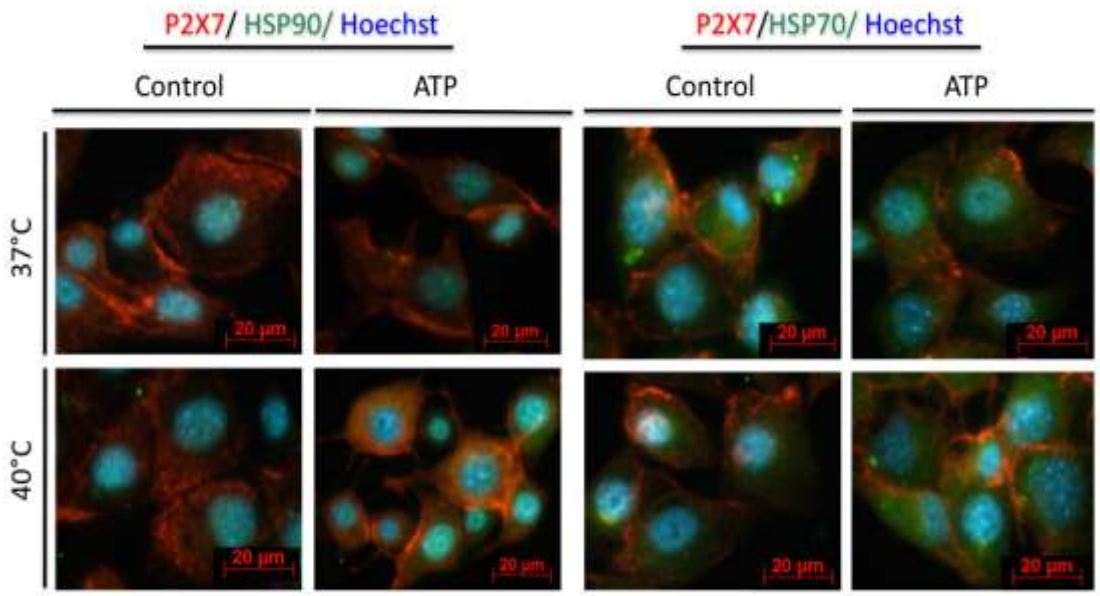
A


37°C
Viability (% in relation to control)
120
100
80
60
40
20
0
Control
GAD 0.5µM
GAD 1µM
GAD 0.5µM + ATP 1mM
GAD 1µM + ATP 1mM
Treatment
Ctrl KD
P2X7 KD
*
*
*

B


Ctrl KD
P2X7 KD
37°C
40°C
37°C
40°C
(-) ATP (-) ATP (-) ATP (-) ATP
HSP40
HSP60
HSP70
HSP90
β-Actin

C


P2X7/ HSP90/ Hoechst
P2X7/HSP70/ Hoechst
Control
ATP
Control
ATP
37°C
40°C
20 μm

D

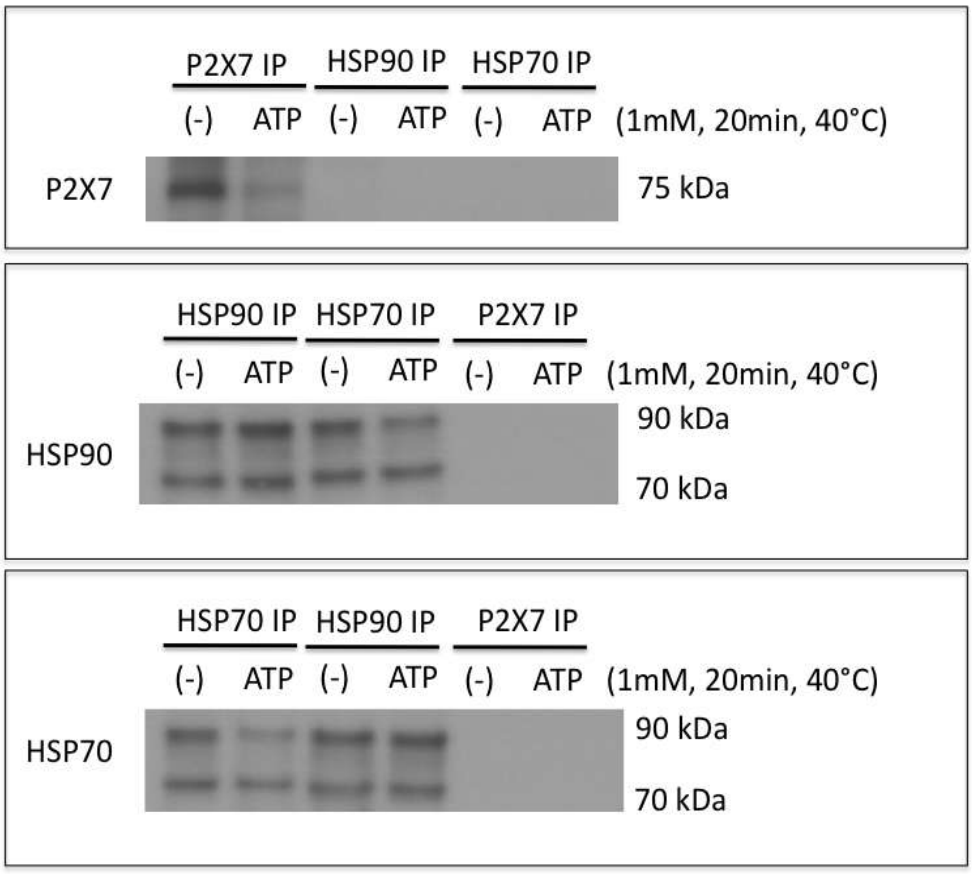
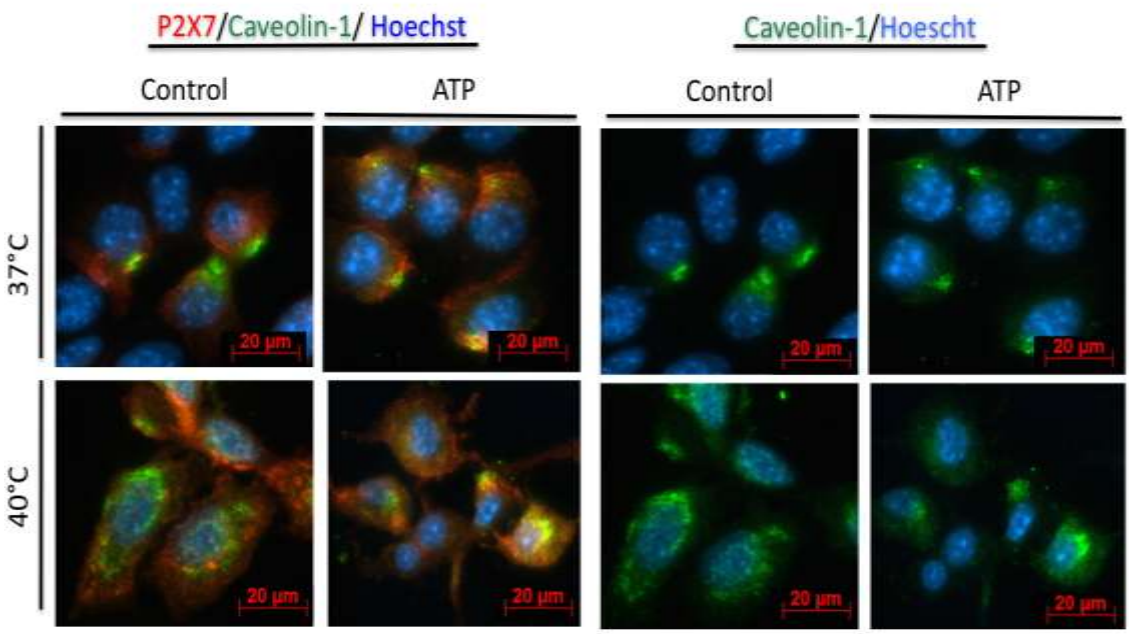
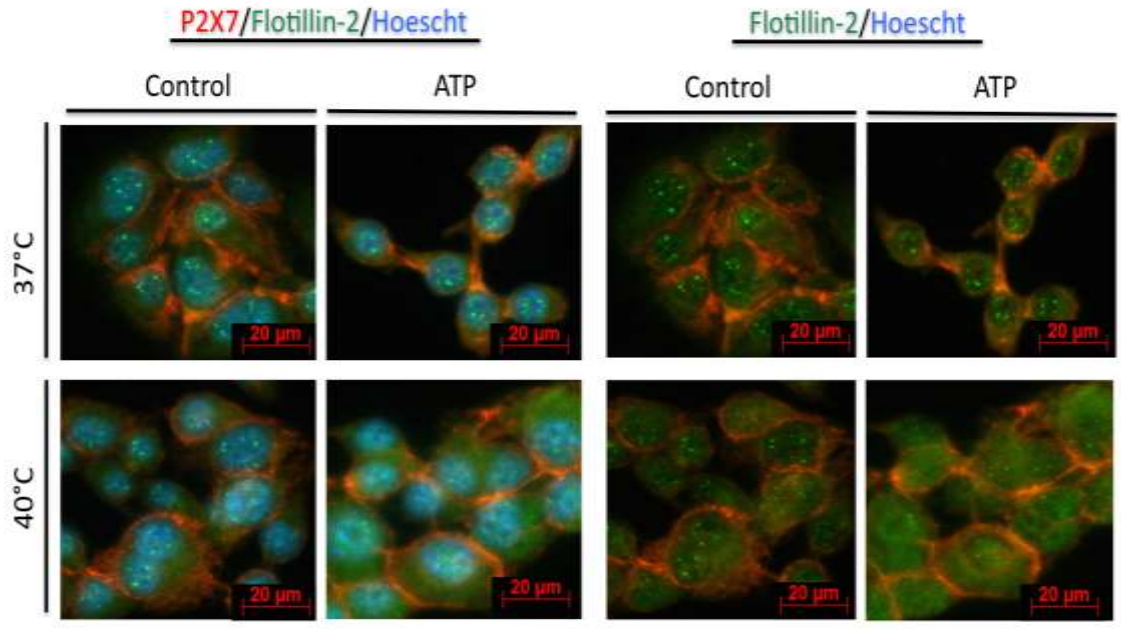

P2X7 IP
HSP90 IP
HSP70 IP
(-) ATP (-) ATP (-) ATP (1mM, 20min, 40°C)
P2X7
75 kDa
HSP90 IP
HSP70 IP
P2X7 IP
(-) ATP (-) ATP (-) ATP (1mM, 20min, 40°C)
HSP90
90 kDa
70 kDa
HSP70 IP
HSP90 IP
P2X7 IP
(-) ATP (-) ATP (-) ATP (1mM, 20min, 40°C)
HSP70
90 kDa
70 kDa

Figure 4

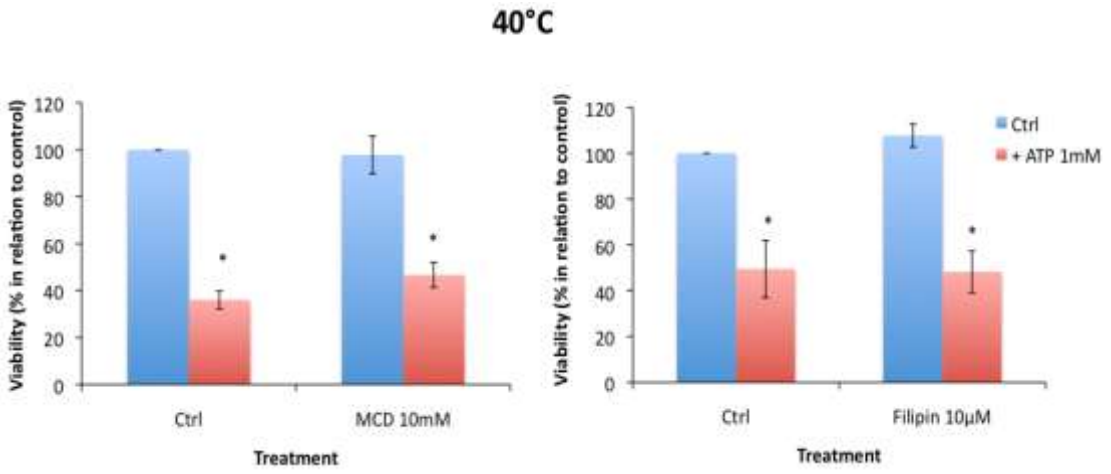
A


P2X7/Caveolin-1/ Hoechst
Caveolin-1/Hoescht
Control
ATP
Control
ATP
37°C
40°C
20 µm

B


P2X7/Flotillin-2/Hoescht
Flotillin-2/Hoescht
Control
ATP
Control
ATP
37°C
40°C
20 µm

C


40°C
Viability (% in relation to control)
120
100
80
60
40
20
0
Ctrl
MCD 10mM
Treatment
Viability (% in relation to control)
Ctrl
Filipin 10µM
Treatment
Ctrl
+ ATP 1mM

D

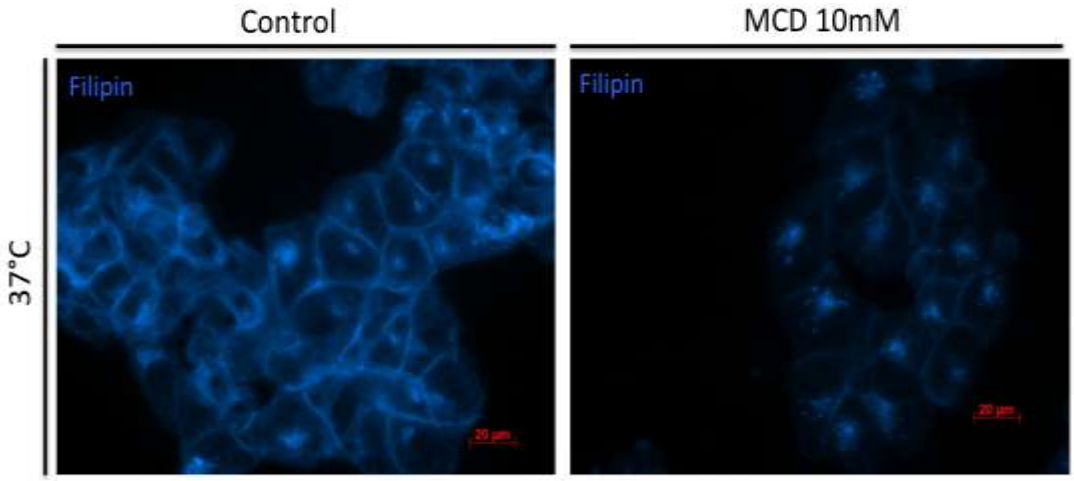
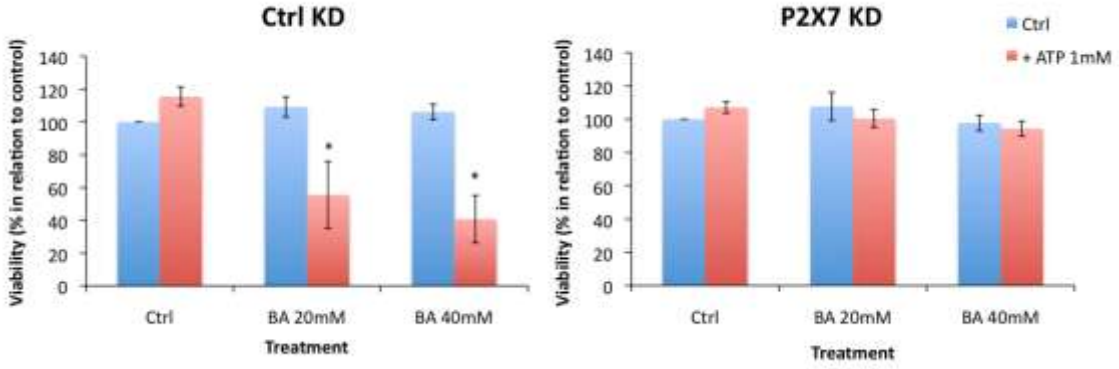
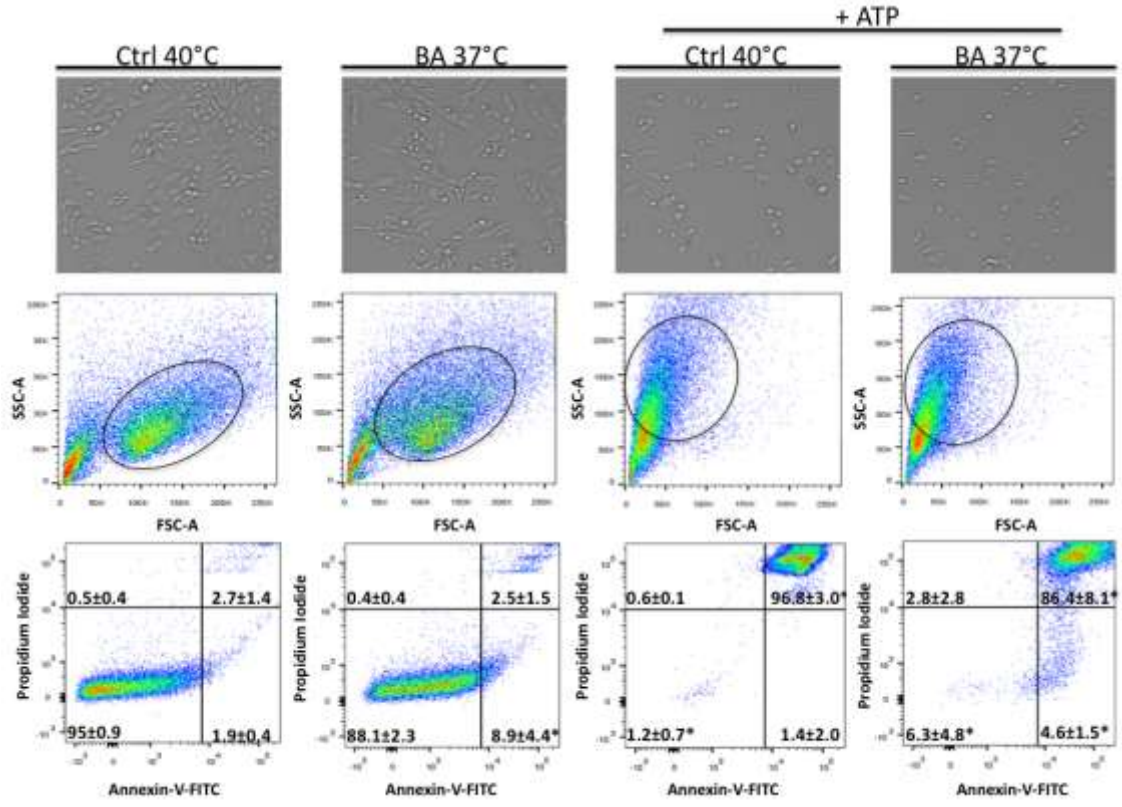

Control
MCD 10mM
37°C
Filipin
Filipin

Figure 5

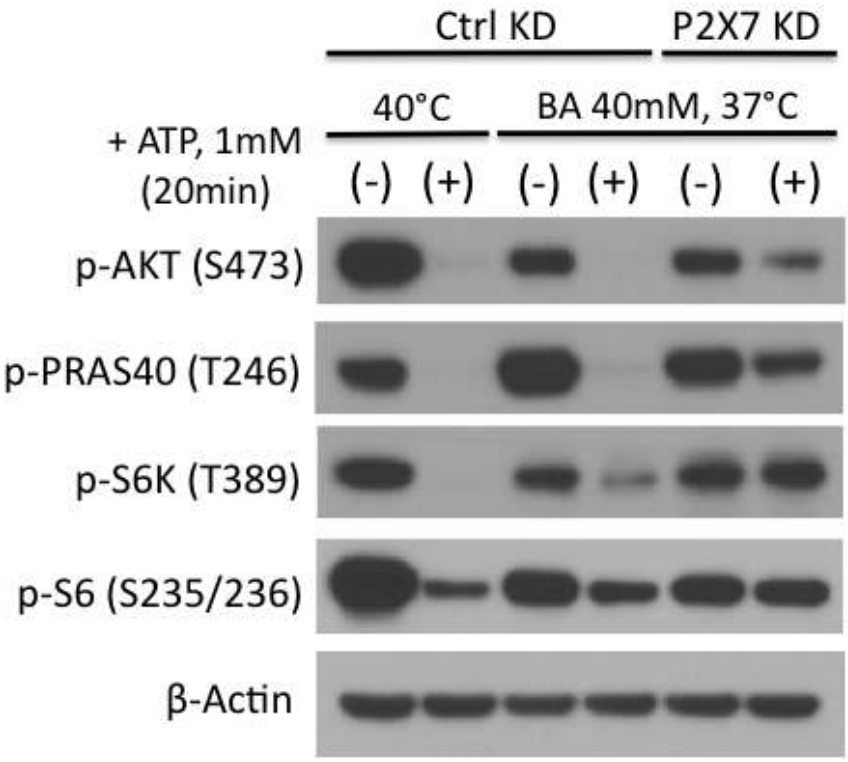
A


Ctrl KD
P2X7 KD
Ctrl
+ ATP 1mM
Viability (% in relation to control)
140
120
100
80
60
40
20
0
Ctrl
BA 20mM
BA 40mM
Treatment
*

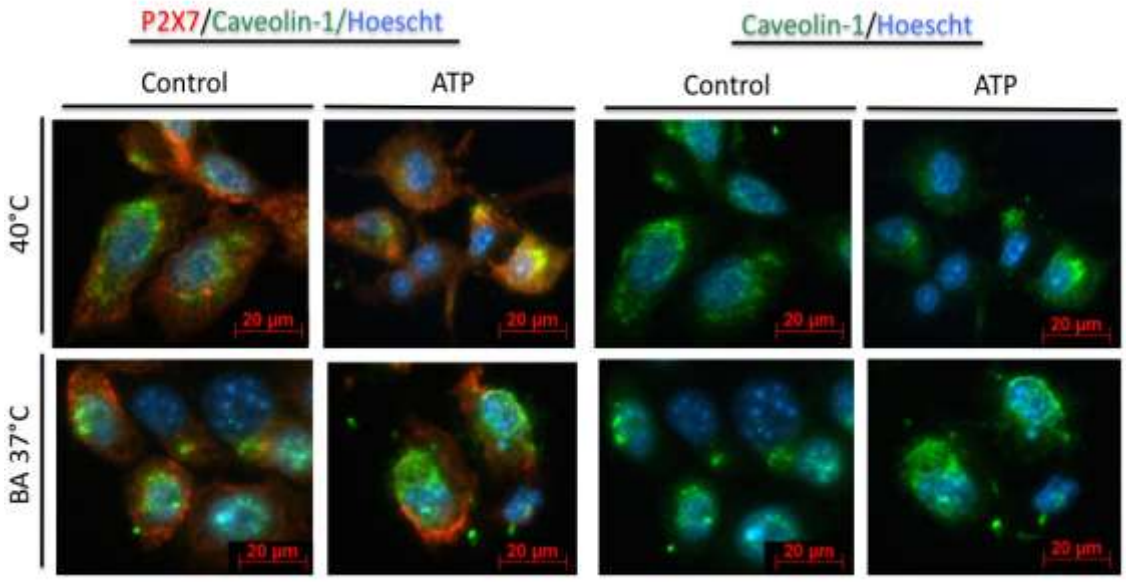
B


+ ATP
Ctrl 40°C
BA 37°C
Ctrl 40°C
BA 37°C
SSC-A
FSC-A
Propidium Iodide
Annexin-V-FITC
0.5±0.4
2.7±1.4
95±0.9
1.9±0.4
0.4±0.4
2.5±1.5
88.1±2.3
8.9±4.4*
0.6±0.1
96.8±3.0*
1.2±0.7*
1.4±2.0
2.8±2.8
86.4±8.1*
6.3±4.8*
4.6±1.5*

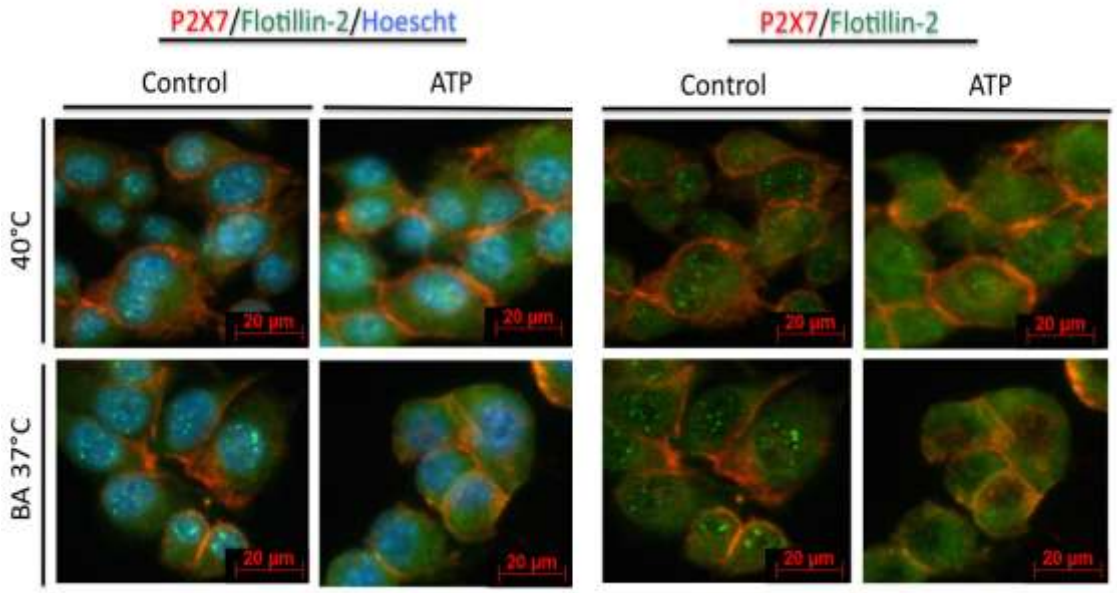
C


Ctrl KD
P2X7 KD
40°C
BA 40mM, 37°C
+ ATP, 1mM
(20min)
(-) (+) (-) (+) (-) (+)
p-AKT (S473)
p-PRAS40 (T246)
p-S6K (T389)
p-S6 (S235/236)
β-Actin

D


P2X7/Caveolin-1/Hoescht
Caveolin-1/Hoescht
Control
ATP
Control
ATP
40°C
BA 37°C
20 μm

E


P2X7/Flotillin-2/Hoescht
P2X7/Flotillin-2
Control
ATP
Control
ATP
40°C
BA 37°C
20 µm

F

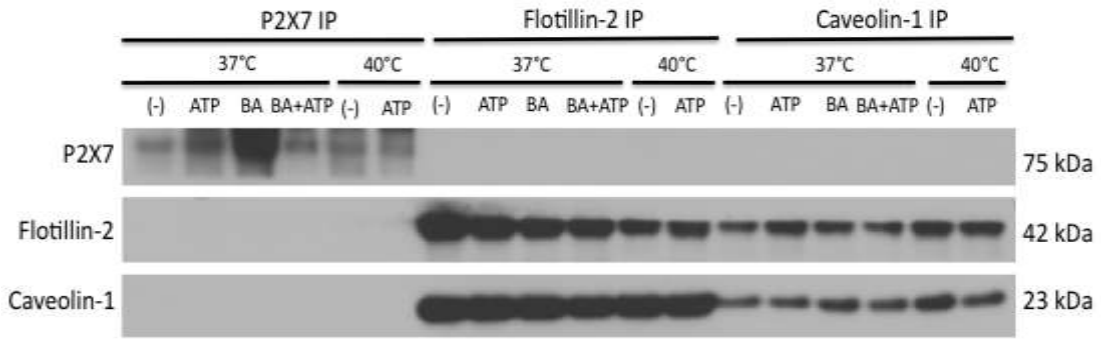
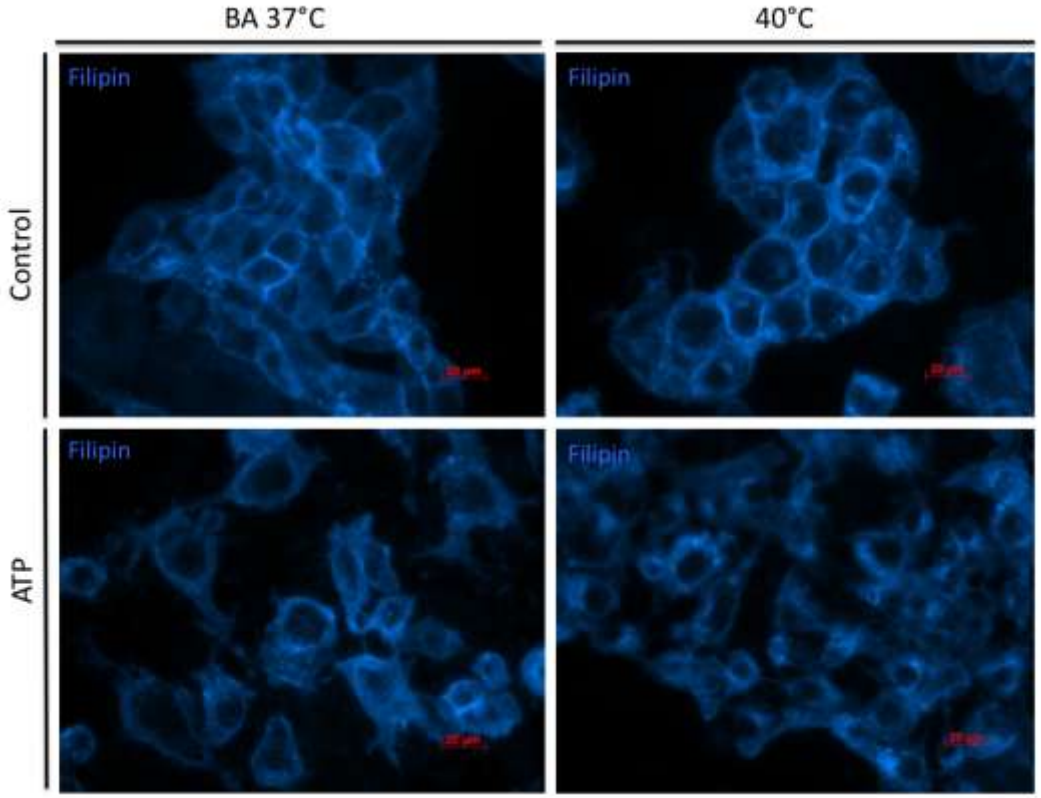
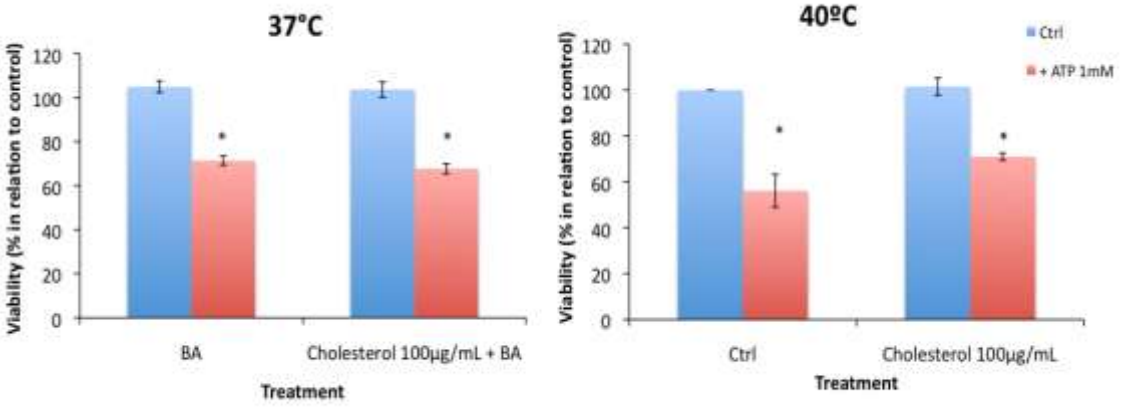

P2X7 IP
Flotillin-2 IP
Caveolin-1 IP
37°C
40°C
(-) ATP BA BA+ATP (-) ATP
P2X7
Flotillin-2
Caveolin-1
75 kDa
42 kDa
23 kDa

Figure 6

A


BA 37°C
40°C
Control
ATP
Filipin
Filipin
Filipin
Filipin

B


37°C
40ºC
Viability (% in relation to control)
120
100
80
60
40
20
0
BA
Cholesterol 100µg/mL + BA
Treatment
Ctrl
Cholesterol 100µg/mL
Treatment
Ctrl
+ ATP 1mM

IV.Discussão Geral

Câncer é um termo genérico utilizado para designar um conjunto de doenças causadas por uma proliferação anormal e acelerada das células do organismo. As células cancerígenas apresentam a capacidade comum de ultrapassar a membrana basal dos tecidos e atingir órgãos distantes do corpo, caracterizando o processo conhecido como metástase (WHO, 2015). É essa capacidade de gerar metástases que faz do câncer uma doença com altas taxas de morbidade e mortalidade, sendo que para o ano de 2012 foram esperados cerca de 14 milhões de novos casos e 8,2 milhões de mortes em todo mundo (GLOBOCAN, 2012). O câncer de pulmão (1,59 milhões), fígado (745.000), estômago (723.000), cólon e reto (694.000), mama (521.000) e esôfago (400.000), foram os que mais levaram pacientes à óbito no ano de 2012 (GLOBOCAN, 2012). A taxa de incidência do câncer nos homens é cerca de 25% maior do que nas mulheres e os principais sítios de desenvolvimento da doença também variam conforme o sexo. Nos homens, o câncer de pulmão, próstata, cólon e reto, estômago e fígado são os mais prevalentes em ordem decrescente; já nas mulheres o perfil muda para o câncer de mama, cólon e reto, pulmão, cérvice e estômago (WHO, 2015).

As causas que levam ao desenvolvimento desta doença são variadas e formadas basicamente pela interação de dois fatores: o genético e o ambiental. Cerca de 80-90% dos casos são relacionados aos fatores ambientais, como tabagismo, hábitos alimentares, alcoolismo, hábitos sexuais, medicamentos, fatores ocupacionais e a radiação solar (INCA, 2014). Dentre todos esses fatores, o uso do tabaco é considerado o fator de maior risco, sendo correlacionado com a morte de 20% dos pacientes com câncer em geral e 70% dos pacientes com câncer de pulmão (WHO, 2015). Apesar do fator genético exercer um papel importante na oncogênese do câncer, a sua contribuição como um fator de risco é menos expressiva, sendo relacionado a alguns tipos de tumor como o retinoblastoma, câncer de mama, estômago e intestino, onde casos familiares são relatados (INCA, 2014).

Alguns tipos de câncer como mama, cervical, oral e colorretal são de fácil tratamento e apresentam altas taxas de cura quando diagnosticados precocemente (WHO, 2015). No entanto, quando detectados em estágios mais

avançados as chances de cura se tornam menores e os tratamentos, como cirurgia, e/ou radioterapia, e/ou quimioterapia, são muitas vezes tóxicos para os pacientes e de alto custo para os planos de saúde. Desta forma, a busca pela prevenção primária, investimentos no diagnóstico precoce e a descoberta de novos e específicos alvos terapêuticos são a fórmula para a redução da incidência e mortalidade dessa doença (Edwards *et al*., 2014).

É na tentativa de encontrar novos e específicos alvos terapêuticos que muitos pesquisadores estudam à gênese do processo neoplásico. Neste, constata-se que alterações em processos intrínsecos como proliferação, diferenciação, morte celular programada e capacidade de controle do micro ambiente, são características que levam ao sucesso do câncer em detrimento do organismo saudável. Desta forma, a análise da proliferação celular e dos eventos relacionados à apoptose, são ferramentas importantes para conhecer o comportamento das células tumorais (Wang *et al*., 2004a).

É nesse contexto que se insere o estudo do sistema purinérgico, visto que a sua sinalização está intimamente relacionada com o controle de processos como o crescimento, proliferação, diferenciação e morte celular (Burnstock & Di Virgilio, 2013). Ainda, estudos têm demonstrado a presença de altos níveis de ATP e adenosina no microambiente tumoral em comparação com os tecidos saudáveis (Ohta *et al.*, 2006; Pellegatti *et al*., 2008), sugerindo um papel desses compostos na gênese e manutenção do tumor (Martin *et al*., 2000; Martin *et al*., 2001). Considerando a complexidade da sinalização purinérgica, a ação final sobre o tumor irá depender do painel de expressão e ativação dos receptores P1 e P2 no tecido, bem como dos níveis de ATP/adenosina no microambiente, que em conjunto irão produzir uma resposta final que pode ser tanto a indução da proliferação, da morte (apoptose/necrose/autofagia) ou da diferenciação celular (Feng *et al*., 2014).

Vários estudos tem demonstrado o efeito anti-tumoral dos nucleotídeos de adenina no câncer, dentre eles podemos citar: câncer de cólon e reto (Hoepfner *et al*., 1999), leucemia (Seetulsingh-Goorah *et al*., 1998), câncer de esôfago (Maaser *et al*., 2002), células de tumor de Erlich (Dubyak & De Young, 1985), células de câncer de pele escamoso (Greig *et al.,* 2003), câncer de pulmão (Schafer *et al*., 2003), câncer cervical (Wang *et al*., 2004a), células H35

de hepatoma (Horstman *et al*., 1986), câncer de próstata (Shabbir *et al*., 2008a), câncer de bexiga (Shabbir *et al*., 2008b), retinoblastoma (Kim *et al*., 2011), neuroblastoma (Gómez-Villafuertes *et al*., 2009), glioma (Suplat-Wypych *et al*., 2010) e melanoma (White *et al*., 2005b; White *et al*., 2009). Diferentes receptores purinérgicos P2 podem estar envolvidos neste efeito citotóxico (P2X5, P2X7, $P2Y_1$, $P2Y_2$ e $P2Y_{11}$) (White & Burnstock, 2006), sendo que a resposta final será diferente de acordo com o tipo celular e os níveis de ATP extracelular (Burnstock & Di Virgilio, 2013).

Assim como o ATP, a adenosina extracelular também parece ter um efeito citotóxico dependendo do tipo tumoral estudado e do receptor purinérgico P1 envolvido. Dentre eles, podemos citar o papel dos receptores A_{2A} em células de câncer de cólon (Caco-2) e de hepatoma (HepG2) (Yasuda *et al*., 2009), mieloma múltiplo e linfoma (Rickles *et al*., 2012), A_1 em células de câncer de cólon (CW2) (Saito *et al*., 2010) e glioblastoma (Synowitz *et al*., 2006) e A_3 em linfoma, melanoma, próstata, pulmão, colon, células de câncer neural e células de carcinoma hepatocelular (HCC) (Fishman *et al*., 2000; Bar-Yehuda *et al*., 2001; Fishman *et al*., 2002; Merighi *et al*., 2002; Madi *et al*., 2003; Fishman *et al*., 2004; Bar-Yehuda *et al*., 2008; Aghaei *et al*., 2011; Aghaei *et al*., 2012; Otsuki *et al*., 2012; Vincenzi *et al*., 2012;). Além de atuar via receptores P1, a adenosina também pode induzir apoptose via recaptação celular através de transportadores específicos de membrana, como foi o caso descrito para as linhagens de câncer de mama (MCF-7) (Tsuchiya *et al.,* 2012), astrocitoma (RCR1) (Sai *et al*., 2006), mesotelioma (Nogi *et al*., 2012), hepatocarcinoma (Yang *et al.,* 2007; Yang *et al.*, 2011) e câncer gástrico (GT3-TKB) (Saitoh *et al*., 2004).

Diferente dos estudos encontrados na literatura, na primeira parte deste trabalho nós mostramos que o efeito citotóxico do ATP extracelular em linhagens celulares humanas de câncer cervical é na verdade mediado pela recaptação da adenosina formada a partir do seu metabolismo e não pela sua ação direta via receptores P2. Isso contrasta com o já descrito mecanismo de indução de apoptose do ATP via ativação do receptor P2X7 em células humanas epiteliais cervicais (Wang *et al*., 2004a). De acordo com este estudo, células epiteliais normais da ectocérvice (hECE) entram em processo de

apoptose após serem expostas a 50 µM de ATP e BzATP, e esse processo é desencadeado exclusivamente pela ativação do receptor P2X7 com conseqüente influxo de cálcio, aumento do $[Ca^{2+}]_i$ e ativação da caspase-9. Ainda, diferente do que foi descrito em nosso trabalho, o tratamento das células epiteliais com ADP ou adenosina não produziu efeito na indução da morte celular, reforçando o papel exclusivo do ATP via receptor P2X7. Considerando-se a concentração dos nucleotídeos utilizada nesse estudo, podemos sugerir que as células epiteliais cervicais são mais susceptíveis ao efeito citototóxico do ATP e BzATP, quando comparado aos resultados obtidos por nós com células de câncer cervical , que necessitaram a concentração de 5mM de ATP para sofrerem um efeito na redução da viabilidade celular. Sendo assim, podemos inferir que as células de câncer cervical desenvolveram um mecanismo de resistência ao efeito citotóxico do ATP ao longo do processo de transformação maligna.

Em concordância com essa suposição, nossos dados mostram que a linhagem de câncer cervical SiHa apresenta uma heterogeneidade com relação a expressão do receptor P2X7. De acordo com o nosso trabalho, as células podem ser subdivididas em duas populações, uma menor subpopulação composta por células que expressam altos níveis do receptor P2X7 e portanto são mortas pela ação do ATP extracelular; e uma maior subpopulação que apresenta baixos níveis do receptor P2X7 e portanto sofre a ação citotóxica da recaptação da adenosina. Essa redução da expressão/funcionalidade do receptor P2X7 como uma forma de escape das células tumorais a apoptose já vem sendo estudada e demonstrada por alguns autores (Huang *et al*., 2013; Li *et al*., 2009). Li e colaboradores (Li *et al*., 2009) reportaram que células tumorais provenientes da ectocérvice (escamosas), endocérvice, endométrio (adenocarcinoma), bexiga (células transicionais) e mama (adenocarcinoma ductal e lobular) apresentavam menores níveis do receptor P2X7 quando comparadas ao tecido normal adjacente. E mais, os níveis do receptor P2X7 já apresentavam-se reduzidos nas células displásicas da ectocérvice e também nas células pré-cancerígenas do endométrio e da bexiga, indicando que uma redução da expressão do receptor P2X7 pode preceder ou coincidir com o desenvolvimento neoplásico dos tecidos estudados.

Ainda com relação a redução na funcionalidade do receptor P2X7, um estudo realizado por Feng e colaboradores (Feng *et al.* 2006b), identificou a presença de uma variante truncada do receptor (P2X7-j) que é expressa endogenamente pelas células de cancer cervical (HT3, HeLa, SiHa e Caski) e é desprovida da região carboxi-terminal sendo portanto incapaz de formar poros e induzir apoptose. Além de ser inativo, P2X7-j também interage com o receptor *full-lengh* inibindo a sua ativação. De acordo com os autores, a interação entre as duas formas do receptor P2X7 representa um mecanismo de resistência celular à apoptose desenvolvido pelas células de câncer cervical. Apesar da linhagem celular SiHa expressar essa variante truncada (em baixos níveis), resultados obtidos pelo nosso grupo demonstram que o receptor P2X7 *full-lenght* é funcional, visto a sua capacidade de indução de apoptose após estímulo pelo BzATP.

Entretanto, o papel do receptor P2X7 na indução da morte celular na linhagem SiHa é pequeno, considerando que o bloqueio da sua ativação pelo uso do antagonista oATP e do seu *knockdown* reduziram a taxa de citotoxicidade do ATP em apenas 20%. Diferentemente, nós observamos que o bloqueio da recaptação da adenosina pelo tratamento com dipiradamol, impediu significativamente a redução da viabilidade celular, sugerindo que a citotoxicidade do ATP não está relacionada ao seu efeito *per se*, mas sim pela ação do seu metabólito adenosina. Muitos estudos descrevem a via de sinalização intrínseca como um dos mecanismos de indução de apoptose pela adenosina (Barry & Lind, 2000; Schrier *et al.*, 2001; Saitoh *et al.*, 2004; Sai *et al*., 2006; Tsuchiya *et al*., 2012). Nesta via a adenosina extracelular é recaptada para o interior das células via os transportadores de membrana e é transformada em AMP pela ação da adenosina kinase, culminando na redução da razão ATP/AMP intracelular com consequente ativação da AMPK e indução de apoptose. O desfecho intracelular na indução de apoptose varia de acordo com o tipo celular estudado e pode ser tanto independente quanto dependente de caspase. No caso da SiHa, a indução de apoptose é independente da caspase e envolve o acúmulo de dATP, o aumento da p53 e ativação da autofagia.

O exato mecanismo pelo qual a entrada de adenosina na célula gera o acúmulo de dATP não foi totalmente esclarecido, mas podemos sugerir que o desequilíbrio causado nos níveis intracelulares de nucleotídeos e nucleosídeos possa estar relacionado. Além disso, já se tem descrito na literatura que o aumento de dATP intracelular está associado ao acúmulo de rupturas nos filamentos de DNA e ativação da proteína p53 pró-apoptótica (Joachims *et al.*, 2008; Johnston, 2011), o que corrobora com os nossos dados. Ainda, Nogi e colaboradores (Nogy *et al.* 2012) também demonstraram, em células de mesotelioma, que a recaptação celular de adenosina e a sua transformação a AMP induz aumento da p53 e leva as células à apoptose de uma forma independente de caspase.

A indução de apoptose, independente ou dependente de caspase, consiste no principal mecanismo de promoção de morte celular entre os agentes quimioterápicos utilizados no tratamento do câncer atualmente. No entanto, a maioria das células tumorais apresentam ou passam a adquirir resistência a esses agentes pró-apoptóticos. Desta forma, a busca por novos compostos que possam promover simultaneamente múltiplos mecanismos indução de morte celular, como apoptose, necrose e autofagia serão a nova fórmula para atingir a máxima eficácia no tratamento do câncer com o mínimo de efeitos adversos (Galluzzi *et al*., 2011).

No nosso trabalho, nós demonstramos pela primeira vez um dado bastante relevante sobre o papel da autofagia no contexto da indução de morte celular pela recaptação da adenosina. A autofagia é um processo celular fisiológico que leva a degradação e reciclagem de componentes do citosol e organelas celulares danificadas a fim de manter a homeostase celular em condições adversas, como privação de nutrientes, presença de patógenos e toxinas (Filippi-Chiela *et al*., 2011). Dependendo do seu grau de ativação a autofagia pode contribuir para a sobrevivência e adaptação celular ou pode levar à morte celular (morte autofágica ou morte celular programada tipo II) (He and Klionsky, 2009; Yang and Klionsky, 2010). A indução da autofagia pode ser monitorada pela expressão de duas proteínas involvidas nesse processo a LC3 e a p62. A proteína LC3 (cadeia leve 3 da proteína 1 associada a microtúbulos) é uma proteína citosólica (LC3 na forma I-LC3-I) que é clivada na região C-

terminal quando sofre um estímulo pró-autofágico, sendo convertida à forma II (LC3-II). Já a proteína p62 marca os componentes celulares que devem ser degradados pelo autofagossomo, sendo portanto reduzida com o estímulo autofágico (Pankiv *et al.*, 2007; Matsumoto *et al.*, 2011). De acordo com o nossos resultados, a indução da autofagia pode ser vista após 48h e 72h de exposição ao ATP 5mM, representado pelo aumento da razão LC3II/I e a diminuição da quantidade de p62. Ainda, esse efeito foi completamente bloqueado pelo pré-tratamento com dipiridamol, reforçando o papel da recaptação da adenosina na indução desse processo. Mais, a avaliação das células marcadas com o laranja de acridina (AO), que indica a formação de autofagolisossomo, mostra que o aumento das células marcadas pelo AO está correlacionado positivamente com o aumento da morte celular, sugerindo a indução da morte autofágica.

O papel da autofagia no desenvolvimento tumoral vem sendo questão de grande interesse nos últimos tempos. Sua ativação parece ter um papel duplo na oncogênese, sendo tanto um fator importante para a indução de morte celular quanto para a progressão tumoral (Baehrecke, 2005; Hippert *et al*., 2006; Galluzzi *et al*., 2008; Eskelinen, 2011). Recentemente, Li e colaboradores (Li *et al*., 2015), apontaram o processo de autofagia como um fator determinante no desenvolvimento do câncer cervical. De acordo com os autores, o vírus HPV promove a carcinogênese por meio da inibição da autofagia na célula hospedeira. Segundo eles, num primeiro momento, quando as células são infectadas pelo vírus, o processo de autofagia é ativado, resultando em morte autofágica com a finalidade de eliminar o invasor e impedir novas infecções. Mas posteriormente, o HPV passa a reprimir a autofagia, bloqueando dessa forma a defesa celular contra o vírus que passa então a proliferar e infectar novas células, culminando na progressão do câncer cervical. Sendo assim, encontrar uma maneira de induzir autofagia, como o uso da adenosina por exemplo, pode ser uma nova estratégia para silenciar o vírus do HPV e prevenir o desenvolvimento do câncer cervical.

Além do uso da adenosina, outras ferramentas também podem ser utilizadas com a finalidade de aumentar a morte celular e prevenir a progressão tumoral. Na segunda parte desse trabalho, utilizando linhagens de câncer de

cólon (MCA38), nós demonstramos que o *heat shock* pode ser utilizado como um novo método para aumentar a sensibilidade do receptor P2X7 ao ATP, ampliando dessa forma a capacidade de indução de morte celular por esse composto. O mecanismo envolvido nesse aumento da funcionalidade do receptor P2X7 parece estar associado a processos de reorganização dos domínios ricos em colesterol da membrana plasmática gerados pelo stress do aquecimento celular.

São grandes os indícios relacionando a resposta celular ao *heat shock* à mudanças na composição e arquitetura das membranas. Na verdade, acredita-se na hipótese de que a membrana plasmática atue como um sensor na interface celular, detectando o sinal de *heat shock* e o transmitindo para o interior da célula (Vigh *et al*., 1998). Alguns pesquisadores propõem que, apesar da mudança do estado físico das membranas *per se,* o aparecimento de microdomínios específicos ou mudanças na composição dos lipídios bem como da interação lipídio-proteína das membranas são potencialmente e igualmente capazes de gerar um estímulo para o interior da célula (Horvath *et al*., 1998; Vigh & Maresca, 2002). Sendo assim, a mudança na estrutura da membrana plasmática causada pelo *heat shock* pode interferir com a atividade de várias moléculas ali presentes, dentre elas os receptores de membrana (Vigh *et al.*, 2005). No caso do receptor P2X7, essa hipótese é apoiada pelo fato de que o agente fluidizador de membrana, benzil álcool (BA), também foi capaz de produzir os mesmos efeitos que o *heat shock*, mas a 37ºC. De acordo com Nagy e colaboradores (Nagy *et al*., 2007), o composto BA atua similarmente como o *heat shock* promovendo uma reorganização dos domínios ricos em colesterol da membrana.

Considerando essa informação, nós avaliamos o papel destes domínios ricos em colesterol de membrana, conhecidos como rafts, no contexto da hiperativação do receptor P2X7 causado pelo BA ou *heat shock*. De acordo com os nossos dados de imunofluorescência e imunoprecipitação, o receptor P2X7 não parece estar interagindo com os rafts planares e as cavéolas. Ainda, quando submetidos ao efeito do *heat shock* ou BA mais o ATP, ambos os rafts de membrana apresentaram uma alteração no seu perfil de distribuição, que não foi vista para o receptor P2X7. Somado a isso, o uso de agentes

depletores de colesterol (MCD e filipinas) ou a adição de colesterol solúvel à membrana plasmática não inibiram o efeito observado sobre o receptor. Esses dados sugerem que o P2X7 não encontra-se associado e não precisa interagir com os rafts lipídicos de membrana nas células MCA38 para exercer a sua função citolítica. Diferentemente, Robinson e colaboradores (Robinson *et al*., 2014) mostraram que, em células humanas (embriônicas de rim) e macrógafos isolados de camundongos, o colesterol apresenta um papel regulador da ativação e sensibilização do receptor P2X7. De acordo com os autores, o uso do agente depletor de colesterol MCD, leva a um aumento significativo da formação do poro do receptor; já a adição de colesterol solúvel à membrana plasmática gera um efeito oposto. Essas discordâncias podem ser explicadas pelas diferença na distribuição/expressão do receptor P2X7 entre as células estudadas (normais x cancerígena).

Recentemente, Bian e colaboradores (Bian *et al*. 2013) descreveram uma nova via de sinalização intracelular envolvida na inibição da proliferação celular das linhagens de câncer de cólon (MCA38) e melanoma (B16/F10). De acordo com os autores, o estímulo do receptor P2X7 por altos níveis de ATP leva a ativação de duas vias, a conhecida PI3K/AKT e a nova AMPK-PRAS40-mTOR, que atuam de forma independente e sinérgica para promover a morte celular através do equilíbrio entre crescimento celular e indução de autofagia. Em concordância com esse estudo, no nosso trabalho a exposição das células MCA38 à altos níveis de ATP em conjunto com o *heat shock* ou BA induziu via P2X7 a ativação das mesmas vias de sinalização, culminando em morte celular. Ainda, a formação do poro de membrana associado com a citotoxicidade do ATP não depende da interação com os canais do tipo conexina/panexina, sugerindo ser uma propriedade exclusiva do receptor P2X7.

Apesar de ser um canal iônico, o receptor P2X7 parece interagir com 11 diferentes proteínas formando um complexo protéico capaz de gerar uma sinalização intracelular (Kim *et al*., 2001). Dentre essas proteínas, três *heat shock proteins* (HSP) foram encontradas: HSP90, HSP70 e HSC71. O verdadeiro papel dessas HSP na ativação do receptor P2X7 ainda não foi totalmente esclarecido, mas segundo Adinolfi e colaboradores (Adinolfi *et al*.

2003), a HSP90 parece ter um efeito regulador sobre a ativação do receptor P2X7. Quando apresenta-se fosforilada na tirosina, a HSP90 atua inibindo a formação e a funcionalidade do complexo P2X7. Desta forma, o bloqueio dessa fosforilação poderia ser uma nova estratégia para aumentar a ativação do receptor. No entanto, o uso de geldanamicina (GDA), um inibidor da HSP90 e da sua fosforilação, apresentou um efeito citotóxico *per se* e independente do receptor P2X7 quando colocado em contato com as células MCA38 do nosso estudo. Estes dados mostram que GAD exerce um efeito particular na indução da morte celular, o que já se foi demonstrado em vários tipos de câncer (Trepel *et al*., 2010; Zhang *et al*., 2015; Grzanka *et al*., 2015). Ainda, nas células MCA38 o receptor P2X7 não parece estar interagindo com a HSP90 e HSP70. E, apesar de o *heat shock* normalmente induzir o aumento dos níveis das HSP intracelulares, no nosso estudo, a temperatura de 40ºC pelo curto período de tempo (20min) não foi suficiente para causar esse efeito. Na verdade, Nagy e colaboradores (Nagy *et al.* 2007) mostraram que um aumento nos níveis protéicos da HSP70 só foram encontrados quando células de melanoma B16/F10 foram expostas no mínimo a 41ºC por 1h. Desta forma, em nosso estudo, o aumento da ativação do receptor P2X7 após o *heat shock* não parece estar relacionado com a ação das *heat shock proteins*.

O uso da hipertermia como um método de tratamento individual ou em combinação com terapias tradicionais, como a quimioterapia, cirurgia e radioterapia vem sendo empregado por muitos pesquisadores com o intuito de potencializar o combate ao câncer (Luk *et al*., 1984; Irish *et al*., 1986; Seegenschmiedt *et al*., 1993). Esta combinação baseia-se no fato de que em regiões tumorais com baixa perfusão, onde o aporte de oxigênio e de nutrientes é escasso, as células cancerígenas são normalmente resistentes ao efeito da quimioterapia e radioterapia, mas por outro lado, são extremamente termossensíveis (Chatterjee *et al*., 2011). Sendo assim, quando usados em associação a eficiência do tratamento pode ser aumentada. Vários estudos clínicos e pré-clínicos avaliando a combinação da hipertermia com a quimioterapia ou a radioterapia estão sendo realizados em câncer de próstata (Van Den Berg *et al*., 2006; Hurwitz *et al.*, 2011), mama (Moros *et al*., 2010; Zagar *et al*., 2010), cérvice (Vasanthan *et al*., 2005; Franckena & Van Der Zee,

2010) e cabeça e pescoço (Huilgol *et al*., 2010a; Huilgol *et al*., 2010b). Resultados preliminares mostram que a adição da hipertermia não aumenta a toxicidade das terapias tradicionais, mas contribui para a estabilização e a cura do câncer e/ou auxilia no tratamento paliativo. No presente trabalho, nós demonstramos pela primeira vez que a hipertermia pode ser utilizada para aumentar a citotoxicidade do ATP extracelular por meio do incremento exclusivo da ativação do receptor P2X7. Considerando-se o contexto do microambinete tumoral, onde altos níveis de ATP extracelular são encontrados (Pellegatti *et al.*, 2008), o uso da hipertermia aumentaria a eficiência terapêutica e possivelmente ajudaria a superar a resistência celular aos tratamentos tradicionais.

Em conclusão, o nosso trabalho fornece evidências adicionais sobre o papel da sinalização purinérgica no contexto da biologia celular tumoral. Ainda, propomos que futuramente o uso de medicamentos à base de purinas possam ser associados à hipertermia como agentes adjuvantes na terapia do câncer. Entretanto, maiores estudos devem ser realizados para comprovação e indicação desta abordagem terapêutica.

V. Conclusões Gerais

Os resultados apresentados nesta tese permitem as seguintes conclusões:

1. O efeito citotóxico do ATP, bem como o papel do receptor P2X7 nesse processo varia de acordo com o tipo de célula tumoral estudada.

2. Em linhagens de câncer cervical, a adenosina formada pelo metabolismo do ATP é o principal agente causador de morte celular. Nesse contexto, o papel do receptor P2X7 parece ser apenas co-adjuvante, visto que o seu silenciamento ou o seu bloqueio reduziram apenas 20% o efeito citotóxico do ATP.

3. Ainda, de acordo com os níveis de expressão do receptor P2X7 as células de câncer cervical parecem responder de forma diferente ao afeito citotóxico do ATP. Células com altos níveis de expressão do receptor parecem morrer via sua ativação pelo ATP, em contrapartida, células com baixa expressão de P2X7 sofrem o efeito citotóxico da recaptação da adenosina.

4. A recaptação da adenosina gera um desequilíbrio nos níveis dos nucleotídeos intracelulares, culminando na ativação da AMPK e da p53 e na indução de autofagia.

5. Em linhagens de câncer de cólon MCA38 o ATP extracelular exerce efeito citotóxico por meio da ativação exclusiva do receptor P2X7, culminando na inibição da via AKT/PRAS40/mTOR.

6. Esse efeito citotóxico pode ser potencializado pela ação do *heat shock*, que parece aumentar a funcionalidade do receptor P2X7 via reorganização na composição e arquitetura da membrana celular.

7. O uso de medicamentos à base de purinas associados à hipertermia podem ser sugeridos como agentes adjuvantes na terapia do câncer.

Mas maiores estudos devem ser realizados para comprovação e indicação desta abordagem terapêutica.

8. O receptor P2X7 poderá ser um potencial alvo molecular no diagnóstico, prognóstico e tratamento do câncer, mas o seu verdadeiro papel no desenvolvimento tumoral ainda precisa ser elucidado.

VI. Perspectivas

No sentido de aprofundar o papel do receptor P2X7 no desenvolvimento/resistência tumoral e avaliar o potencial terapêutico do uso dos nucleotídeos/nucleosídeos de adenina em associação com a hipertermia como uma forma de aumentar a ativação do receptor P2X7 no microambiente tumoral, algumas perspectivas são sugeridas:

1. Comparar a resistência à quimioterapia, capacidade de proliferação, e marcadores da angiogênese, entre a linhagens de câncer cervical SiHa silenciada e não silenciada para o gene do receptor P2X7, por meio de estudos *in vitro* e *in vivo*;

2. Descobrir o exato mecanismo pelo qual o *heat shock* está levando a ativação do receptor P2X7 na linhagem MCA38;

3. Avaliar o efeito do *heat shock* na ativação do receptor P2X7 em outras linhagens celulares, como as de câncer cervical;

4. Realizar estudos *in vivo*, avaliando o efeito local da hipertermia no tratamento de tumores malignos implantados em associação com radioterapia e/ou quimioterapia.

VII. Referências

ADINOLFI, E.; KIM, M.; YOUNG, M. T.; DI VIRGILIO, F.; SURPRENANT A. tyrosine phosphorylation of HSP90 within the P2X7 receptor complex negatively regulates P2X7 receptors. *The journal of biological chemestry,* v.278, p.37344-51, 2003.

ADINOLFI, E.; CALLEGARI, M. G.; FERRARI, D.; BOLOGNESI, C.; MINELLI, M.; WIECKOWSKI, M. R.; PINTON, P.; RIZZUTO, R.; DI VIRGILIO, F. Basal activation of the P2X7 ATP receptor elevates mitochondrial calcium and potential, increases cellular ATP levels, and promotes serum-independent growth. *Molecular Biology of the Cell*, v.16, p.3260–3272, 2005.

ADINOLFI, E.; RAFFAGHELLO, L.; GIULIANI, A. L.; CAVAZZINI, L.; CAPECE, M.; CHIOZZI, P.; BIANCHI, G.; KROEMER, G.; PISTOIA, V.; DI VIRGILIO, F. Expression of P2X7 receptor increases in vivo tumor growth. *Cancer Research*, v.72, p.2957–2969, 2012.

AGHAEI, M.; KARAMI-TEHRANI, F.; PANJEHPOUR, M.; SALAMI, S.; FALLAHIAN F. Adenosine induces cell-cycle arrest and apoptosis in androgen-dependent and -independent prostate cancer cell lines, LNcap-FGC-10, DU-145, and PC3. *The Prostate*, v.72, p.361-75, 2012.

AGHAEI, M.; PANJEHPOUR, M.; KARAMI-TEHRANI, F.; SALAMI, S. Molecular mechanisms of A3 adenosine receptor-induced G1 cell cycle arrest and apoptosis in androgen-dependent and independent prostate cancer cell lines: involvement of intrinsic pathway. *Journal of Cancer Research and Clinical Oncology*, v.137, p.1511-23, 2011.

AGTERESCH, H. J.; DAGNELIE, P. C.; VAN DER GAAST, A.; STIJNEN, T.; WILSON, J. H. Randomized clinical trial of adenosine 50-triphosphate in patients with advanced non-small-cell lung cancer. *Journal of the National Cancer Institute*,v.92, p.321–8, 2000.

ANTONIOLLI, L.; BLANDIZZI, C.; PACHER, P.; HASKO, G. Immunity, inflammation and cancer: a leading role for adenosine. *Nature Reviews. Cancer*, v.13, n.12, p.842-57, 2013.

BAEHRECKE, E. H. Autophagy: dual roles in life and death. *Nature Reviews. Molecular Cell Biology,* v.6, p.505-10, 2005.

BARRY, C. P.; LIND, S. E. Adenosine-mediated killing of cultured epithelial cancer cells. *Cancer Research,* v.60, p.1887–94, 2000.

BAR-YEHUDA, S.; BARER, F.; VOLFSSON, L.; FISHMAN, P. Resistance of muscle to tumor metastases: a role for a3 adenosine receptor agonists. *Neoplasia*, v.3, p.125-31, 2001.

BAR-YEHUDA, S.; STEMMER, S. M.; MADI, L.; CASTEL, D.; OCHAION, A.; COHEN, S.; BARER, F.; ZABUTTI, A.; PEREZ-LIZ, G.; DEL VALLE, L.; FISHMAN, P. The A3 adenosine receptor agonist CF102 induces apoptosis of hepatocellular carcinoma via de-regulation of the Wnt and NF-kappaB signal transduction pathways. *International Journal of Oncology*, v.33, p.287-95, 2008.

BEIJER, S.; HUPPERETS, P. S.; VAN DEN BORNE, B. E.; EUSSEN, S. R.; VAN HENTEN, A. M.; VAN DEN BEUKEN-VAN EVERDINGEN, M.; DE GRAEFF, A.; AMBERGEN, T. A.;

VAN DEN BRANDT, P. A.; DANGNELIE, P. C. Effect of adenosine 50-triphosphate infusions on the nutritional status and survival of preterminal cancer patients. *Anti-cancer Drugs*, v.20, p.625–33, 2009.

BIAN, S.; SUN, X.; BAI, A.; ZHANG, C.; LI, L.; ENJYOJI, K.; JUNGER, W. G.; ROBSON, S. C.; WU, Y. P2X7 integrates PI3K/AKT and AMPK-PRAS40-mTOR signaling pathways to mediate tumor cell death. *PLoS One,* v.; 8, p.e60184, 2013.

BURNSTOCK, G.; DI VIRGILIO, F. Purinergic signalling and cancer. *Purinergic Signalling*, v.9, p.491-540, 2013.

CALVERT, R. C.; SHABBIR, M.; THOMPSON, C. S.; MIKHAILIDIS, D. P.; MORGAN, R. J.; BURNSTOCK, G.Immunocytochemical and pharmacological characterisation of P2-purinoceptor-mediated cell growth and death in PC-3 hormone refractory prostate cancer cells. *Anticancer Research*, v.24, p.2853-9, 2004.

CASAS-PRUNEDA, G.; REYES, J. P.; PEREZ-FLORES, G.; PEREZ-CORNEJO, P.; ARREOLA, J. Functional interactions between P2X4 and P2X7 receptors from mouse salivary epithelia. *The Journal of Physiology*, v.587, p.2887–2901, 2009.

CHATTERJEE, D. K.; DIAGARADJANE, P.; KRISHNAN, S. Nanoparticle-mediated hyperthermia in cancer therapy. *Therapeutic Delivery*, v. 2, p.1001-14, 2011.

CHEN, Y.; CORRIDEN, R.; INOUE, Y.; YIP, L.; HASHIGUCHI, N.; ZINKERNAGEL, A. NIZET, V.; INSEL, P. A.; JUNGER, W. G. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science*, v.314, p.1792–1795, 2006.

CORRIDEN, R.; CHEN, Y.; INOUE, Y.; BELDI, G.; ROBSON, S. C.; INSEL, P. A.; JUNGER, W. G. Ectonucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1/CD39) regulates neutrophil chemotaxis by hydrolyzing released ATP to adenosine. The Journal of Biologycal Chemistry, v.283, p.28480–28486, 2008.

COUTINHO-SILVA, R.; STAHL, L.; CHEUNG, K. K.; DE CAMPOS, N. E.; DE OLIVEIRA SOUZA, C.; OJCIUS, D. M.; BURNSTOCK, G. P2X and P2Y purinergic receptors on human intestinal epithelial carcinoma cells: effects of extracellular nucleotides on apoptosis and cell proliferation. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, n.288, p.G1024-35, 2005.

DELI, T.; CSERNOCH, L. Extracellular ATP and cancer: an overview with special reference to P2 purinergic receptors. *Pathology Oncology Research*, v.14, p.219-31, 2008.

DEAGLIO, S.; ROBSON, S. C. Ectonucleotidases as regulators of purinergic signaling in thrombosis, inflammation, and immunity. *Advances in pharmacology*, v.61, p.301-32, 2011.

DI VIRGILIO, F. Liaisons dangereuses: P2X(7) and the inflammasome. *Trends in Pharmacological Science*, v.28, p465–472, 2007.

DI VIRGILIO, F. Purines, Purinergic Receptor, and Cancer. *Cancer Research*, v. 72, p.5441-5447, 2012.

DI VIRGILIO, F.; FERRARI, D.; ADINOLFI, E. P2X(7): a growth-promoting receptor-implications for cancer. *Purinergic Signalling*, v.5, p.251–256, 2009.

DIXON, C. J.; BOWLER, W. B.; FLEETWOOD, P.; GINTY, A. F.; GALLAGHER, J. A. Extracellular nucleotides stimulate proliferation in MCF-7 breast cancer cells via P2-purinoceptors. *British Journal of Cancer*, v.75, n.1, p.34-9, 1997.

DUBYAK, G. R.; DE YOUNG, M. B. Intracellular Ca2+ mobilization activated by extracellular ATP in Ehrlich ascites tumor cells. *The Journal of Biological Chemistry*, v.260, p.10653–10661, 1985.

EDWARDS, B. K.; NOONE, A. M.; MARIOTTO, A. B.; SIMARD, E.P.; BOSCOE, F. P.; HENLEY, S. J.; JEMAL, A.; CHO, H.; ANDERSON, R. N.; KOHLER, B. A.; EHEMAN, C. R.; WARD, E. M.; Annual Report to the Nation on the status of cancer, 1975-2010, featuring prevalence of comorbity and impacton survival among persons with lung, colorectal, breast, or prostate cancer. *Cancer,* v.120, p.1290-314, 2014.

ELTZSCHIG, H. K.; SITKOVSKY, M. V.; ROBSON, S. C. Purinergic signaling during inflammation. *The New England Journal of Medicine*, v.367, p.2322-33, 2012.

ESKELINEN, E. L; SCHMIDT, C. K.; NEU, S.; WILLENBORG,M.; FUERTES, G.; SALVADOR, N.; TANAKA, Y.; LULLMANN-RAUCH, R; HARTMANN, D.; HEEREN, J.; VON FIGURA, K.; KNECHT, E.; SAFTIG, P. Disturbed Cholesterol Traffic but Normal Proteolytic Function in LAMP-1/LAMP-2 Double-deficient Fibroblasts. *Molecular Biology of the Cell*, v.15, p.3132-3145, 2004.

ESKELINEN, E. L. The dual role of autophagy in cancer. *Current Opinion in Pharmacology*, v.11, p.294-300, 2011.

FANG, W. G.; PIRNIA, F.; BANG, Y. J.; MYERS, C. E.; TREPEL, J. B. P2-purinergic receptor agonists inhibit the growth of androgen-independent prostate carcinoma cells. *The Journal of Clinical Investigation*, v.89, p.191-6, 1992.

FENG, L.; SUN, X., CSIZMADIA, E.; HAN, L.; BIAN, S.; MURAKAMI, T.; WANG, X.; ROBSON, S. C.; WU, Y. Vascular CD39/ENTPD1 directly promotes tumor cell growth by scavenging extracellular adenosine triphosphate. *Neoplasia*, v.13, p.206-16, 2011.

FENG, L.; TAPPER, E. B.; SUN, X.; GEHRING, M.; ROBSON, S. C.; WU, Y. Purinegic Modulation and CD39/ENTPD1 in Cancer. *Frontiers in Anti-Cancer Drug Discovery*, Chapter 5, v.4, p.229-292, 2014.

FENG, Y. H.; LI, X.; ZENG, R.; GORODESKI, G. I. Endogenously expressed truncated P2X7 receptor lacking the C-terminus is preferentially upregulated in epithelial cancer cells and fails to mediate ligand-induced pore formation and apoptosis. *Nucleosides, Nucleotides & Nucleic Acids*, v.25, p.1271–1276, 2006a.

FENG, Y. H.; LI, X.; WANG, L.; ZHOU, L.; GORODESKI, G. I. A truncated P2X7 receptor variant (P2X7-j) endogenously expressed in cervical cancer cells antagonizes the full-length P2X7 receptor through hetero-oligomerization. *The Journal of Biological Chemistry*, v.281, p17228–17237, 2006b.

FILIPPI-CHIELA, E. C.; VILLODRE, E. S.; ZAMIN, L. L.; LENZ, G. Autophagy Interplay with Apoptosis and Cell Cycle Regulation in the Growth Inhibiting Effect of Resveratrol in Glioma Cells. *Plos One*, v.6, p.e20849, 2011.

FERRARI, D.; CHIOZZI, P.; FALZONI, S.; DAL SUSINO, M.; MELCHIORRI, L.; BARICORDI, O. R.; DI VIRGILIO, F. Extracellular ATP triggers IL-1 beta release by activating the purinergic P2Z receptor of human macrophages. *Journal of Immunology*, v.159, p.1451–1458, 1997.

FISHMAN, P.; BAR-YEHUDA, S.; OHANA, G.; BARER, F.; OCHAION, A.; ERLANGER, A.; MADI, L. An agonist to the A3 adenosine receptor inhibits colon carcinoma growth in mice via modulation of GSK-3 beta and NF-kappa B. *Oncogene,* v.23, p.2465-71, 2004.

FISHMAN, P.; BAR-YEHUDA, S.; OHANA, G.; PATHAK, S.; WASSERMAN, L.; BARER, F.; MULTANI, A. S. Adenosine acts as an inhibitor of lymphoma cell growth: a major role for the A3 adenosine receptor. *European Journal of Cancer,* v.36, p.1452-8, 2000.

FISHMAN, P.; MADI, L.; BAR-YEHUDA, S.; BARER, F.; DEL VALLE, L.; KHALILI, K. Evidence for involvement of Wnt signaling pathway in IB-MECA mediated suppression of melanoma cells. *Oncogene*, v. 21, p.4060-4, 2002.

FRANCKENA, M.; VAN DER ZEE, J. Use of combined radiation and hyperthermia for gynecological cancer. *Current Opinion in Obstetrics & Gynecology*, v. 22, p.9–14, 2010.

FU, W.; MCCORMICK, T.; QI, X.; LUO, L.; ZHOU, L.; LI, X.; WANG, B. C.; GIBBONS, H. E.; ABDUL-KARIM, F. W.; GORODESKI, G. I. Activation of P2X(7)-mediated apoptosis inhibits DMBA/TPA-induced formation of skin papillomas and cancer in mice. BMC Cancer, v.9, p.114, 2009.

GALLUZZI, L.; VICENCIO, J. M.; KEPP, O.; TASDEMIR, E.; MAIURI, M. C.; KROEMER, G. To die or not to die: that is the autophagic question. *Current Molecular Medicine*, v.8, p.78-91, 2008.

GALLUZZI, L.; VITALE, I.; VACCHELLI, E.; KROEMER, G. Cell death signaling and anticancer therapy*. Frontiers in Oncology*, v.1, article 5, 2011.

GARCIA-MARCOS, M.; POCHET, S.; MARINO, A.; DEHAYE, J. P. P2X7 and phospholipid signalling: the search of the “missing link” in epithelial cells. *Cellular Signalling*, v.18, p.2098–2104, 2006.

GARTLAND, A.; BUCKLEY, K. A.; HIPSKIND, R. A.; BOWLER, W. B.; GALLAGHER, J. A. P2 receptors in bone-modulation of osteoclast formation and activity via P2X7 activation. *Critical Reviews in Eukaryotic Gene Expression*, v.13, p.237–242, 2003.

GARTLAND, A.; HIPSKIND, R. A.; GALLAGHER, J. A.; BOWLER, W. B. Expression of a P2X7 receptor by a subpopolation of human osteoblasts. *Journal of Bone and Mineral Research*, v.16, p.846–856, 2001.

GARTLAND, A.; ORRISS, I. R.; RUMNEY, R. M.; BOND, A. P.; ARNETT, T.; GALLAGHER, J. A. Purinergic signalling in osteoblasts. *Frontiers in Bioscience (Landmark Edition)*, v.17, p.16–29, 2012.

GEHRING, M. P.; PEREIRA, T. C.; ZANIN, R. F.; BORGES, M. C.; BRAGA FILHO, A.; BATTASTINI, A. M.; BOGO, M. R.; LENZ, G.; CAMPOS, M. M.; MORRONE, F. B. P2X7 receptor activation leads to increased cell death in a radiosensitive human glioma cell line. *Purinergic Signalling*, v.8, p.729-39, 2012.

GHIRINGHELLI, F.; APETOH, L.; TESNIERE, A.; AYMERIC, L.; MA, Y.; ORTIZ, C.; VERMAELEN, K.; MIGNOT, G.; ULLRICH, E.; PERFETTINI, J. L.; SCHLEMMER, F.; TASDEMIR, E.; UHL, M.; GENIN, P.; CIVAS, A.; RYFFEL, B.; KANELLOPOULOS, J.; TSCHOPP, J.; ANDRE, F.; LIDEREAU, R.; MCLAUGHLIN, N. M.; HAYNES, N. M.; SMYTH, M. J.; KROEMER, G.; ZITVOGEL, L. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nature Medicine*, v.15, p.1170–8, 2009.

GLOBOCAN 2012. Estimated Cancer Incidence, Mortality and Prevalence Worlwide in 2012. Disponível em: < http://globocan.iarc.fr/Default.aspx> Acesso em: 06 out. 2015.

GREIG, A. V.; LINGE, C.; HEALY, V.; LIM, P.; CLAYTON, E.; RUSTIN, M. H.; MCGROUTHER, D. A.; BURNSTOCK, G. Expression of purinergic receptors in non-melanoma skin cancers and their functional roles in A431 cells. *The Journal of Investigative Dermatology*, v.121, n.2, p.315-27, 2003.

GRZANKA, D.; KOWALCZYK, A. E.; IZDEBSKA, M.; KLIMASZEWSKA-WISNIEWSKA, A.; GAGAT, M. The interactions between SATB1 and F-actin are important for mechanisms of active cell death. *Folia Histochemica et Cytobiologica*, v.53, p.152-61.

GÓMEZ-VILLAFUERTES, R.; DEL PUERTO, A,; DÍAZ-HERNÁNDEZ, M.; BUSTILLO, D.; DÍAZ-HERNÁNDEZ, J. I.; HUERTA, P. G.; ARTALEJO, A. R.; GARRIDO, J. J.; MIRAS-PORTUGAL, M. T. Ca2+/calmodulin-dependent kinase II signalling cascade mediates P2X7 receptordependent inhibition of neuritogenesis in neuroblastoma cells. *The FEBS Journal*, v.276, p.5307–5325, 2009.

HASKO, G.; LINDEN, J.; CRONSTEIN, B; PACHER, P. Adenosine receptors: therapeutics aspects for inflammatory and immune diseases. *Nature Reviews. Drug Discovery*, v.7, p.759-770, 2008.

HATTORI, F.; OHSHIMA, Y.; SEKI, S.; TSUKIMOTO, M.; SATO, M.; TAKENOUCHI, T.; SUZUKI, A.; TAKAI, E.; KITANI, H.; HARADA, H.; KOJIMA, S. Feasibility study of B16 melanoma therapy using oxidized ATP to target purinergic receptor P2X7. European Journal of Pharmacology, v.695, p.20–26, 2012.

HE, C.; KLIONSKY, D. J. Regulation mechanisms and signaling pathways of autophagy. *Annual Review of Genetics*, v.43, p.67–93, 2009.

HILLMAN, K. A.; JOHNSON, T. M.; WINYARD, P. J.; BURNSTOCK, G.; UNWIN, R. J.; WOOLF, A. S. P2X(7) receptors are expressed during mouse nephrogenesis and in collecting duct cysts of the cpk/cpk mouse. *Experimental Nephrology*, v.10, p.34–42, 2002.

HIPPER, M. M.; O'TOOLE, P. S.; THORBURN, A. Autophagy in Cancer: Good, Bad or Both. *Cancer Research*, v.66, p.9349-5, 2006.

HOPFNER, M.; LEMMER, K.; JANSEN, A.; HANSKI, C.; RIECKEN, E.O.; GAVISH, M.; MANN B.; BUHR, H. GLASSMEIER, G.; SCHERUBL, H. Expression of functional P2-purinergic receptors in primary cultures of human colorectal carcinoma cells. *Biochemical and Biophysical Research Communications*, v.251, p.811-7, 1998.

HOEPFNER, M.; KAP, H.; JANSEN, A.; LEMMER, K.; HANSKI, C.; RIECKEN, E. O.; SCHERUEBL, H. Extracellular ATP induces apoptosis and inhibits growth of colorectal carcinomas. *Gastroenterology,* v.116, p.A423, 1999.

HORSTMAN, D. A.; TENNES, K. A.; PUTNEY, J. W. JR. ATP-induced calcium mobilization and inositol 1,4,5-triphosphate formation in H-35 hepatoma cells. *FEBS Letters*, v.204,p.189–192,1986.

HORVATH, I.; GLATZ, A.; VARVASOVSZKI, V.; TOROK, Z.; PALI, T.; BALOGH, G.; KOVACS, E.; NADASDI, L.; BENKO, S.; JOO, F.; VIGH, L. Membrane physical state controls the signaling mechanism of the heat shock response in Synechocystis PCC 6803: identification of hsp17 as a "fluidity gene". *Proceedings of the National Academy of Sciences of The United States of America*, v.95, p.3513–3518, 1998.

HUANG, S.; CHEN, Y.; WU, W.; OUYANG, N.; CHEN, J.; LI, H.; LIU, X.; SU, F.; LIN, L.; YAO, Y. miR-150 promotes human breast cancer growth and malignant behavior by targeting the pro-apoptotic purinergic P2X7 receptor. *PLoS One*, v.8, p.e80707, 2013.

HUILGOL, N. G.; GUPTA, S.; SRIDHAR, C. R. Hyperthermia with radiation in the treatment of locally advanced head and neck cancer: a report of randomized trial. *Journal of Cancer Research and Therapeutics*, v.6, p.492–496, 2010a.

HUILGOL, N. G.; GUPTA, S.; DIXIT, R. Chemoradiation with hyperthermia in the treatment of head and neck cancer. *International Journal of Hyperthermia*, v.26, p.21–25, 2010b.

HURWITZ, M. D.; HANSEN, J. L.; PROKOPIOS-DAVOS, S.; MANOLA, J.; WANG, Q.; BORNSTEIN, B. A.; HYNYNEN, K.; KAPLAN, I. D. Hyperthermia combined with radiation for the treatment of locally advanced prostate cancer: long-term results from Dana-Farber Cancer Institute study 94–153. *Cancer,* v.117, p.510–516, 2011.

IDE, S.; NISHIMAKI, N.; TSUKIMOTO, M.; KOJIMA, S. Purine receptor P2Y6 mediates cellular response to gamma-ray-induced DNA damage. *The Journal of Toxicological Science*, v.39,p.15-23, 2014.

IDZKO, M.; FERRARI, D.; RIEGEL, A. K.; ELTZSCHIG, K. Extracellular nucleotide and nucleoside signaling in vascular and blood disease. *Blood,* v.124, n.7, p.1029-37, 2014.

INCA. Instituto Nacional do Câncer. Estatísticas do Câncer, Vigilância do Câncer e de Fatores de Risco. Disponível em: < http://www.inca.gov.br/estimativa/2014/> Acesso em: 06 out. 2015.

IRISH, C. E.; BROWN, J.; GALEN, W. P.; GALLUCCI, J. J.; HYMAN, M. D.; HOROWITZ, I. J.; SNEDECOR, P. A.; BAKER, H. W. Thermoradiotherapy for persistent cancer in previously irradiated fields. *Cancer*, v.57, p.2275–2279,1986.

JELASSI, B.; ANCHELIN, M.; CHAMOUTON, J.; CAYUELA, M. L.; CLARYSSE, L.; LI, J.; GORE, J.; JIANG, L.H.; ROGER, S. Anthraquinone emodin inhibits human cancer cell invasiveness by antagonizing P2X7 receptors. *Carcinogenesis*, v.34, p.1487–1496, 2013.

JELASSI, B.; CHANTOME, A.; ALCARAZ-PEREZ, F.; BAROJA-MAZO, A.; CAYUELA, M. L.; PELEGRIN, P.; SURPRENANT, A.; ROGER, S. P2X(7) receptor activation enhances SK3 channels-and cystein cathepsin-dependent cancer cells invasiveness. *Oncogene*, v.30, p.2108–2122, 2011.

JIANG, L. H.; BALDWIN, J. M.; ROGER, S.; BALDWIN, S. A. Insights into the Molecular Mechanisms Underlying Mammalian P2X7 Receptor Functions and Contributions in Diseases, Revealed by Structural Modeling and Single Nucleotide Polymorphisms. *Frontiers in Pharmacology*, v.4, p.55, 2013.

JOACHIMS, M. L.; MARBLE, P.; KNOTT-CRAIG, C.; PASTUSZKO, P.; BLACKBURN, M. R.; THOMPSON, L. F. Inhibition of deoxynucleoside kinases in human thymocytes prevents dATP accumulation and induction of apoptosis. *Nucleosides, Nucleotides & Nucleic Acids*, v.27, p.816–820, 2008.

JOHNSTON, J. B. Mechanism of action of pentostatin and cladribine in hairy cell leukemia. *Leukemia & Lymphoma*, v.52, p.43–45, 2011.

KALHAN, A.; GHARIBI, B.; VAZQUEZ, M.; JASANI, B.; NEAL, J.; KIDD, M.; MODLIN, I. M.; PFRAGNER, R.; REES D. A.; HAM, J. Adenosine A2A and A2B receptor expression in neuroendocrine tumours: potential targets for therapy. *Purinergic Signalling*, v.8, p.265-74, 2012.

KHAKH, B. S.; NORTH, R. A. P2X receptors as cell-surface ATP sensors in health and disease. Nature, V.442, p.527-32, 2006.

KIM, M.; JIANG, L.H.; WILSON, H. L.; NORTH, R. A.; SURPRENANT, A. Proteomic and functional evidence for a P2X7 receptor signalling complex. *The EMBO Journal,* v.20, p.6347-58, 2001.

KIM, S. G.;, GAO, Z. G.; SOLTYSIAK, K. A.; CHANG, T. S.; BRODIE, C.; JACOBSON, K. A. P2Y6 nucleotide receptor activates PKC to protect 1321N1 astrocytoma cells against tumor

necrosis factor induced apoptosis. *Cellular and Molecular Neurobiology*, v.23, p.401-18, 2003.

KIM, N. H.; PARK, K. S.; SOHN, J. H.; YEH, B. I.; KO, C. M.; KONG, I. D. Functional expression of P2Y receptors in WERI-Rb1 retinoblastoma cells. Korean J Physiol Pharmacol, v.15, p.61–66, 2011.

KONERU, M.; SCHAER, D.; MONU, N.; AYALA, A.; FREY, A. B. Defective proximal TCR signaling inhibits CD8+ tumor-infiltrating lymphocyte lytic function. *Journal of Immunology*, v.174, p.1830–1840, 2005.

LI, X.; QI, X.; ZHOU, L.; CATERA, D.; ROTE, N. S.; POTASHKIN, J.; ABDUL-KARIM, J. F. W.; GORODESKI, G. I. Decreased expression of P2X7 in endoltelial epithelial pre-cancerous and cancer cells. *Gynecologic Oncology*, v.106, p.233–243, 2007.

LI, X.; QI, X.; ZHOU, L.; FU, W.; ABDUL-KARIM, F. W.; MACLENNAN, G.; GORODESKI, G. I. P2X(7) receptor expression is decreased in epithelial cancer cells of ectodermal, uro-genital sinus, and distal paramesonephric duct origin. *Purinergic Signalling*, v.5, p.351–368, 2009.

LI, X.; ZHOU, L.; FENG, Y. H.; ABDUL-KARIM, F. W.; GORODESKI, G. I. The P2X7 receptor: a novel biomarker of uterine epithelial cancers. *Cancer Epidemiology Biomarkers & Prevention*, v.15, p.1906-13, 2006.

LI, X.; GONG, Z.; ZHANG, L.; ZHAO, C.; ZHAO, X.; GU, X.; CHEN, H. Autophagy knocked down by high-risk HPV infection and uterine cervical carcinogenesis. *International Journal of Clinical and Experimental Medicine*, v.8, p.10304-14, 2015.

LINDEN, J. Adenosine metabolism and cancer. Focus on “Adenosine downregulates DPPIV on HT-29 colon cancer cells by stimulating protein tyrosine phosphatases and reducing ERK1/2 activity via a novel pathway”. *American Journal of Physiology. Cell Physiology,* v.291, p. C405-C406, 2006.

LUK, K. H.; FRANCIS, M. E.; PEREZ, C. A.; JOHNSON, R. J. Combined radiation and hyperthermia: comparison of two treatment schedules based on data from a registry established by the Radiation Therapy Oncology Group (RTOG). *International Journal of Radiation Oncology, Biology, Physics*, v.10, p.801–809, 1984.

MAASER, K.; HOPFNER, M.; KAP, H.; SUTTER, A. P.; BARTHEL, B.; VON LAMPE, B.; ZEITZ, M.; SCHERUBL, H. Extracellular nucleotides inhibit growth of human oesophageal cancer cells via P2Y(2)-receptors. *British Journal of Cancer*, v.86, p.636-44**,** 2002.

MADI, L.; BAR-YEHUDA, S.; BARER, F.; ARDON, E.; OCHAION, A.; FISHMAN P. A3 adenosine receptor activation in melanoma cells: association between receptor fate and tumor growth inhibition. *The Journal of Biological Chemestry*, v.278, p.42121-30, 2003.

MATHEW, R.; KARANTZA-WADSWORTH, V.; WHITE, E. Role of autophagy in cancer. *Nature Reviews. Cancer,* v.7, p.961-7, 2007.

MARTIN, D. S.; BERTINO, J. R.; KOUTCHER, J. A. ATP depletion + pyrimidine depletion can markedly enhance cancer therapy: fresh insight for a new approach. *Cancer Research*, v.60, p.6776–6783, 2000.

MARTIN, D. S.; SPRIGGS, D.; KOUTCHER, J. A. A concomitant ATPdepleting strategy markedly enhances anticancer agent activity. *Apoptosis*, v.6, p.125–131, 2001.

MATSUMOTO, G.; WADA, K.; OKUNO, M.; KUROSAWA, M.; NUKINA, N. Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins. *Molecular Cell* , v.44, p.279–289, 2011.

MEDIAVILLA-VARELA, M.; LUDDY, K.; NOYES, D.; KHALIL, F. K.; NEUGER, A. M.; SOLIMAN, H.; ANTONIA, S. Antagonism of adenosine A2A receptor expressed by lung adenocarcinoma tumor cells and cancer associated fibroblasts inhibits their growth. *Cancer Biology & Therapy*, v.14, n.9, p.860-8, 2013.

MERIGHI, S.; MIRANDOLA, P.; MILANI, D.; VARANI, K; GESSI, S.; KLOTZ, K. N.; LEUNG, E.; BARALDI, P. G.; BOREA, P. A. Adenosine receptors as mediators of both cell proliferation and cell death of cultured human melanoma cells. *The Journal of Investigative Dermatology*, v.119, p.923-33, 2002.

MICHAUD, M.; MARTINS, I.; SUKKURWALA, A. Q.; ADJEMIAN, S.; MA, Y.; PELLEGATTI, P.; SHEN, S.; KEPP, O.; SCOAZEC, M.; MIGNOT, G.; RELLO-VARONA, S.; TAILLER, M.; MENGER, L.; VACCHELLI, E.; GALLUZZI, L.; GHIRINGHELLI, F.; DI VIRGILIO, F.; ZITVOGEL, L.; KROEMER, G. Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. *Science*, v.334, p.1573-7, 2011.

MONU, N., FREY, A. B. Suppression of proximal T cell receptor signaling and lytic function in CD8+ tumor-infiltrating T cells. Cancer Research, v.67, p.11447–11454, 2007.

MOROS, E. G.; PENAGARICANO, J.; NOVAK, P.; STRAUBE, W. L.; MYERSON, R. J. Present and future technology for simultaneous superficial thermoradiotherapy of breast cancer. *International Journal of Hyperthermia*, v.26, p.699–709, 2010.

NAGY, E.; BALOGI, Z.; GOMBOS, I.; AKERFELT, M.; BJORKBOM, A.; BALOGH, G.; TOROK, Z.; MASLYANKO, A.; FISZER-KIERZKOWSKA, A.; LISOWSKA, K.; SLOTTE, P. J.; SISTONEN, L.; HORVATH, I.; VIGH, L. Hyperfluidization-coupled membrane microdomain reorganization is linked to activation of the heat shock response in a murine melanoma cell line. *Proceedings of the National Academy of Sciences of the United of States of America*, v.104, p.7945-7950, 2007.

NISHIMAKI, N.; TSUKIMOTO, M.; KITAMI, A.; KOJIMA, S. Autocrine regulation of gammairradiation-induced DNA damage response via extracellular nucleotides-mediated activation of P2Y6 and P2Y12 receptors. *DNA Repair (Amst),* v.11, p.657-65, 2012.

NOGI, Y.; KANNO, T.; NAKANO, T.; FUJITA, Y.; TABATA, C.; FUKUOKA, K.; GOTOH, A.; NISHIZAKI, T. AMP converted from intracellularly transported adenosine upregulates p53 expression to induce malignant pleural mesothelioma cell apoptosis. *Cellular Physiology and Biochemistry*, v.30, p.61–74, 2012.

OHSHIMA, Y.; TSUKIMOTO, M.; TAKENOUCHI, T.; HARADA, H.; SUZUKI, A.; SATO, M.; KITANI, H.; KOJIMA, S. gamma-Irradiation induces P2X(7) receptor-dependent ATP release from B16 melanoma cells. *Biochimica et Biophysica Acta*, v.1800, p.40–46, 2010.

OTHA, A. *et al*. A2A adenosine receptor protects tumors from antitumor T cells. *Proceedings of the National Academy of Science of the United States of America,* v.103, n.35, p.13132-13137, 2006.

OTSUKI, T.; KANNO, T.; FUJITA, Y.; TABATA, C.; FUKUOKA, K.; NAKANO, T.; GOTOH, A.; NISHIZAKI, T. A3 adenosine receptor-mediated p53-dependent apoptosis in Lu-65 human lung cancer cells. *Cellular Physiology and Biochemistry*, v.30, p. 210-20, 2012.

PANKIV, S.; CLAUSEN, T. H.; LAMARK, T.; BRECH, A.; BRUUN, J. A.; OUTZEN, H.; ØVERVATN, A.; BJØRKØY, G.; JOHANSEN, T. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *The Journal of Biological Chemistry*, v.282, p.24131–24145, 2007.

PELLEGATTI, P.; RAFFAGHELLO, L.; BIANCHI, G.; PICCARDI, F.; PISTOIA, V.; DI VIRGILIO, F. Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. *PLos One*, v.3, n.7, p.e2599, 2008.

PINES, A.; PERRNONE, L.; BIVI, N.; ROMANELLO, M.; DAMANTE, G.; GULISANO, M.; KELLEY, M. R.; QUADRIFOGLIO, F.; TELL, G. Activation of APE1/Ref-1 is dependent on reactive oxygen species generated after purinergic receptor stimulation by ATP. *Nucleic Acids Research,* v.33, n.14, p.4379-94, 2005.

POCHET, S.; GARCIA-MARCOS, M.; SEIL, M.; OTTO, A.; MARINO, A.; DEHAYE, J. P. Contribution of two ionotropic purinergic receptors to ATP responses in submandibular gland ductal cells. Cellular Signalling, v.19, p.2155–2164, 2007.

RAPAPORT, E. Treatment of human tumor cells with ADP or ATP yields arrest of growth in the S phase of the cell cycle. *Journal of Cellular Physiology*, v.114, p. 279-83, 1983.

RICKLES, R. J.; TAM, W. F.; GIORDANO, T. P.; PIERCE, L. T.; FARWELL, M.; MCMILLIN, D. W.; NECHEVA, A.; CROWE, D.; CHEN, M.; AVERY, W.; KANSRA, V.; NAWROCKI, S. T.; CAREW, J. S.; GLIES, F. J.; MITSIADES, C. S.; BORISY, A. A.; ANDERSON, K. C.; LEE, M. S. Adenosine A2A and beta-2 adrenergic receptor agonists: novel selective and synergistic multiple myeloma targets discovered through systematic combination screening. *Molecular Cancer Therapeutics*, v.11, p.1432-42, 2012.

ROBINSON, L. E.; SHRIDAR, M.; SMITH, P.; MURRELL-LAGNADO, R. D. Plasma Membrane Cholesterol as a Regulator of Human and Rodent P2X7 Receptor Activation and Sensitization. *The Journal of Biological Chemistry*, v.289, p.31983-94, 2014.

ROGER, S.; JELASSI, B.; COUILLIN, I.; PELEGRIN, P.; BRESSON, P.; JIANG, L.H. Understanding the roles of P2X7 receptor in solid tumor progression and therapeutic perspectives. *Biochimica et Biophysica Acta*, v.1848, n.10PtB, p.2584-602, 2014.

ROGER, S.; PELEGRIN, P. P2X7 receptor antagonism in the treatment of cancers. *Expert Opinion on Investigational Drugs,* v.20, n.7, p.875-80, 2011.

SAI, K.; YANG, D.; YAMAMOTO, H.; FUJIKAWA, H.; YAMAMOTO, S.; NAGATA, T.; SAITO, M.; YAMAMURA, T.; NISHIZAKI, T. A1 adenosine receptor signal and AMPK involving caspase-9/-3 activation are responsible for adenosine-induced RCR-1 astrocytoma cell death. *Neurotoxocology*, v.27, p.458-467, 2006.

SAITOH, M.; NAGAI, K.; NAKAGAWA, K.; YAMAMURA, T.; YAMAMOTO, S.; NISHIZAKI, T. Adenosine induces apoptosis in the human gastric cancer cells via an intrinsic pathway relevant to activation of AMP-activated protein kinase. *Biochemical Pharmacology*, v.67, p. 2005–2011, 2004.

SAITO, M.; YAGUCHI, T.; YASUDA, Y.; NAKANO, T.; NISHIZAKI, T. Adenosine suppresses CW2 human colonic cancer growth by inducing apoptosis via A(1) adenosine receptors. *Cancer Letters,* v.290, p.211-5, 2010.

SAUER, A. V.; BRIGIDA, I.; CARRIGLIO, N.; HERNANDEZ, R. J.; SCARAMUZZA, S.; CLAVENNA, D.; SANVITO, F.; POLIANI, P. L.; GAGLIANI, N.; CARLUCCI, F.; TABUCCHI, A.; RONCAROLO, M. G.; TRAGGIAI, E.; VILLA, A.; AIUTI, A. Alterations in the adenosine metabolism and CD39/CD73 adenosinergic machinery cause loss of Treg cell function and autoimmunity in ADA-deficient SCID. *Blood*, v.119, p.1428–39, 2012.

SCHAFER, R.; SEDEHIZADE, F.; WELTE, T.; REISER, G. ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells. American *Journal of Physiology. Lung Cellular and Molecuar Physiology*, v.285, n.2, p.L376-85, 2003.

SCHIFFMAN, M.; WENTZENSEN, N.; WACHOLDER, S.; KINNEY, W.; GAGE, J. C.; CASTLE, P. E. Human Papillomavirus Testing in the Prevention of Cervical Cancer. *Journal of the National Cancer Institute,* v.103, p.368–383, 2011.

SCHRIER, S. M.; VAN TILBURG, E. W.; VAN DER MEULEN, H.; IJZERMAN, A. P.; MULDER, G. J.; NAGELKERKE, J. F. Extracellular adenosine-induced apoptosis in mouse neuroblastoma cells: studies on involvement of adenosine receptors and adenosine uptake. *Biochemical Pharmacology*, v.61, p.417–2, 2001.

SERRA, S.; HORENSTEIN, A. L.; VAISITTI, T., BRUSA, D.; ROSSI, D.; LAURENTI, L.; D'ARENA, G.; COSCIA, M.; TRIPODO, C.; INGHIRAMI, G.; ROBSON, S. C.; GAIDANO, G.; MALAVASI, F.; DEAGLIO, S. CD73-generated extracellular adenosine in chronic lymphocytic leukemia creates local conditions counteracting drug-induced cell death. *Blood*, v.118, p.6141-52, 2011.

SEEGENSCHMIEDT, M. H.; SAUER, R.; MIYAMOTO, C.; CHALAL, J. A.; BRADY, L. W. Clinical experience with interstitial thermoradiotherapy for localized implantable pelvic tumors. *American Journal of Clinical Oncology*, v.16, p.210–222, 1993.

SEETULSINGH-GOORAH, S. P.; STEWART, B. W. Growth inhibition of HL-60 cells by extracellular ATP: concentration-dependent involvement of a P2 receptor and adenosine generation. *Biochemical and Biophysical Research Communications,* v.250, p.390–396, 1998.

SHABBIR, M.; BURNSTOCK, G. Purinergic receptor-mediated effects of adenosine 5'-triphosphate in urological malignant diseases. *International Journal of Urology*, v.16, p.143-50, 2009.

SHABBIR, M.; THOMPSON, C. S.; JARMULOWICZ, M.; MIKHAILIDIS, D. P.; BURNSTOCK, G. Effect of extracellular ATP on the growth of hormone refractory prostate cancer in vivo. *BJU International,* v.102, p.108–112, 2008a.

SHABBIR, M.; RYTEN, M.; THOMPSON, C.; MIKHAILIDIS, D.; BURNSTOCK, G. Purinergic receptor-mediated effects of ATP in high-grade bladder cancer. *BJU International*, v.101, n.1, p.106-12, 2008b.

SODELARO BILBAO P.; BOLAND, R. Extracellular ATP regulates FoxO family of transcription factors and cell cycle progression through PI3K/Akt in MCF-7 cells. *Biochimica at Biophysica Acta,* v.1830, p.4456-69, 2013.

SUPLAT-WYPYCH, D,; DYGAS, A.; BARAÑSKA, J. 2′,3′-O-(4- benzoylbenzoyl)-ATP-mediated calcium signaling in rat glioma C6 cells: role of the P2Y2 nucleotide receptor. *Purinergic Signalling,* v.6, p317–325, 2010.

SOUZA, C. O.; SANTORO, G. F.; FIGLIUOLO, V. R.; NANINI, H. F.; DE SOUZA, H. S.; CASTELO-BRANCO, M. T.; ABALO, A. A.; PAIVA, M. M.; COUTINHO, C. M.; COUTINHO-SILVA, R. Extracellular ATP induces cell death in human intestinal epithelial cells. Biochimica at Biophysica Acta, v.1820, p.1867-78, 2012.

STAGG, J.; DIVISEKERA, U.; DURET, H.; SPARWASSER, T.; TENG, M. W.; DARCY, P. K.; SMYTH, M. J. CD73-deficient mice have increased antitumor immunity and are resistant to experimental metastasis. *Cancer Research*, v.71, p.2892–900, 2011.

SUN, X.; HAN, L.; SETH, P.; BIAN, S.; LI, L.; CSIZMADIA, E.; JUNGER, W. G.; SCHMELZLE, M.; USHEVA, A.; TAPPER, E. B.; BAFFY, G.; SUKHATME, V. P. WU, Y.; ROBSON, S. C. Disordered purinergic signaling and abnormal cellular metabolism are associated with development of liver cancer in Cd39/Entpd1 null Mice. *Hepatology*, v.57, p.205–216, 2013.

SYNOWITZ, M.; GLASS, R.; FARBER, K.; et al. A1 adenosine receptors in microglia control glioblastoma-host interaction. *Cancer Research,* v.66, p.8550-7, 2006.

TAFANI, M.; DI VITO, M.; FRATI, A.; PELLEGRINI, L.; DE SANTIS, E.; SETTE, G.; ERAMO, A.; SALE, P.; MARI, E.; SANTORO, A.; RACO, A.; SALVATI, M.; DEMARIA, R.; RUSSO, M. A. Pro-inflammatory gene expression in solid glioblastoma microenvironment and in hypoxic stem cells from human glioblastoma. *Journal of Neuroinflammation*, v.8, p.32, 2013.

TAKAI, E.; TSUKIMOTO, M.; HARADA, H.; KOJIMA, S. Autocrine signaling via release of ATP and activation of P2X7 receptor influences motile activity of human lung cancer cells. *Purinergic Signalling*, 2014.

TAKAI, E.; TSUKIMOTO, M.; HARADA, H.; SAWADA, K.; MORIYAMA, Y.; KOJIMA, S. J. Autocrine regulation of TGF-ß1-induced cell migration by excocytosis of ATP and activation of P2 receptors in human lung cancer cells. *Journal of Cell Science*, v.125, p.5051–5060, 2012.

TAMAJUSUKU, A. S.; VILLODRE, E. S.; PAULUS, R.; COUTINHO-SILVA, R.; BATTASSTINI, A. M.; WINK, M. R.; LENZ, G. Characterization of ATP-induced cell death in the GL261 mouse glioma. Journal of Cellular Biochemistry, v.109, p.983–991, 2010.

TREPEL, J.; MOLLAPOUR, M.; GIACCONE, G.; NECKERS, L. Targeting the dynamic HSP90 complex in cancer. *Nature Reviews. Cancer*, v. 10, p.537-49, 2010.

TSUCHIYA, A.; KANNO, T.; SAITO, M.; MIYOSHI, Y.; GOTOH, A.; NAKANO, T.; NISHIZAKI T. Intracellularly transported adenosine induces MCF-7 human breast cancer cells by accumulating AMID in the nucleus. *Cancer Letters*, v. 321, p.65-72, 2012.

VAN DEN BERG, C. A.; VAN DE KAMER, J. B.; DE LEEUW, A. A.; JEUKENS, C. R.; RAAVMAKERS, B. W.; VAN VULPEN, M.; LAGENDIJK, J. J. Towards patient specific thermal modeling of the prostate. *Physics in Medicine and Biology*, v.51, p.809–825, 2006.

VASANTHAN, A.; MITSUMORI, M.; PARK, J. H.; ZHI-FAN, Z.; YU-BIN, Z.; OLIYNYCHENKO, P.; TATSUZAKI, H.; TANAKA, Y.; HIRAOKA, M. Regional hyperthermia combined with radiotherapy for uterine cervical cancers: a multi-institutional prospective randomized trial of the international atomic energy agency. International Journal of Radiation Oncology, Biology, Physics, v.61, p.145–153, 2005.

VARANI, K., MANIERO, S.; VINCENZI, F.; TARGA, M.; STEFANELLI, A.; MANISCALCO, P.; MARTINI, F.; TOGNON, M.; BOREA. P. A. A3 receptors are overexpressed in pleura from patients with mesothelioma and reduce cell growth via Akt/ nuclear factor-kB pathway. *American Journal of Respiratory and Critical Care Medicine*, v.183, p.522–30, 2011.

VAZQUEZ-CINTRON, E. J.; MONU, N. R.; BURNS, J. C.; BLUM, R.; CHEN, G.; LOPES, P.; MA, J.; RADOJA, S.; FREY, A. B. Protocadherin-18 is a novel differentiation marker and an inhibitory signaling receptor for CD8+ effector memory T cells. *PLoS One,* v.7, p.e36101, 2012.

VAZQUEZ-CUEVAS, F. G.; MARTINEZ-RAMIREZ, A. S.; ROBLES-MARTINEZ, L.; GARAY, E.; GARCIA-CARRANCA, A.; PEREZ-MONTIEL, D.; CASTANEDA-GARCIA, C.; ARELLANO, R. O. J. Paracrine stimulation of P2X7 receptor by ATP activates a proliferative pathway in ovarian carcinoma cells. *Journal of Cellular Biochemistry*, v.115, p.1955-66, 2014.

VIGH, L.; MARESCA, B.; HARWOOD, J. Does the membrane's physica state control the expression of heat shock and other genes. *Trends in Biochemical Sciences*, v.23, p.369–373, 1998.

VIGH, L.; MARESCA, B. Dual role of membranes in heat stress: As thermosensors modulate the expression of stress genes and by interacting with stress proteins, re-organize their own lipid order and functionality. In*: Cell and Molecular Responses to Stress*, (eds) Storey, K. B.; Storey, J. M. (Elsevier, Amsterdam), p.173–188, 2002.

VIGH, L.; ESCRIBA, P.; SONNLEITNER, A.; SONNLEITNER, M.; PIOTTO, S.; MARESCA, B.; HORVATH, I.; HARWOOD, L. J. The significance of lipid composition for membrane activity: new concepts and ways of assessing function. *Progress in Lipid Research*, v.44, p.303–344, 2005.

VINCENZI, F.; TARGA, M.; CORCIULO, C.; GESSI, S.; MERIGHI, S.; SETTI, S.; CADOSSI, R.; BOREA, P. A.; VARANI, K. The anti-tumor effect of A3 adenosine receptors is potentiated by pulsed electromagnetic fields in cultured neural cancer cells. *PLoS One*, v.7, n.6, p.e39317, 2012.

VOLONTE, C.; AMADIO, S.; D'AMBROSI, N.; COLPI, M.; BURNSTOCK, G. P2 receptor web: complexity and fine-tuning. *Pharmacology & Theraputics*, v.112, p.264-80, 2006.

WANG, Q.; WANG, L.; FENG, Y. H.; LI, X.; ZENG, R.; GORODESKI, G. I. P2X7 receptor-mediated apoptosis of human cervical epithelial cells. *American Journal of Physiology Cell Physiology*, v.287, p.C1349-58, 2004a.

WANG, X.; ARCUINO, G.; TAKANO, T.; LIN, J.; PENG, W.G.; WANG, P.; LI, P.; XU, Q.; LIU, Q.S.; GOLDMAN, S.A.; NEDERGAARD, M. P2X7 receptor inhibition improves recovery after spinal cord injury. *Nature Medicine,* v.10, n.8, p.821-7, 2004b.

WEBER, F. C.; ESSER, P. R.; MULLER, T.; GANESAN, J.; PELLEGATTI, P.; SIMON, M. M.; ZEISER, R.; IDZKO, M.; JAKOB, T.; MARTIN, S. F. Lack of the purinergic receptor P2X(7) results in resistance to contact hypersensitivity. *The Journal of Experimental Medicine*, v.207, p.2609-19, 2010.

WEI, Q.; COSTANZI, S.; BALASUBRAMANIAN, R.; GAO, Z. G.; JACOBSON, K. A. A2B adenosine receptor blockade inhibits growth of prostate cancer cells. *Purinergic Signalling*, v.9, p.271-80, 2013.

WEI, W.; RYU, J. K.; CHOI, H. B.; MCLARNON, J. G. Expression and function of the P2X(7) receptor in rat C6 gliomas cells. *Cancer Letters*, v.260, p.79–87, 2008.

WHITE, N.; BURNSTOCK, G. P2 Receptors and cancer. *Trends in Pharmacological Science*, v.27, n.4, p.211-7, 2006.

WHITE, N.; RYTEN, M.; CLAYTON, E.; BUTLER, P.; BURNSTOCK, G. P2Y purinergic receptors regulate the growth of human melanomas. *Cancer letters*, v.224, n.1, p.81-91, 2005a.

WHITE, N.; BUTLER, P. E.; BURNSTOCK, G. Human melanomas express functional P2X(7) receptors. *Cell and Tissue Research*, n.321, p.411-8, 2005b.

WHITE, N.; KNIGHT, G. E.; BUTLER, P. E.; BURNSTOCK, G. An in vivo model of melanoma: treatment with ATP. *Purinergic Signalling*, v.5, p.327–333, 2009.

WHO. World Heath Organization. Disponível em: <http://www.who.int/mediacentre/factsheets/fs297/en/> Acesso em: 15 out. 2015

WILHELM, K.; GANESAN, J.; MULLER, T.; DURR, C.; GRIMM, M.; BEILHACK, A.; KREMPL C. D.; SORICHTER, S.; GERLACH, U. V.; JUTTNER, E.; ZERWECK, A.; GARTNER, F.; PELLEGATTI, P.; DI VIRGILIO, F.; FERRARI, D.; KAMBHAM, N.; FISCH, P.; FINKE, J.; IDZKO, M.; ZEISER, R. Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R. *Nature Medicine*, v.16, p.1434-8, 2010.

WU, P. Y.; LIN, Y. C.; CHANG, C. L.; LU, H. T.; CHIN, C. H. T.; HSU, T.; CHU, D.; SUN, S. H. Functional decreases in P2X7 receptors are associated with retinoic acid-induced neuronal differentiation of Neuro-2a neuroblastoma cells. *Cellular Signalling*, v.21, p.881–891, 2009.

WU, Y.; SUN, X.; KACZMAREK, E.; DWYER, K. M.; BIANCHI, E.; USHEVA A.; ROBSON, S. C. RanBPM associates with CD39 and modulates ecto-nucleotidase activity. The Biochemical Journal, v.396, p.23–30, 2006.

YANG, D.; YAGUCHI, T.; YAMAMOTO, H.; NISHIZAKI, T. Intracellularly transported adenosine induces apoptosis in HuH‐7 human hepatoma cells by downregulating c‐FLIP expression causing caspase‐3/-8 activation. *Biochemical Pharmacology*, v.73, p.1665–1675, 2007.

YANG, Z.; KLIONSKY, D. J. Mammalian autophagy: core molecular machinery and signaling regulation. *Current Opinion in Cell Biology*, v.22, p.2124–131, 2010.

YANG, D.; YAGUCHI, T.; NAKANO, T.; NISHIZAKI, T. Adenosine activates AMPK to phosphorylate Bcl‐XL responsible for mitochondrial damage and DIABLO release in HuH‐7 cells. *Cellular Physiology and Biochemistry*, v.27, p.71–78, 2011.

YASUDA, Y.; SAITO, M.; YAMAMURA, T.; YAGUCHI, T.; NISHIZAKI, T. Extracellular adenosine induces apoptosis in Caco-2 human colonic cancer cells by activating caspase-9/-3 via A(2a) adenosine receptors. *Journal of Gastroenterology*, v.44, p.56-65, 2009.

YAMADA, K.; FUKAO, Y.; HAYASHI, M.; FUKAZAWA, M.; SUZUKI, I.; NISHIMURA, M. Cytosolic HSP90 Regulates the Heat Shock Response That Is Responsible for Heat Acclimation in Arabidopsis thaliana. *The Journal of Biological Chemestry*, v. 282, p. 37794–37804, 2007.

ZAGAR, T. M.; OLESON, J. R.; VUJASKOVIC, Z.; DEWHIRST, M. W.; CRACIUNESCU, O. I.; BLACKWELL, K. L.; PROSNITZ, L. R.; JONES, E. L. Hyperthermia combined with radiation therapy for superficial breast cancer and chest wall recurrence: a review of the randomised data. International Journal of Hyperthermia, v.26, p.612–617, 2010.

ZHANG, Z.; XIE, Z.; SUN, G.; YANG, P.; LI, J.; YANG, H.; XIAO, S.; LIU, Y.; QIU, H.; QIN, L.; ZHANG, C.; ZHANG, F.; SHAN, B. Reversing drug resistance of cisplatin by hsp90 inhibitors in human ovarian cancer cells. *International Journal of Clinical and Experimental Medicine*, v.8, p.6687-701, 2015.

ZHOU, J. Z.; RIQUELME, M. A.; GAO, X.; ELLIES, L. G.; SUN, L. Z.; JIANG, J. X. Differential impact of adenosine nucleotides released by osteocytes on breast cancer growth and bonoe metastasis. *Oncogene*, v.34, p.1831-42, 2014.

ZHOU, L.; QI, X.; POTASHKIN, J. A.; ABDUL-KARIM, F. W.; GORODESKI, G. I. MicroRNAs miR-186 and miR-150 down-regulate expression of the pro-apoptotic purinergic P2X7 receptor by activation of instability sites at the 3'-untranslated region of the gene that decrease steady-state levels of the transcript. *The Journal of Biological Chemistry*, v.283, p.28274–28286, 2008.