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

Influência do tratamento com imatinibe sobre a sinalização purinérgica em linhagem celular de Leucemia Mielóide Crônica e avaliação do efeito citotóxico de sua combinação com derivado do ácido betulínico

JÚLIA BIZ WILLIG

PORTO ALEGRE, 2019

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RESUMO

A leucemia mielóide crônica (LMC) é uma neoplasia mieloproliferativa caracterizada pela oncoproteína BCR-ABL. A primeira linha de tratamento é o mesilato de imatinibe, o qual atua como um inibidor seletivo da oncoproteína com diversos casos descritos de resistência e baixa aderência. Considerando-se a importância do ATP no desenvolvimento tumoral e o mecanismo de ação do mesilato de imatinibe, no capítulo I desse trabalho avaliamos a influência do tratamento com mesilato de imatinibe sobre o sistema purinérgico de células K-562 sensíveis e resistentes ao imatinibe através da hidrólise dos nucleotídeos de adenina e UDP, bem como da expressão das *ENTPDs*. Para suplantar as dificuldades enfrentadas pelo uso do fármaco, tem se intensificado a busca por novas moléculas baseadas em produtos naturais ou semissintéticos. Nesse contexto, no capítulo II verificamos a atividade citotóxica e possíveis mecanismos de ação do composto derivado do ácido betulínico nas linhagens celulares K-562, bem como o efeito da combinação do mesilato de imatinibe com este composto. De acordo com nossos resultados, a linhagem K-562 apresenta a expressão de todas *ENTPDs* e *NT5E*. No entanto, quando tratada com mesilato de imatinibe por 24 horas houve um aumento nas expressões de *ENTPD1, ENTPD2, ENTPD3* e *ENTPD5*, juntamente com o aumento da atividade de hidrólise, que poderia ser causada pela ativação dessas enzimas em resposta ao acúmulo de ATP induzido por imatinibe. Em células K-562 resistentes ao imatinibe ocorreu a diminuição da expressão de *ENTPD1* e *ENTPD5,* e hidrólise de nucleotídeos. Na avaliação do composto derivado, observou-se que o mesmo apresentou citotoxicidade nas células sensíveis e resistentes. A apoptose observada foi desencadeada pela via intrínseca com ativação das caspases 3 e 9 e o ciclo celular mostrou parada em G0/G1. Além disso, o derivado levou ao aumento da produção de espécies reativas de oxigênio e à indução da autofagia, aumentando também a expressão dos genes de LC3II e Beclin-1. Nesse contexto, esse estudo fornece evidências em relação à importância e influência do imatinibe sobre a sinalização purinérgica e abre novas perspectivas para possíveis opções de tratamento como agente adjuvante na LMC. **Palavra chaves:** Leucemia mieloide crônica, sinalização purinérgica, NTPDases, mesilato de imatinibe, derivado do ácido betulínico, sinergismo.

ABSTRACT

Chronic myeloid leukemia (CML) is a myeloproliferative disease characterized by the BCR-ABL oncoprotein. The first line of treatment is imatinib mesylate, which acts as a selective inhibitor of the oncoprotein, though several cases of resistance and low adherence are already described. Considering the importance of ATP in tumor development and the mechanism of action of imatinib mesylate, in chapter I of this work we evaluated the influence of treatment with imatinib mesylate on the hydrolysis of adenine and UDP nucleotides, as well as on the expression of *ENTPDs* in imatinib-sensitive and -resistant K-562 cells. To overcome the difficulties faced using the drug, the search for new molecules based on natural or semi-synthetic products has intensified. In this context, in chapter II we verified the cytotoxic activity and possible mechanisms of action of the betulinic acid derivative in the K-562 cell lines, as well as the effect of the combination of imatinib mesylate and this derivative compound. According to our results, K-562 lineage presents the expression of all *ENTPDs* and *NT5E*. However, when treated with imatinib mesylate for 24 hours there was an increase in the expressions of *ENTPD1*, *ENTPD2*, *ENTPD3* and *ENTPD5*, together with increased hydrolysis activity, which could be caused by the activation of these enzymes in response to the accumulation of ATP induced by imatinib mesylate. In imatinib-resistant K-562 cells the expression of *ENTPD1* and *ENTPD5* and nucleotide hydrolysis decreased. In the evaluation of the derivative compound, it showed cytotoxicity in the sensitive and resistant cells. The observed apoptosis was triggered by the intrinsic pathway with activation of caspases 3 and 9, and the cell cycle showed a G0/G1 arrest. In addition, the derivative led to an increase in the production of reactive oxygen species and the induction of autophagy, also increasing the levels of proteins LC3II and Beclin-1. In this context, this study provides evidence regarding the importance and influence of imatinib mesylate on purinergic signaling and opens new perspectives for possible treatment options as an adjuvant agent for CML.

Key words: Chronic myeloid leukemia, purinergic signaling, NTPDases, imatinib mesylate, betulinic acid derivative, synergism.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

ABL – *Abelson leukemia Vírus*

AB – Ácido Betulínico

AP – Ecto fosfatase alcalina

ADA – Adenosina deaminase

ADP- Adenosina difosfato

AMP - Adenosina monofosfato

ATP- Adenosina trifosfato

BCR – *Breakpoint Cluster Region*

CI – Índice de combinação

CMSP – Células mononucleares de sangue periférico

CD39 – Ectonucleósideo trifosdato difosfohidrolase 1

CD73 – Ecto-5'nucleotidases

DNA – Ácido Desoxirribonucleico

FDA – *Food and Drug Administration*

FISH – Hibridização *in situ* Fluorescente

HPLC – *High Performance Liquid Cromatography*

INCA – Instituto Nacional do Câncer

IARC – *International Agency for Research on Cancer*

ITQ – Inibidores de tirosina quinase

K-562 – Linhagem celular humana de leucemia mielóide crônica

K-562R – Linhagem celular humana de leucemia mieloide crônica com resistencia induzida ao imatinibe

LLA – Leucemia Linfocítica Aguda

LLC – Leucemia Linfocítica Crônica

LMC – Leucemia Mielóide Crônica

LSCs – *Leukemic stem cells* (Células tronco leucêmicas quiescentes)

NTPDases – Ectonucleosideo trifosfato difosfohidrolase

NPPs – Ectonucleotídeo pirofosfatase/fosfodiesterases

Ph+ - Cromossomo *Philadelphia*

PDGR – *Platelet-derived growth receptor* (Receptor de crescimento derivado de plaqueta)

PKC – *Protein Kinase C* (Proteína Quinase C)

RNA – Ácido Ribonucleico

SCF – *Stem Cell Factor* (Fator estimulante das células germinativas)

UDP – Uridina difosfato

UTP – Uridina trifosfato

SUMÁRIO

I. INTRODUÇÃO

I.1 Leucemia Mielóide Crônica

As leucemias são um conjunto de doenças malignas que afetam principalmente os glóbulos brancos, geralmente de origem desconhecida, que têm como principal característica o acúmulo de células anormais na medula óssea (INCA, 2018). Além do amadurecimento anormal das células, elas são capazes de se dividir mais rapidamente que o normal, e escapar dos processos de morte celular. As células leucêmicas deixam a medula óssea e entram na corrente sanguínea, fazendo com que o número de glóbulos brancos aumente no sangue (AMERICAN CANCER SOCIETY, 2016). Segundo dados da IARC (do inglês, *International Agency for Research on Cancer*), são estimados 437.033 novos casos de leucemias no mundo para o ano de 2018 (IARC, 2018). No Brasil, a estimativa é de 10.800 casos para o biênio 2018-2019 (INCA, 2018).

A Leucemia Mielóide Crônica (LMC) é uma neoplasia hematológica mieloproliferativa caracterizada pelo acúmulo excessivo de eritrócitos, granulócitos e plaquetas na medula óssea, sangue periférico e tecidos (WAGLE et al., 2016; JABBOUR E. et al.,2016). Nos Estados Unidos são esperados 8.430 novos casos de LMC para o ano de 2018 (SIEGEL; MILLER; JEMAL, 2017) e dados para o Brasil ainda são desconhecidos. A principal característica dessa doença é formação do cromossomo Philadelphia (Ph+) através da fusão do gene ABL (do inglês, *Abelson leukemia virus*), na região q34, no cromossomo 9 com o gene BCR (do inglês, *breakpoint cluster region*), na região q11 do cromossomo 22, gerando um gene híbrido BCR-ABL t(9;22)(q34;q11) (TAMASCAR; RAMANARAYANAN, 2009; ZHOU; MEDEIROS; HU, 2018) (Figura 1). Essa anomalia citogenética resulta numa oncoproteína que possui atividade tirosina quinase desregulada a qual interage com diversas vias de sinalização envolvidas na sobrevivência celular, inibição de apoptose e ativação de fatores de transcrição como JAK/STAT, PI3K/AKT e RAS/MEK (SINCLAIR; LATIF; HOLYOAKE, 2013; HOLYOAKE; VETRIE, 2017). A identificação do cromossomo Ph+ é realizada por análise molecular convencional ou por hibridização *in situ* com fluorescência (FISH) e está presente em 95% dos pacientes com LMC (ZHOU; MEDEIROS; HU, 2018).

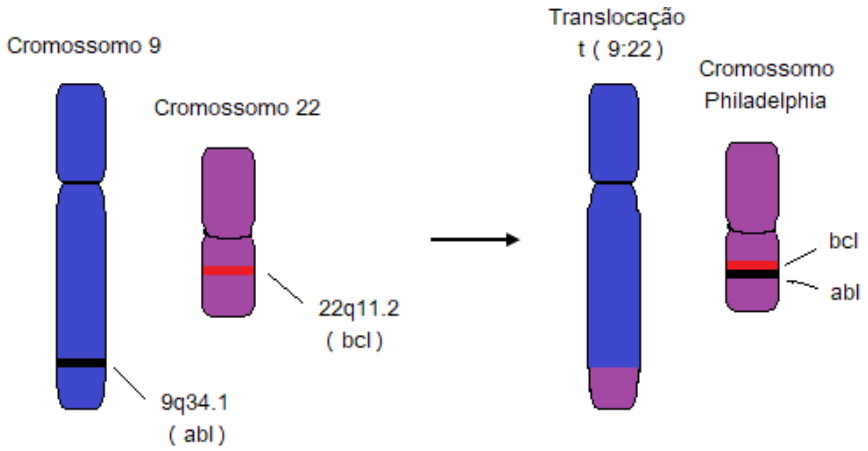

Figura 1. Translocação do cromossomo 9 e 22, com a formação do cromossomo Philadelphia. Construção da figura foi obtida através do site: https://smart.servier.com/.

A LMC apresenta três fases distintas de evolução da doença. A fase crônica, etapa onde a maioria dos pacientes é diagnosticada, tem uma progressão lenta, marcada por hiperplasia medular e capacidade de maturação das células mielóides. Essa etapa apresenta os inibidores de tirosina quinase (ITQ) como terapia de escolha. Na fase acelerada, ocorre uma transição gradual da fase crônica para a blástica, com uma duração média de 2 a 15 meses, e se caracteriza por um aumento do número de blastos no sangue periférico (10-19%), leucocitose, basofilia (>20%) e trombocitopenia. Essa fase é importante no prognóstico individual, pois necessita de intervenções terapêuticas mais agressivas. Na fase crise blástica, acontece a agudização da leucemia, na qual a doença adquire atributos de uma leucemia aguda, presença maior de 20% de blastos no sangue periférico ou medula óssea (VARDIMAN; HARRIS; BRUNNING, 2002).

I.2 Tratamento da Leucemia Mieloide Crônica

Os primeiros tratamentos para LMC incluíam quimioterápicos, como bussulfan e hidroxiureia, que eram empregados somente como tratamento paliativo para os sintomas, pois melhoravam a qualidade de vida dos pacientes, mas sem curá-los. Com o passar do tempo, outras opções de tratamento surgiram, como interferon-α, inibidores de tirosina quinase (ITQ) e transplante de células tronco hematopoéticas, sendo este último a única forma de curar a doença (TAMASCAR; RAMANARAYANAN, 2009). A introdução dos ITQ

modificou drasticamente o tratamento dos pacientes com LMC melhorando a taxa de sobrevida em aproximadamente 10 anos (CORTES; REA; LIPTON, 2018).

O primeiro ITQ aprovado pelo *Food and Drug Administration* (FDA), mesilato de imatinibe (Glivec®) (figura 2), consiste na primeira linha de tratamento para LMC.

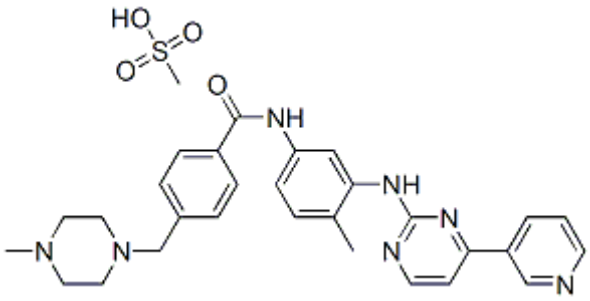

Figura 2. Estrutura química do Mesilato de Imatinibe. Construção da figura foi obtida através www.chemistry.com.

Seu mecanismo de ação ocorre através da competição pelo receptor celular de ATP no domínio tirosina quinase de ABL, impedindo a habilidade desse cromossomo em transferir grupos fosfato do ATP e resíduos de tirosina fosforilada, o que previne a transdução de sinais para a proliferação celular e apoptose (MITCHELL et al., 2012) (Figura 3). Este medicamento inibe outras proteínas envolvidas na sinalização, como o receptor de crescimento derivado de plaquetas (PDGR) e fator estimulante das células germinativas pluripotentes (SCF), mas não inibe outras tirosinas quinases, como as proteínas que apresentam mutação em T315I de Abl, uma das responsáveis pela resistência terapêutica (DEININGER et al., 2013).

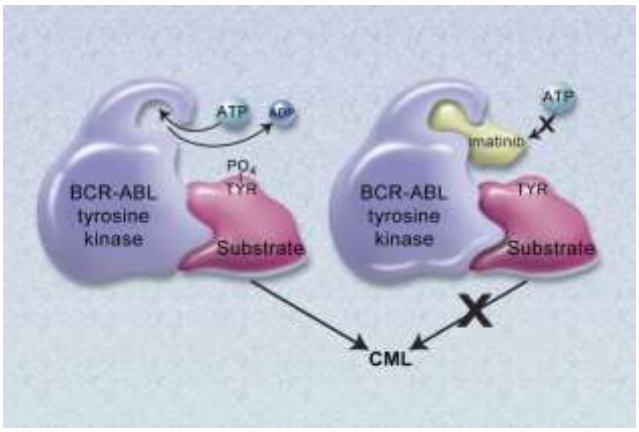

Figura 3. Mecanismo de ação do mesilato de imatinibe. O fármaco se liga ao sítio de ligação do ATP da tirosina-quinase BCR-ABL bloqueando a ligação do ATP e consequentemente inibe a atividade quinase de BCR-ABL. Cópia autorizada por *Blood Journal*, número da licença 501447836.

O objetivo do tratamento com mesilato de imatinibe compreende em alcançar uma resposta hematológica completa (contagem de plaquetas <450.000/mm^3, leucócitos <10.000/mm^3 sem granulócitos imaturos e menos de 5% de basófilos) aos 3 primeiros meses de tratamento, seguida de resposta citogenética maior (0-35% de cromossomos Ph+ detectáveis) ou completa (cromossomos Ph+ não detectáveis na MO) aos 6 meses e por fim, resposta molecular completa, sem transcritos bcr/abl não-detectáveis com 12 a 18 meses de tratamento. Se ao longo do tratamento o paciente não atingir essas condições, alternativas são necessárias, como a utilização de ITQ de segunda geração, desatinibe, nilotinibe e bosutinibe ou terceira geração, ponatinibe (ROSSARI; MINUTOLO; ORCIUOLO, 2018).

Contudo, a única forma de tratamento capaz de curar pacientes com LMC é o transplante alogênico, o qual tem como objetivo erradicar o clone maligno e reestabelecer a hematopoese normal através da infusão de medula óssea ou de células-tronco hematopoiéticas normais de doadores sadios (GRIBBEN, 2018). Entretanto, esse tipo de tratamento ainda apresenta elevadas taxas de morbimortalidades (PAVKOVIC; ANGELKOVIC; POPOVA-SIMJANOVSKA, 2015).

I.3 Resistência Terapêutica

Apesar do grande avanço no tratamento da LMC com a introdução dos ITQ, 25% dos pacientes apresentam resistência terapêutica (ARRIGONI et al., 2018), que pode ser classificada como primária ou secundária. Na resistência primária, o paciente apresenta ausência de resposta ao tratamento, além de apresentar mecanismos que são ditos como independentes de BCR-ABL, onde a proteína oncogênica não está inibida de forma eficaz ou apresenta mutações secundárias que causem a proliferação celular. Na resistência secundária, também conhecida como resistência adquirida ou ainda BCR-ABL dependente, o paciente apresenta uma resposta inicial, mas em seguida torna-se refratário à terapia. As causas da resistência ainda não estão completamente esclarecidas, entretanto diversos estudos relacionam com mutações de ponto no domínio quinase de BCR-ABL (ARRIGONI et al., 2018), superexpressão do gene BCR-ABL, superexpressão de transportadores de membrana ABC, como Glicoproteína-P/MDR1 ou ABCG2 (GOTTESMAN; FOJO; BATES, 2002; ILLMER et al., 2004 BEGICEVIC; FALASCA, 2017) além de modificações epigenéticas como acetilação ou metilação de histonas, metilação de DNA e presença de miRNAS (KOSCHMIEDER; VETRIE, 2018).

O tratamento com os ITQ não erradica as células-tronco leucêmicas quiescentes (LSCs), fazendo com que essas modifiquem suas características fenotípicas e genotípicas ao longo do tratamento. As células-tronco são células indiferenciadas capazes de promover proliferação descontrolada e auto-renovação, apresentam marcadores de adesão celular (CD34, CXCR4, CD44, CD133) (ARRIGONI et al., 2018; CURTARELLI et al., 2018), marcadores de pluripotência (OCT4, Nanog, Sox2) que são influenciados também pelo tratamento contínuo com imatinibe (BONO; DELLO SBARBA; LULLI, 2018).

I.4 Sistema purinérigo

O sistema purinérgico é um sistema de sinalização celular mediado principalmente por nucleotídeos e nucleosídeos, enzimas e receptores nas quais interajam entre si e desempenham diversas funções celulares (BURNSTOCK; DI VIRGILIO, 2013). Os nucleotídeos antigamente eram conhecidos por apresentar ação intracelular, entretanto atualmente sabe-se que essas moléculas são capazes de agir também no meio

extracelular, atuando como mensageiros extracelulares. As moléculas de purinas (ATP, ADP e adenosina) e pirimidinas (UTP e UDP) que são responsáveis por essas funções. Esses nucleotídeos podem ser liberados para o meio extracelular através de mecanismos fisiológicos como abertura de canais de membrana, via transportadores, exocitose ou também por difusão (DI VIRGILIO et al., 2018). O ATP extracelular pode exercer duas funções no meio extracelular: ser substrato de enzimas que metabolizam os nucleotídeos (ectonucleotidases) ou interagir com seus respectivos receptores (Figura 4).

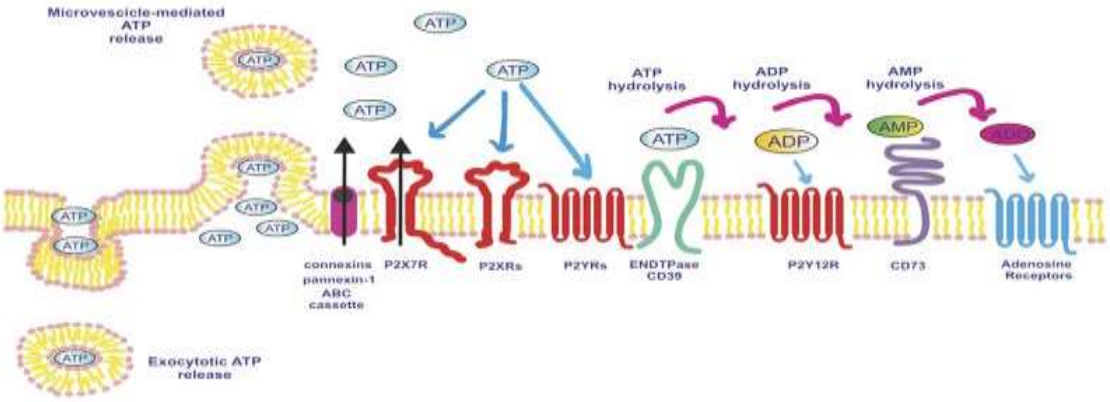

Figura 4. Figura esquemática da sinalização purinérica. Os nucleotídeos são liberados para o meio extracelular através de canais de membrana, via transportadores, exocitose ou também por difusão. O ATP extracelular pode ser substrato para as enzimas que metabolizam nucleotídeos (ectonucleotidases) (DI VIRGILIO; ADINOLFI, 2017). Imagem sob licença de *Creative Commons*, no qual atribuições não comerciais/educacionais não precisam de permissão adicional pela editora Springer Nature.

Os nucleotídeos interagem através da ligação com receptores purinérgicos P2. Os receptores P2 se subdividem em duas classes: receptores P2X na qual são subclassificados de 1 a 7 e são ligados a canais iônicos e P2Y na qual já foram identificados 8 subtipos, 1,2,4,6,11 a 14 onde são acoplados à proteína G. Os receptores P2Y são ativados por nucleotídeos de adenina e uracila, entretanto os receptores P2X são ativados somente por ATP.

Além da ligação com receptores, a concentração de nucleotídeo extracelular também é controlada por múltiplas ações da família das enzimas ectonucleotidases. Essa família é constituída por NTPDases (ecto-nucleosídeo trifosfato difosfohidrolase), as

quais hidrolisam nucleotídeos tri e difosfatados; NPPS (ecto-nucleotídeo pirofosfatase/fosfodiesterase); AP (ecto-fosfatase alcalina) e enzima ecto-5'nucleotidase (CD73), que hidrolisa o AMP gerado pela hidrólise de ATP e ADP formando adenosina. (ZIMMERMANN, 2001). A adenosina, entretanto, pode atuar tanto nos receptores purinérgicos P1, no qual se subdividem em A1, A2A, A2B e A3, ser captada para o interior das células por meio de transportadores ou ainda ser degrada pela enzima adenosina deaminase (ADA) em inosina (LISA GIULIANI; CLARA SARTI; DI VIRGILIO, 2018).

As NTPDases constituem uma família de 8 membros já clonados e caracterizados. Elas possuem cinco regiões altamente conservadas na sequência de aminoácidos relacionados com o sítio catalítico e apresentam dependência de cátions divalentes, como Ca^{2+} e Mg^{2+}, para sua atividade máxima. Dentro dessa família, as NTPDase1, -2, -3 e -8 possuem 2 domínios transmembrana e apresentam sítio catalítico voltado ao meio extracelular. A NTPDase1 hidrolisa ATP e ADP na mesma proporção, já NTPDase2 apresenta grande preferência pelo ATP. As NTPDase3 e -8 possuem preferências intermediárias de ATP, causando um leve acúmulo de difosfonucleosídeos. As NTPDase5 e -6 possuem somente um domínio transmembrana, estão localizadas no meio intracelular e podem ser clivadas proteoliticamente e liberadas para o meio extracelular. A NTPDase5 apresenta preferência pelos nucleotídeos difosfatados e baixa afinidade para degradação dos nucleotídeos trifostatados. As NTPDase4 e -7 estão localizadas exclusivamente no meio intracelular (ROBSON; SÉVIGNY; ZIMMERMANN, 2006). A ecto-5'nucleotidase (CD73) possui dois domínios transmembrana e é responsável pela degradação de AMP em seu respectivo nucleosídeo. Além disso, também tem sua atividade potencializada por cátions divalentes, principalmente por Mg^{2+}.

I.5 Câncer e NTPDases

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 neoplásicas apresentam a capacidade comum de ultrapassar as membranas dos tecidos e atingir órgãos distantes, processo conhecido como metástase (WHO, 2015). É nesse contexto de progressão tumoral que as NTPDases estão relacionadas, visto que essas enzimas estão envolvidas com processos biológicos como estimulação ou inibição de

morte celular, proliferação, migração, diferenciação, secreção de fatores de crescimento e quimiocinas inflamatórias (BURNSTOCK; DI VIRGILIO, 2013; DI VIRGILIO et al., 2018).

Diversos estudos têm avaliado o papel das NTPDases no câncer, dentre eles podemos citar: câncer gástrico (CAI et al., 2018), células de câncer cervical (BECKENKAMP et al., 2014), câncer de endométrio (ALIAGAS et al., 2014), tumores cerebrais (glioblastoma e meduloblastoma) (CAPPELLARI et al., 2012; XU et al., 2013), células tumorais de bexiga (STELLA et al., 2010), câncer na glândula da tireoide (BERTONI et al., 2018), câncer hepatocelular (CAI et al., 2016), entre outros. Em tumores hematológicos está descrito a participação das enzimas em leucemias linfocíticas crônicas e agudas (SCHETINGER et al., 2007).

A NTPDase1, também conhecida como CD39, está expressa tanto em células normais como células natural killer, células T ativadas e monócitos, tendo seu papel importante no sistema imunológico, além de apresentar diferentes níveis de expressão em células neoplásicas. Atualmente, a participação em conjunto das enzimas CD39 e CD73 está sendo investigada no sentindo de que o equilíbrio entre ATP/ADP e AMP/adenosina nas células tumorais possa influenciar o microambiente tumoral, gerando respostas imunossupressoras ou imunoativação (DI VIRGILIO et al., 2018). As NTPDase2 e NTPDase3 não apresentam muitos estudos avaliando seu papel nas neoplasias, entretanto em estudo realizado *in vivo* avaliou a superexpressão da NTPDase2 demonstrou que essa enzima está relacionada com o aumento do crescimento tumoral, além de modulação de produção de citocinas pró-inflamatórias e reativação plaquetária (BRAGANHOL et al., 2012). Já a NTPDase3 está expressa em células epiteliais, incluindo rins e bexiga, vias aéreas e sistema digestivo. No câncer está relacionada com progressão tumoral em células da bexiga, associada com a fase inicial do desenvolvimento tumoral (STELLA et al., 2010). A NTPDase5 também está expressa de forma alterada em neoplasias, apresentando níveis controversos dependendo do tipo celular envolvido. Em linhagens celulares tumorais de laringe, apresentam níveis elevados de expressão quando comparados com células saudáveis (BLÁNQUEZ et al., 2002). Entretanto, em células de tumor do cólon, à medida que a malignidade aumenta a expressão da NTPDase5 diminui (MIKULA et al., 2011). A NTPDase6 foi descrita por

apresentar expressão em tecidos musculares e cardíacos, entretanto sua principal referência na literatura está na avaliação desse gene *ENTPD6* como um supressor tumoral e sua influência no processo de resistência quimioterápica à cisplatina em câncer de testículo (TADA et al., 2011).

Atualmente, sabe-se que o ATP extracelular é um dos principais componentes do microambiente tumoral e através do desenvolvimento de sondas conseguiu-se demostrar que a concentração de ATP em tumores sólidos está na faixa de 100-500 µmol/L, o que é muito maior do que a concentração no interstício de tecidos saudáveis (10-100 nmol/L) (DI VIRGILIO et al., 2018). Entretanto, seu papel ainda é controverso na modulação do desenvolvimento do câncer, podendo ter ação antitumoral com características imunogênicas ou pró-tumoral, influenciando migração e proliferação das células tumorais. Sua influência no microambiente tumoral vai depender do mecanismo pelo qual esse nucleotídeo consegue interagir com células tumorais (ROGER et al., 2015).

I.6 Tumores Hematológicos e NTPDases

Em tumores hematológicos, o papel da CD39 e CD73 parece estar relacionado com desenvolvimento tumoral em Leucemia Linfocítica Aguda (LLA) e Leucemia Linfocítica Crônica (LLC) (DULPHY et al., 2014; SCHETINGER et al., 2007). Várias publicações têm associado a expressão de CD39 e CD73 à produção de adenosina, criando um ambiente imunossupressor, no qual linfócitos T helper inibem respostas imunes contra o tumor (ALLARD; CHROBAK; STAGG, 2015; VAISITTI; ARRUGA; DEAGLIO, 2018). Cai e colaboradores correlacionaram também a produção de adenosina com a resistência quimioterápica, sugerindo que a adenosina protegeria as células tumorais de sofrerem apoptose (CAI; FENG; WANG, 2018). Estudos mais detalhados correlacionaram a expressão de CD39 com estágios avançados e desfecho clínico negativo em pacientes com LLC, além de associarem a expressão dessa enzima com marcadores já característicos da doença, como CD38 e ZAP-70 (ABOUSAMRA et al., 2015).

A expressão de CD73 também foi avaliada em células nucleadas da medula óssea em vários subtipos de leucemias, no qual a expressão estava aumentada em células de pacientes com LLA do tipo B e LLA pré-B. A expressão da enzima foi associada ao

subtipo, diferenciação e desenvolvimento das leucemias (Zhao Sx et al., 2011). Wang e colaboradores sugeriram que CD73 seria um marcador de monitoramento de doença residual mínima para pacientes com LLA do tipo B (WANG et al., 2016). O impacto das ectonucleotidases também já foi relacionado com a rejeição de aloenxertos e doença do enxerto contra hospedeiro (CHERNOGOROVA; ZEISER, 2012).

I.7 Sinalização purinérgica como biomarcador e alvo terapêutico

Tendo em vista a relação do sistema purinérgico na carcinogênese, esse sistema vem sendo investigado como possível biomarcador e alvo terapêutico em diversos tipos tumorais (ABOUSAMRA et al., 2015; CAI et al., 2016; MOSAAD ZAKI et al., 2018; PAN et al., 2011; WANG et al., 2016). Por exemplo, o nível de ATP em carcinoma de mama foi proposto para detectar níveis de proliferação celular e, portanto, usado como um marcador de agressividade e potencial metastático, sugerindo que o nível de ATP poderia representar um biomarcador de diagnóstico, prognóstico e alvo terapêutico no câncer de mama (PAN et al., 2011).

Vários modelos de estudo mostram a influência da adenosina na imunossupressão no qual o bloqueio das enzimas CD39 e CD73, pelo uso de inibidores ou anticorpos monoclonais, parece ser uma estratégia terapêutica para controlar o equilíbrio dos nucleotídeos e a consequência deles para o microambiente tumoral (GAO; DONG; ZHANG, 2014; ALLARD; CHROBAK; STAGG, 2015; DI VIRGILIO et al., 2018). Todavia, na LMC o sistema purinérgico ainda precisa ser melhor estudado, apesar de existirem indícios que a expressão de receptores de adenosina A3Rs possam ser correlacionados com a progressão da doença (DI VIRGILIO; ADINOLFI, 2017).

I.7 Produtos Naturais

Os produtos naturais são utilizados há séculos na medicina e apresentam elevado potencial terapêutico. Além de serem empregados na medicina popular, inúmeros trabalhos consistentes mostram sua grande importância nas áreas de câncer e doenças infecciosas (CRAGG; PEZZUTO, 2016)

Atualmente, fármacos de origem natural e seus derivados semissintéticos são usados na terapia contra o câncer, como por exemplo, taxol e derivados semissintéticos

(docetaxel, paclitaxel), alcalóides da vinca (vimblastina, vincristina), podofilotoxinas e análogos (etoposídeo e teniposídeo) e camptotecina e seus derivados (topotecano e irinotecano), representando cerca de 60% dos fármacos utilizados na quimioterapia (BALUNAS et al., 2008; CRAWFORD, 2013).

Nosso grupo de pesquisa tem se dedicado a entender a influência de antineoplásicos sobre o sistema purinérgico, em especial em modelos celulares leucêmicos. Além disso, vem realizando colaborações com outros grupos que apresentam elevada experiência na semissíntese a partir de produtos naturais, visando a busca de novas moléculas ativas com capacidade antitumoral. Nesse contexto, também nos propomos a investigar o efeito *in vitro* na terapia antineoplásica de LMC de um composto derivado do ácido betulínico (AB), no qual foi adicionado um anel hidroxietil-2-hidroxi-fenólico na posição C-28 através de uma ligação éster (Figura 4).

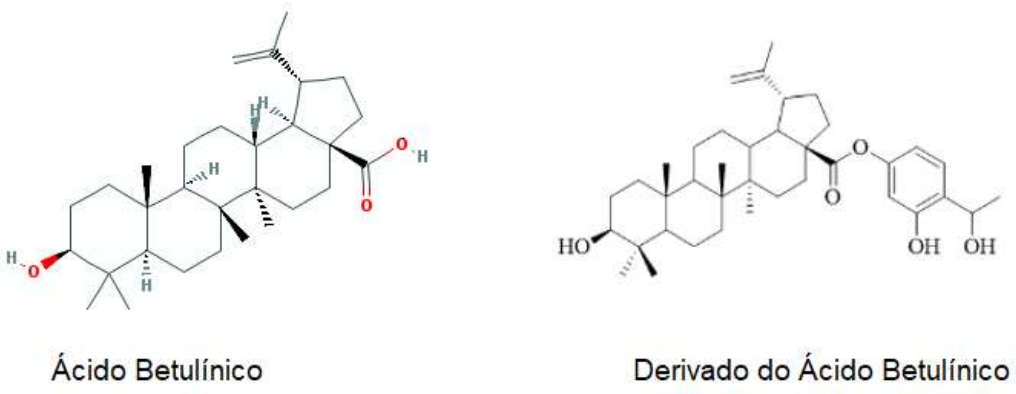

Figura 5. Esquema química do Ácido Betulínico e seu derivado. Construção da figura foi obtida através www.chemistry.com.

O AB é um triterpeno pentacíclico encontrado em diversas plantas, frutos e vegetais, o qual apresenta diversas atividades terapêuticas já demonstradas como: anti-inflamatória, antiviral, antibacteriana, antimalárica, imunomoduladora, antidepressiva e anticâncer (KOMMERA et al., 2010; BAI et al., 2012; DA SILVA et al., 2013). No tratamento do câncer está descrito como um candidato promissor em diversas linhagens celulares e estudos *in vivo*: melanoma, neuroblastoma, glioblastoma, cólon,

hepatocelular, pulmão, próstata, mama, ovário e carcinoma cervical (FULDA, 2009). Seu principal mecanismo de ação ocorre através da indução da via de morte celular por apoptose (via extrínseca e/ou intrínseca), modulação de proteína quinase, inibição da DNA topoisomerase entre outros (FULDA, 2009). Na LMC, o AB e seus derivados semissintéticos demonstraram atividade nas células K-562 (Ph+) e a inibição de proteossoma humano 20S parece ser o principal alvo terapêutico (WAECHTER et al., 2017).

Segundo resultados preliminares, o composto derivado do AB apresentou atividade antitumoral frente a linhagens celulares de câncer de mama e câncer cervical (Couto NM., e colaboradores dados não publicados). Nesse sentido, esses resultados promissores nos estimularam a investigar seu potencial frente a modelos de LMC.

I.8 Interação farmacológica

O uso de vários fármacos com mecanismos de ação diferentes pode direcionar o efeito para um único alvo ou doença resultando numa terapia mais eficaz. Existem duas classes diferentes de interação de fármacos: sinergismo e antagonismo.

O sinergismo é um tipo de interação obtida através da associação de dois ou mais medicamentos cujo resultado é maior do que a simples soma dos efeitos isolados de cada medicamento (CHOU; MARTIN, 2005). O sinergismo pode ser subdividido em aditivo, no qual os medicamentos possuem o mesmo mecanismo de ação, e somação, por agir em diferentes modos ou ainda por potencialização nos quais agem em diferentes receptores farmacológicos (BERENBAUM,M.C, 1977). Os resultados favoráveis incluem o aumento da eficácia do tratamento, a diminuição da dosagem, mantendo ou aumentando a eficácia, evitando toxicidade, minimizando ou diminuindo a resistência aos fármacos (CHOU, 2016). Já no antagonismo, a resposta farmacológica é suprimida ou reduzida na presença de outro fármaco, muitas vezes pela competição desses medicamentos pelo mesmo sítio de receptor (CHOU, 2016).

A combinação de imatinibe com outros agentes anticancerígenos tem sido descrita para contornar a resistência aos medicamentos e melhorar a resposta terapêutica no tratamento da LMC. Em linhagem celular K-562, o efeito do imatinibe combinado com cisplatina ou oxaliplatina demonstrou efeito sinérgico, aumentando a morte por apoptose

e o dano ao DNA (WEI; TO; AU-YEUNG, 2015). Além da associação de dois antineoplásicos, o sinergismo também está sendo avaliado para a possibilidade de combinação do imatinibe com compostos naturais. A criptotansinona, diterpeno quinoide, isolada das raízes de *Salvia militiorrhiza,* apresentou sinergismo com imatinibe aumentando a indução de morte celular por apoptose, modulando a expressão de proteínas pró-apoptóticas e diminuindo a expressão de BCR-ABL de maneira concentração-dependente (GE et al., 2015).

II. OBJETIVOS

Considerando a importância da sinalização purinérgica no desenvolvimento tumoral e o mecanismo de ação do mesilato de imatinibe sobre o sitio ativo de ligação do ATP na proteína BCR-ABL, o objetivo geral desse trabalho é avaliar o efeito do mesilato de imatinibe na caracterização da expressão e na funcionalidade de membros da família das NTPDases e ecto-5'nucleotidase (CD73) em linhagem celular K-562 (Ph+) e em uma fração de células mononucleares de sangue periférico, além da busca de novas moléculas para o tratamento da LMC.

Assim, os seguintes objetivos específicos foram propostos:

1- Identificar a expressão das NTPDases e ecto-5'nucleotidase (CD73);
2- Padronizar a hidrólise dos nucleotídeos de adenina e UDP para caracterizar a funcionalidade das enzimas purinérgicas em linhagem celular de LMC e em uma fração de células mononucleares;
3- Investigar o efeito do mesilato de imatinibe sobre a hidrólise dos nucleotídeos e expressão das NTPDases e ecto-5'nucleotidase (CD73);
4- Investigar o perfil das NTPDases em células já resistentes ao imatinibe;
5- Analisar o metabolismo do ATP através da análise de HPLC;
6- Avaliar a atividade antitumoral e possível mecanismo de ação de um composto derivado do ácido betulínico em linhagem celular de LMC, além da avaliação se sua interação farmacológica com mesilato de imatinibe.

Os resultados desta dissertação foram organizados em dois capítulos na forma de manuscritos distintos conforme figura 6.

Capítulo	Objetivo geral	Questões de Pesquisa	Objetivo específico	Métodos
• Capítulo I	• Caracterizar a expressão e funcionalidade das NTPDases e Ecto-5'nucleotidase em linhagem celular K-562 e K-562R.	• Qual a expressão das NTPDases na LMC? • Apresenta atividade de hidrólise dos nucleotídeos de adenina e UDP? • Em relação a células mononucleares de sangue periférico apresenta expressão e atividade diferente? • Qual o efeito do mesilato de imatinibe na expressão e atividade das NTPDases e em uma linhagem resistente ao imatinibe?	• Identificar a expressão das NTPDases e Ecto-5'nucleotidase (CD73); • Padronizar a hidrólise dos nucleotídeos de adenina e UDP para caracterizar a funcionalidade das enzimas purinérgicas em linhagem celular de LMC e em uma fração de células mononucleares; • Investigar o efeito do mesilato de imatinibe sobre a hidrólise dos nucleotídeos e expressão das NTPDases e ecto-5'nucleotidase (CD73); • Investigar o perfil das NTPDases em células já resistentes ao imatinibe;	• Indução da resistência ao imatinibe; • PCR convencional e qPCR; • Atividade de hidrólise – Verde de Malaquita; • Análise por HPLC.
Capítulo	Objetivo geral	Questões de Pesquisa	Objetivo específico	Métodos
• Capítulo II	• Investigar o mecanismo envolvido na ação citotóxica induzida por composto derivado do ácido betulínico em linhagem celular K-562 e K-562R.	• Como o composto derivado do ácido betulínico age sobre a linhagem celular de K-562 e K-562R? • Esse composto apresenta sinergismo com mesilato de imatinibe nas células K-562? • Qual o mecanismo de morte celular envolvido?	• Avaliar a citotoxicidade de um composto derivado do ácido betulínico em K-562 e K-562R; • Avaliar a interação entre composto derivado e mesilato de imatinibe em K-562; • Avaliar o mecanismo de morte envolvido.	• Citometria de fluxo – avaliação da citotoxidade *in vitro*; • Ensaio ciclo celular; • Ensaio com kit de anexina V/iodeto de propídio; • Ensaio com colaração de DAPI; • qPCR (caspases 3,8,9); • Ensaio com laranja de acridina; • qPCR (LC3-II e Beclin-1) • Ensaio com DCF-DA;

Figura 6: Estrutura de organização dos resultados da dissertação.

II. CAPÍTULO I

III. CAPÍTULO 1 – Júlia Biz Willig, Débora Renz Barreto Vianna, Aline Beckenkamp, Liziane Raquel Beckenkamp, Jean Sévigny, Márcia Rosangêla Wink, Andréia Buffon, Diogo André Pilger. Imatinib mesylate affects extracellular ATP catabolism and expression of NTPDases in a Chronic Myeloid Leukemia cell line

Manuscrito será submetido ao periódico Purinergic Signalling (3.190).

Imatinib mesylate affects extracellular ATP catabolism and expression of NTPDases in a Chronic Myeloid Leukemia cell line

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Abstract

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm, characterized by the occurrence of the t(9;22)(q34;q11) translocation. First line therapy for CML consists of treatment with imatinib mesylate, which selectively inhibits the BCR-ABL protein by competing for its ATP-binding site. Adenine nucleotide signaling is modulated by the ectonucleotidases and this pathway is related to tumorigenic processes. Considering the relationship between ATP and cancer, we aimed to evaluate the influence of imatinib mesylate on the expressions and functions of the NTPDase and ecto-5'-nucleotidase (CD73) enzymes in imatinib-sensitive and -resistant K-562 cell lines. Findings demonstrate that K-562 cells express all *ENTPDs* and *NT5E*. However, when treated with imatinib mesylate for 24 hours, expressions of *ENTPD1, -2, -3 and -5* increased, together with hydrolysis activity, possibly mediated by the activation of these enzymes in response to imatinib-induced ATP accumulation. HPLC analysis identified increased ATP degradation in cells after 24 hours of treatment, with consequent ADP and AMP formation. Imatinib-resistant K-562 cells presented decreased nucleotide hydrolysis and expressions of *ENTPD1* and *-5*, possibly due to the mechanism of action of imatinib mesylate at the ATP-binding site of the BCR-ABL protein and decreased intracellular ATP accumulation. Since these enzymes have important catalytic activities, the modulation of ectonucleotidases in CML cells may represent an important therapeutic approach to regulate levels of extracellular adenine nucleotides.

Keywords: Chronic Myeloid Leukemia, purinergic signaling, ectonucleotidases, imatinib mesylate, resistance.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm, characterized by the clonal expansion of pluripotent hematopoietic stem cells and leading to the accumulation of myeloid cells in the bone marrow and peripheral blood [1]. This disease, in most cases, is characterized by the presence of a specific cytogenetic abnormality, the Philadelphia chromosome (Ph^+), resulting from the t(9; 22)(q34; q11) translocation between the *ABL* oncogene on chromosome 9 and the *BCR* gene on chromosome 22 [2]. This chromosomal fusion results in an oncoprotein with potentialized and deregulated tyrosine kinase activity, responsible for the differentiation and proliferation of malignant cells [3].

The introduction of tyrosine kinase inhibitors (TKIs) substantially modified the treatment of patients with CML. Imatinib mesylate (Glivec®), the first tyrosine kinase inhibitor approved by the Food and Drug Administration (FDA), is the first line of treatment for CML [4]. It induces hematological remission in 99% of patients and a cytogenetic response in 74% after 12 months of treatment. Imatinib mesylate acts through competition for the ATP-binding site in the tyrosine kinase domain of ABL, inhibiting the ability of this protein to transfer ATP phosphate groups to tyrosine residues of target proteins, which is necessary for signal transduction for cell proliferation and apoptosis [5]. Despite the therapeutic success of target therapy, the occurrence of resistance to imatinib mesylate has led to the development of second- and third-generation TKIs. Several studies are investigating resistance to imatinib, however no specific mechanism has been identified; studies have, thus far, evaluated mutations in the oncoprotein, overexpression of resistance genes and have looked at efflux pumps [6–8] .

Extracellular adenine nucleotides, such as ATP (adenosine 5'-triphosphate) and ADP (adenosine 5'-diphosphate), act as signaling molecules through their binding to P2 purinergic receptors (P2X and P2Y subtypes) [9]. In cancer, adenine nucleotides are associated with several biological processes, such as growth factor production, secretion of inflammatory chemokines, stimulation or inhibition of cell death, cell differentiation,

migration and proliferation [10]. The role of nucleotides in the immune system is also under study, where they can act by modulating immunosuppression or immunoactivation [11]. The levels of these nucleotides are modulated by a hydrolysis cascade consisting of several enzymes, called the ectonucleotidases. NTPDases are a family of eight members that have already been cloned and characterized. NTPDase1 (CD39) hydrolyzes ATP and ADP at the same ratio, while NTPDase2 has a higher affinity for ATP. NTPDase3 and -8 have intermediate preference for ATP, causing a slight accumulation of diphosphonucleosides. In contrast, NTPDase5 and -6 present preference for the hydrolysis of nucleoside diphosphates. [12], while ecto-5'-nucleotidase (CD73) is responsible for the degradation of AMP to its respective nucleoside, adenosine [13].

A role for purinergic signaling has been reported in several types of cancers, such as in cervical cancer cells, gastric tumors, bladder tumor cells, and thyroid gland tumor, among others [14–17]. In hematologic malignancies, CD39 and CD73 appear to be associated with tumor development in acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL). CD73 expression has been evaluated in nucleated bone marrow cells in various subtypes of leukemias, where increased expression of this enzyme was observed in cells of patients with type B ALL. Enzyme expression of CD39 has been associated with the subtype, differentiation and development of leukemias [18–20].

Considering the importance of purinergic signaling in the development of cancer and the action of imatinib mesylate on the ATP-binding site in Ph+ leukemic cells, the objective of this study is to evaluate the influence of imatinib mesylate treatment on the expression and function of the NTPDases and CD73 in a human cell line derived from Ph+ CML (K-562). Expressions were compared with those of imatinib-resistant K-562 cells and with peripheral blood mononuclear cells (PBMNCs).

Materials and methods

Cell Culture

The human chronic myeloid leukemia cell line, K-562 (Ph^+), was obtained from the Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil). Peripheral blood mononuclear fraction cells (PBMNCs) from healthy donators were obtained by centrifuging peripheral blood over a Histopaque®-1077 (Sigma-Aldrich, USA) density gradient and used as control cells [21]. Cells were maintained in RPMI-1640 medium (Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (Life Technologies, USA), 0.5% penicillin/streptomycin (Sigma-Aldrich, USA) and 1% amphotericin B (Sigma-Aldrich, USA), at 37°C in an atmosphere of 5% CO_2. The development of the imatinib-resistant K-562 cell line (K-562 R) was adapted from methodology described by Wang et al., 2015 [22]. K-562 cells were exposed to increasing concentrations of imatinib mesylate, starting with 0.125 µM, which were doubled every 10 days until 10 µM. Every four weeks, the cells were exposed to different concentrations of imatinib mesylate (0-50.0µM) for resistance determination. Cell counts were performed on the FACSVerse™ cytometer equipped with a 488nm laser and flow sensor (BD Biosciences, USA). To maintain resistance, the cells were exposed weekly to 2µM of imatinib mesylate. This project was approved by Ethics Committee of UFRGS (n.1.979.570).

Determination of the IC_{50} of imatinib mesylate

K-562 cells were plated ($1x10^3$cells/well) in 96-well plates, and after 24 hours they were treated with different concentrations of Imatinib mesylate (0.1µM - 10.0µM) for 48 hours. Cell counts were performed by flow cytometry (FACSVerse™ Cytometer). The IC_{50} was determined in 48 hours (0.64µM). Imatinib mesylate was used in all experiments but the work concentration was determined considering the proportion of free base imatinib.

Cell treatments

Cells were seeded in culture flasks (25 cm^2), at densities of 1×10^6 cells/flask in a volume of 5 mL of culture medium. After 1 day, cells were treated or not with imatinib mesylate (0.64 µM) for 24 or 48 hours as described:

K-562 control group: cells maintained with culture medium only;
K-562 24h group: cells treated with imatinib mesylate for 24 hours;
K-562 48h group: cells treated with imatinib mesylate for 48 hours;
K-562R group: imatinib-resistant cells maintained with culture medium for the previous 3 days.
PBMNCs control group: cells maintained with culture medium only;
PBMNCs 24h group: cells treated with imatinib mesylate for 24 hours;
PBMNCs 48h group: cells treated with imatinib mesylate for 48 hours.

Determination of enzymatic activities

Specific activities of enzymes were determined by the measurement of the release of inorganic phosphate (Pi) using a colorimetric assay [23]. Cells from the treated and control groups were washed three times with the same incubation buffer and the Bradford method was used to determine the amount of protein [24]. Cell suspensions were diluted to a final protein concentration of 4µg/mL and fifty microliters were added to the reaction medium containing 120 mM NaCl, 5 mM KCl, 60 mM Glucose, 0.5 mM $CaCl_2$, 50 mM Tris-HCl, pH=8 (for ATP, ADP and UDP) and 120 mM NaCl, 5 mM KCl, 60 mM Glucose, 1 mM $MgCl_2$, 50 mM Tris-HCl, pH=8 (for AMP) and preincubated for 10 min at 37ºC. The reaction was started by the addition of ATP, ADP and UDP (final concentration of 3.0 mM) to determine the activity of the NTPDases, and AMP (final concentration of 3.0 mM) for the Ecto-5'nucleotidase (CD73) assay. The reaction was stopped after 60 min by the addition of 10% trichloroacetic acid (TCA). Samples were incubated on ice for 10 min prior to the inorganic phosphate (Pi) release assay using malachite green as a colorimetric reagent. To eliminate the non-enzymatic hydrolysis of the substrates, controls were carried out containing only the nucleotides and reaction buffers [25]. Before and after incubations, cell integrity was assessed using the dye exclusion method with trypan blue and no changes in cell viability were observed. Specific activity was expressed as nmol Pi/mg protein/min.

RT-PCR Analysis

Total RNA was isolated from cells using Trizol LS (Life Technologies, USA), according to the manufacturer's instructions. The cDNA was synthesized with M-MLV reverse transcriptase (Invitrogen, Brazil) from 5µg of total RNA in a total volume of 25µl with a random hexamer primer, according to the manufacturer's instructions. Glyceraldehyde-3- phosphate dehydrogenase (*GAPDH*) amplification was used as an endogenous control. The PCR contained 1.0µL of the RT reaction product (cDNA), 1.0µL of each primer pair and 1.25 units of Taq DNA Polymerase (Invitrogen, Brazil) in a final volume of 25 µL. The primers used in this study are described in Table S1. The PCR was run for 35 cycles and the cycling conditions were as follows: 1min at 95°C, 1 min at 94°C, 1 min at annealing temperature, 1 min at 72°C and a final 10 min extension at 72°C. The PCR product was analyzed on a 1.5% agarose gel containing GelRed® (Biotium, USA) and visualized under ultraviolet light. Non-template controls were performed with distilled water as a template and for positive controls cell lines that had previously been described in the literature as expressing *ENTPDs* and *NT5E* [14]. The HaCaT cell line was used as the control for *ENTPD 1,3, 6* and *NT5E* and the SiHa cell line for *ENTPD2* and *5*. Semi-quantification was performed through ImageJ software and expression by the ratio between marker expression/GAPDH.

Real-time RT-PCR (RT-qPCR)

Total RNA and cDNA were generated as described in RT-PCR analysis. All SYBR Green I-based real-time PCR mixtures were prepared using the GoTaq® qPCR Master Mix (Promega, USA), following the manufacturer's recommendation. One µL of cDNA was diluted to a final volume of 25µL in the reaction mix using Rotor-Gene Q (Qiagen, Germany). Reaction conditions were; 95°C for 1 min and 40 cycles of 10 s at 95°C, 15 s at 60°C or 63°C, and 20 s at 72°C. For the relative quantification ($2^{-\Delta\Delta CT}$) of *ENTPDs* and *NT5E*, real-time PCR were performed in duplicate using *GAPDH* as the endogenous control.

HPLC analysis

Cell suspensions were diluted to a final protein concentration of 4µg/mL and fifty microliters were added to the reaction medium and preincubated for 10 min at 37ºC. The reaction was started by the addition of ATP (100 µMol). To stop the reaction, the tube was transferred to ice and centrifuged at 4ºC for 30 min at 16 000g. Aliquots of 20µl were applied to a reverse phase HPLC system using a C18 Shimadzu column (Shimadzu, Japan) with absorbance measured at 250 nm. The mobile phase was 60mM KH_2PO_4, 5mM tetrabutyl ammonium chloride, pH 5.0 in methanol 30%. Retention times were assessed using standard samples of nucleotide and purines and concentrations are expressed as µMol of nucleotide (mean ± S.E.).

Flow cytometry

After treatment with imatinib mesylate, $1x10^6$ cells were centrifuged for 5min at 500xg and washed twice with phosphate buffered saline (PBS) plus 1% bovine serum albumin (BSA). The pellets were suspended and incubated for 45 min with primary antibodies (mouse or rabbit polyclonal anti-human antibodies) against NTPDase1 (hN1-8L5), NTPDase2 (hN2-2L5) and NTPDase3 (hN3H10s) (1:200 dilution; http://www.ectonucleotidases-ab.com), followed by incubation with a FITC-conjugated anti-rabbit or Alexa Fluor-conjugated anti-mouse secondary antibody (Life Technologies, USA) for 30 min. These antibodies were obtained from ectonucleotidases-ab, Université Laval, Québec, QC, Canada, and the specificity was previously characterized [26, 27]. Ecto-5'nucleotidase expression analysis used 1µL mouse anti-human CD73-PE (559257-BD). After incubation, cells were centrifuged and washed twice with PBS. The same number of cells was incubated with secondary antibody as a control. All samples were analyzed using a BD FACSVerse™ cytometer.

Statistical Analysis

Results were analyzed by one-way analysis of variance (ANOVA), followed by Tukey test (GraphPad Prism v5.0 software). Values were considered significant when $p < 0.05$.

Results

Induction of resistance to Imatinib mesylate

Acquired resistance to imatinib mesylate was determined after 6 months of continuous exposure to the drug, as described in material and methods. A dose-response curve was performed with different concentrations of imatinib mesylate (0.1 μM to 50.0 μM) and the IC_{50} was determined for both K-562 sensitive and imatinib-resistant (K-562R) cells. The IC_{50} of imatinib mesylate was 0.64 μM and 41.01 μM in K-562 sensitive and resistant cells, respectively.

The final IC_{50} for imatinib was 64.08-fold higher for K-562R than for sensitive K-562 (Fig. 1a). As the exposure to imatinib mesylate increased, a change in the phenotypic profile of K-562 cells was observed (Fig. 1b). K-562R cells acquired a spheroid morphology and started to grow in clusters. In order to confirm this resistant profile, the expression of differentiation markers, cell surface markers and the expressions of multidrug resistance genes were evaluated. The K-562R cell line showed increased expression of the major specific markers of undifferentiation, such as *Nanog* and *SOX-2*, and adhesion markers such as *CD44* and *CD133* (Fig. 1c and 1d). In addition, resistance to imatinib altered the expressions of the *ABCB1/MDR1* and *ABCG2* genes, responsible for drug efflux pumps production.

Ectonucleotidase activity

The ability of cells to hydrolyze adenine nucleotides and UDP was determined for the K-562 tumor line and compared that of PBMNCs (Table 1). Results demonstrate a significant increase in the hydrolysis activity of all the nucleotides tested in the tumor line, compared to PBMNCs cells. In order to evaluate the influence of imatinib on the hydrolysis of adenine nucleotides and UDP, cells were treated with imatinib mesylate for 24 and 48 hours (Table 2). After 24 hours of treatment, there was an increase in the hydrolysis of ATP, ADP, AMP and UDP, when compared to K-562 cells without imatinib mesylate. After 48 hours of treatment, this activity decreased in relation to the 24-hour treatment. The hydrolysis of UDP in 48 hours was significantly decreased when compared to the hydrolysis in control K-562.

We also evaluated whether resistance to imatinib could change the hydrolysis of nucleotides. Decreases in the hydrolysis of ATP, ADP and UDP were observed in K-562R, when compared to the K-562 cell line. In addition, the increase in enzymatic activity in K-562, observed after treatment with imatinib mesylate, did not occur in PBMNCs, since the effects of imatinib mesylate are dependent on the binding of this drug to the active site of BCR- ABL, which is not present in PBMNCs (Table S2).

Gene expressions of *ENTPDs* and *NT5E*

To confirm the difference found in nucleotide hydrolysis, levels of *ENTPD* and *NT5E* mRNAs were determined. The K-562 cell line expressed all the enzymes investigated, furthermore, *ENTPD 2,3,5* and *6* present higher expressions in K-562, when compared to PBMNCs (Figure S1), corroborating results found for nucleotide hydrolysis activities (Table 1). In order to analyze the effect of treatment with imatinib mesylate on the expressions of *ENTPDs* and *NT5E*, screening was performed using RT-PCR to observe which enzymes were influenced (Fig. 2a). RT-PCR demonstrated that treatment with imatinib mesylate modulated the expressions of the *ENTPD1, ENTPD2, ENTPD3, ENTPD5* and *NT5E* genes and consequently these genes were analyzed by RT-qPCR. To confirm this observation, the mRNA was quantified using the $2^{-\Delta\Delta CT}$ method, where the expression was compared to the untreated K-562 cells. Figure 2b shows that after 24 hours of treatment with imatinib mesylate, increases in the gene expressions of *ENTPD1*, *ENTPD2*, *ENTPD3*, *ENTPD5* and *NT5E* were observed in relation to control cells. After 48 hours of treatment with imatinib mesylate, only the expression of *ENTPD5* was significantly reduced, consistent with the alteration in the hydrolysis rate of UDP at 48 hours. The expressions of *ENTPD1* and *ENTPD5* were decreased in K-562R cells.

HPLC analysis

The extracellular ATP metabolism profiles were similar in the K-562 and K-562R cells and were similar in K-562 cells treated with imatinib mesylate for 24 and 48 hours (Fig. 3a). ADP and AMP formation occurred in all groups tested. During treatment with imatinib mesylate, ADP (27.05 µM ± 1.27) and AMP (4.56 µM ± 0.04) increased in K-562

at 24 hours, in comparison with K-562 (ADP = 14.96 ± 0.57 and AMP = 1.35 ± 0.02), especially at 60 min (Fig. 3b). In contrast, the K-562R cells presented a slight reduction in the concentration of nucleotides formed (ADP = 12.96 µM ± 0.77 and AMP = 1.10 µM ± 0.44).

Determination of NTPDase 1-3 and CD73 expression by flow cytometry

To assess whether imatinib mesylate influenced the expression of the NTPDases 1-3 and CD73 at the protein level, flow cytometry was performed. As shown in Figure 4, significant increases in expression (MFI) were observed after 24 hours of imatinib treatment for NTPDase 1 (1,900 ± 40.30 increased to 2,803 ± 65.75), NTPDase 2 (1,857 ± 37.4 to 3,104 ± 86.67), NTPDase 3 (169 ± 3.53 to 258 ± 19.01) and CD73 (631 ± 36.06 to 723 ± 59.40). In contrast, these increases in enzyme expressions were not observed after 48 hours of treatment. For the K-562R cell line, a decrease in the expression of NTPDase 1, in comparison to K-562, was observed (1900 ± 40.30 to 1029 ± 9.19), consistent with previous results showing that K-562R present decreased hydrolysis of ATP and ADP, and a decrease in the mRNA expression of *ENTPD1*.

Discussion

An association of ectonucleotidase activities with different cancers and their possible role in the immune response, via modulation of extracellular ATP, has been previously described [28]. However, the role of ATP in leukemias has not yet been fully elucidated. In cell lines and primary cultures of acute myeloid leukemia, ATP inhibits proliferative activity, which can be reversed using aspirase [29]. Another study, evaluating the role of ATP in the cell proliferation of the Jurkat acute myeloid leukemia cell line, found three times the concentrations of ATP and ADP in the extracellular medium, when compared to healthy cells [30].

In this study, K-562 cells demonstrated a higher rate of nucleotide hydrolysis and NTPDase mRNA expression, when compared to PBMNCs. NTPDase1 expression, however, was higher in PBMNCs, and *NT5E* expression was not significant changed. These findings are in agreement with reports in the literature showing the presence of

NTPDase1 in the mononuclear cells of healthy patients [25]. Our results indicate that purinergic signaling is altered in CML and we, thus, evaluated the influence of imatinib on purinergic signaling in imatinib -sensitive and -resistant CML cell lines.

The K-562R cell line, in addition to forming spheroid groups, presented an increase in the expressions of *SOX-2* and *Nanog*, as described in the literature [31]. Nanog is associated with various types of human cancers, including gastric , colon, and breast carcinomas, among others [32–35]. The expression of this gene is associated with decreased survival, and the development of therapeutic resistance and metastasis of tumors [36]. we also observed an increased expression of cell surface markers, such as *CD44* and *CD133*, on these cells, which are characteristic of leukemia stem cells [37, 38]. The increased expressions of efflux pump markers, such as *ABCB1/MDR1* and *ABCG2,* have been suggested to participate in the development of resistance to imatinib, as patients presenting increased expressions of these genes demonstrate an increased risk of developing resistance and treatment relapse [6, 39, 40].

The influence of imatinib mesylate on purinergic signaling was assessed in 4 different cell groups: untreated K-562 cells, K-562 cells treated with imatinib mesylate for 24 hours and 48 hours and in imatinib-resistant cells (K-562R). There was a significant increase in the hydrolysis of all nucleotides tested after 24 hours of imatinib mesylate treatment, and this was followed by an increase in the expressions of ectonucleotidase mRNA and protein. In previous studies, patients with lung cancer, treated with gemcitabine or cisplatin, displayed increased ATP and ADP hydrolyzing activities and increased CD39 expression, when compared to healthy individuals [41]. Another study that administered methotrexate to C6 rat glioblastoma cell cultures for 24 hours demonstrated increases in CD73 activity and expression. This study of the glioblastoma microenvironment also demonstrated that rats treated with methotrexate nanoparticles demonstrated increased CD39 expression in $CD3^+CD8^+$ cells [42]. Such an increase in ATP was not observed at 48 hours in our study, possibly because of the short-term stability of ATP in extracellular medium.

In Figure 5, we describe the influence of imatinib mesylate on the signaling of the NTPDases enzymes. Panel 5 shows the presence of NTPDases in the leukemic cells

before treatment with imatinib mesylate. When cells were treated with imatinib mesylate for 24 hours (fig. 5b), these enzymes exhibited increased activity and expression, possibly due to the inhibition of ATP binding to BCR-ABL by imatinib mesylate, leading to the intracellular accumulation of ATP with consequent transportation to the extracellular medium via microvesicles, connexins or pannexin-1 [11]. Upon reaching the extracellular medium, ATP may modulate these enzymes leading to their increased expression and enzymatic activity. This is not observed in K-562R, since imatinib mesylate is unable to interact with the ATP-active site and there is no intracellular accumulation of ATP. Accordingly, in the resistant phenotype, we observed decreased activities and expressions of these enzymes, especially of NTPDase 1 and 5 (Fig. 5c).

In K-562R cells, we found decreases in the mRNA expression of *ENTPD1* and in the protein expression of NTPDase1, confirming the results of the enzymatic and HPLC analyses, since nucleotide hydrolysis was lower on the surface of these cells. The imatinib-resistant cells also showed a decrease in the expression of *ENTPD5*, in association with a decrease in the enzymatic activity of UDP. We postulate that the modulation of NTPDase expression may contribute to resistance development; however, this finding should be further investigated. The role of the NTPDases in the development of resistance has been poorly explored in the literature; however, in cisplatin-resistant testicular tumor cells (NEC-8/DDP), the expression of ENTPD6 was found to be decreased [43].

NTPDase1, also known as CD39, has been studied as a possible prognostic marker. In chronic lymphocytic leukemia, a study showed a significantly increased expression of this enzyme in peripheral blood T lymphocytes, when compared to healthy controls [19]. The increased expression of NTPDase1 plays an important role in the overall survival of patients with gastric cancer and could act as a prognostic biomarker [15]. Increased ATP hydrolysis may also be due to the enzymatic activities of NTPDase2 and -3, which were expressed in all groups evaluated, especially in the K-562 24h group. Few studies have investigated these NTPDases in cancer; findings indicate that NTPDase2 is highly expressed in rat astrocytes and, when overexpressed in a C6 glioma cell line, promotes tumor growth and is associated with malignant characteristics in a rat glioma

model [44–46]. In addition, the co-expression of NTPDase1 and -2 is associated with tumor growth in Walker's Tumor, and the co-expression of NTPDase2 and -5 is also observed in Walker 256 cells [47, 48]. However, NTPDase2 and -3 appear to play an important role in purinergic system signaling in CML.

NTPDase5 is the principal enzyme with preference for diphosphate nucleotides and has low affinity for the degradation of the nucleoside triphosphates. Our results show that treatment of K-562 cells with imatinib mesylate for 24 hours increased UDP hydrolysis in association with an increase in NTPDase5 expression, while treatment for 48 hours and the resistance protocol decreased NTPDase5 expression. Of the enzymes investigated, only NTPDase5 is reportedly a proto-oncogene, also known as PCPH [49]. This enzyme presents an important association with the PI3K/PTEN and AKT signaling pathway [50]. These two pathways are related to the malignant transformation mediated by the BCR-ABL protein in CML. The proteomic and transcriptomic decrease of *ENTPD5* was first identified during colon tumor progression from a normal colon through to colon adenoma and then to adenocarcinoma [51].

Ectonucleotidases are currently being studied as promising candidates for cancer immunotherapy and several studies have reported on the development of antibodies and inhibitors of CD39 and CD73 in animal models [11, 20, 52]. Our study also highlights the importance of understanding the role of purinergic signaling in CML, since its components could be modulated as a form of treatment. However, we emphasize that this study provides the initial step towards understanding purinergic signaling in this leukemia. Further studies investigating patient samples, as well as the fate of nucleotides and ectonucleotidases in resistance development are necessary.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures in this study were approved by the Ethics Committee of UFRGS (CEP/UFRGS) of the Universidade Federal de Rio Grande do Sul, under protocol number 1.979.570.

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Tables

Table 1**.** Specific activities for the hydrolysis of ATP, ADP, AMP and UDP in K-562 cells and peripheral blood mononuclear cells (PBMNCs).

Cell line	ATP	ADP	AMP	UDP
PBMNCs	25 ± 4	21 ± 2	5 ± 3	12 ± 2
K-562	55 ± 1*	33 ± 5*	10 ± 4*	55 ± 4*

Specific activities are expressed as Mean ± S.D. of three different experiments (nmol of Pi liberated/min/mg of protein). Data were compared by ANOVA, followed by Tukey's test. *P<0.05, represents statistical significance, when comparing K-562 cells with PMNCs.

Table 2. Specific activities for the hydrolysis of ATP, ADP, AMP and UDP in K-562 cells, K-562 cells after treatment with imatinib mesylate and K-562R.

Cell line	ATP	ADP	AMP	UDP
K-562	55 ± 1	33 ± 5	10 ± 4	55 ± 4
K-562 24h	84 ± 5*	88 ± 5*	25 ± 2*	64 ± 3*
K-562 48h	60 ± 2	51 ± 8	19 ± 6*	20 ± 1*
K-562R	46 ± 2*	24 ± 5*	10 ± 2	30 ± 1*

Specific activities are expressed as Mean ± S.D. of three different experiments performed in triplicate (nmol of Pi liberated/min/mg of protein). Data were compared by ANOVA, followed by Tukey's test. *$p<0.05$ represents statistical significance, when comparing K-562 treated with imatinib mesylate for 24h (K-562 24h), 48hs (K-562 48h) and imatinib-resistant K-562 (K-562R) to untreated K-562 (K-562).

Figures and Legends

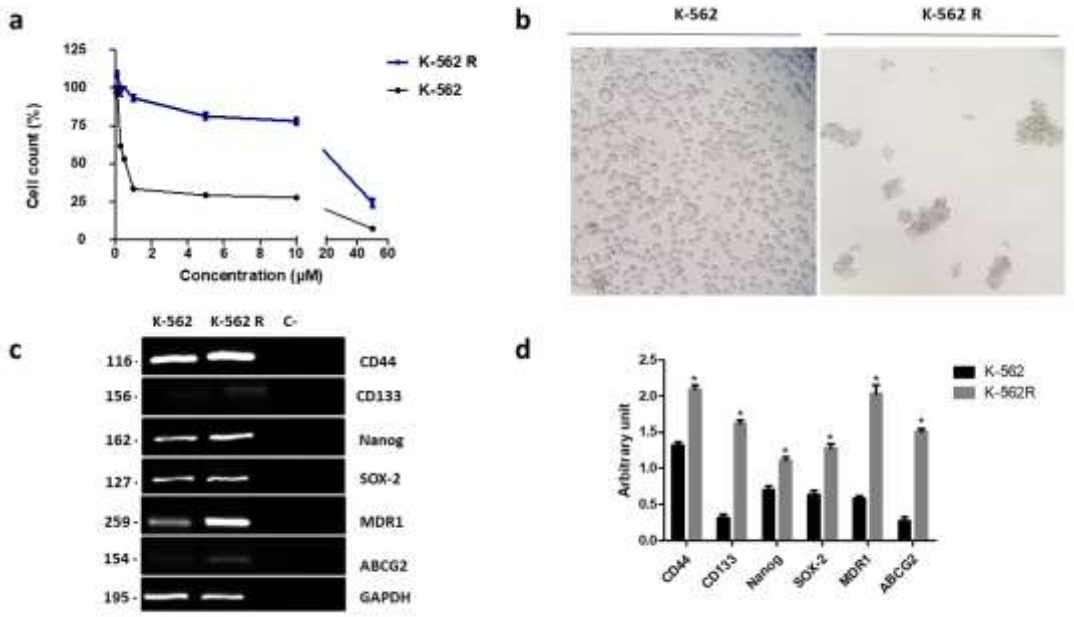

Fig. 1 Characterization of resistance to imatinib mesylate. (a) Cell counts were performed by flow cytometry to evaluate treatments with different concentrations (0-50.0μM) of imatinib mesylate in K-562 (sensitive) and K-562R (resistant) cells (b) Comparison of the phenotypic characteristics of K-562 cells and K-562R after long-term exposure to imatinib mesylate. 200x magnification. (c) Expression of differentiation markers, cell surface markers and expression of multidrug resistance genes in K-562 and K-562R (RT-PCR). (d) Semi-quantification of the fragments and expression ratio of the markers/GAPDH. Data were compared by ANOVA followed by Tukey's test. *$p<0.05$ represents statistical significance

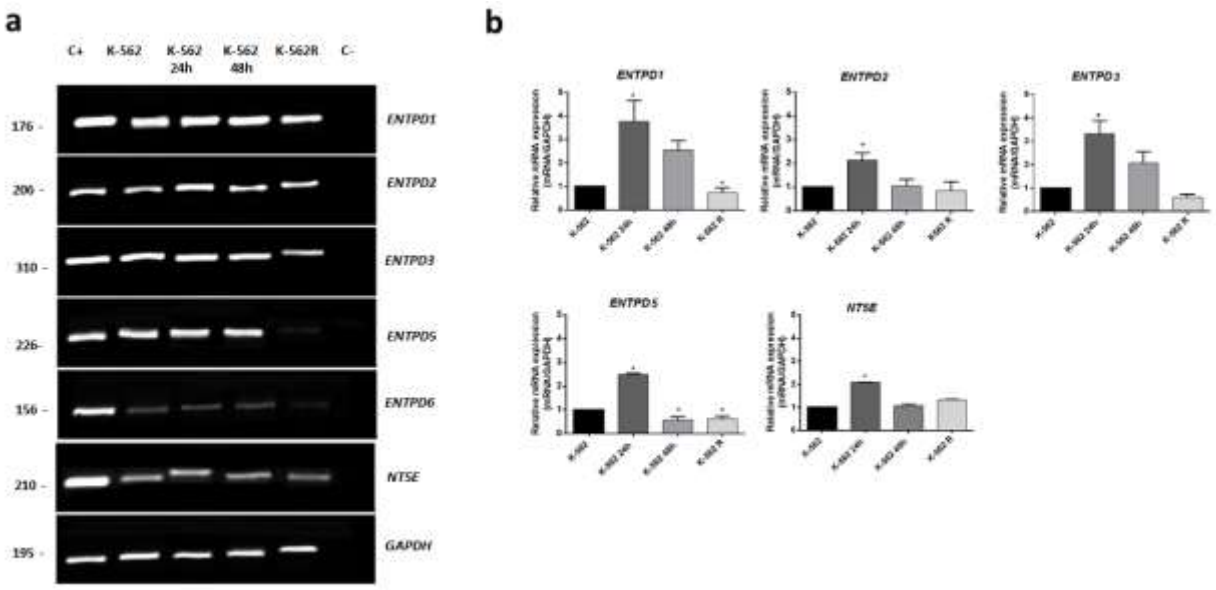

Fig.2 Expressions of *ENTPDs* and *NT5E* in K-562 cells, K-562 cells after treatment with imatinib mesylate for 24 hours (K-562 24h) and 48 hours (K-562 48h) and imatinib-resistant K-562 cells (K-562R). (a) Analysis by RT-PCR. Cell lines that already express these enzymes (as described in the literature) were used as positive controls (C+) and template controls (C-) were performed with distilled water. The lengths (bp) of the PCR products obtained with each pair of primers are given in each figure. (b) Quantitative expressions of *ENTPDs* and *NT5E* were analyzed by real-time PCR. Comparative analysis was performed using the $2^{-\Delta\Delta CT}$ method, where the expression was compared to the untreated K-562 cells using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* as the endogenous control. The experiment was performed three times (n = 3) with samples in duplicate. Data were compared by ANOVA followed by Tukey's test. *$p<0.05$ represents statistical significance

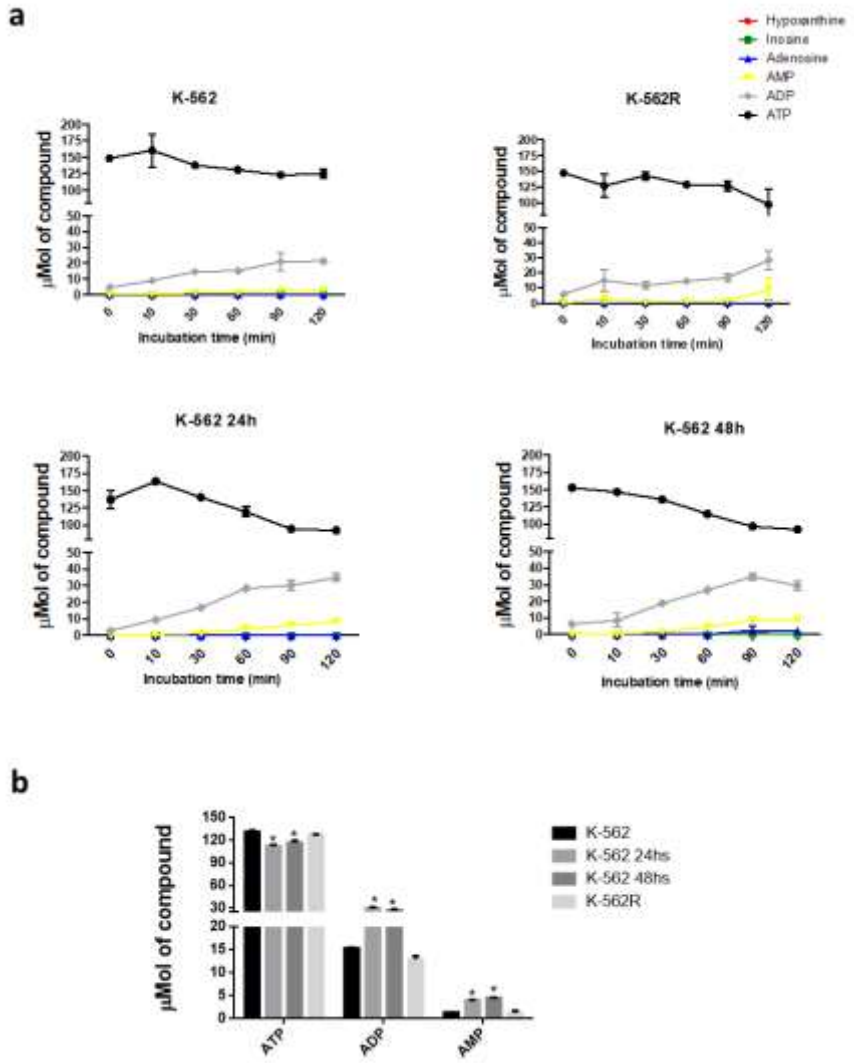

Fig. 3 Hydrolysis of extracellular ATP and product formation by K-562 cells, K-562 cells after treatment with imatinib mesylate for 24 hours (K-562 24h) and 48 hours (K-562 48h) and imatinib-resistant K-562 cells (K-562R). (a) Cells were incubated with 100 µM ATP and supernatant aliquots were collected after 0, 10, 30, 60, 90 and 120min. The nucleotides were quantified by HPLC. (b) Amounts of ATP, ADP and AMP formed after 60 min of incubation. Values are representative of two different experiments. Data were compared by one-way ANOVA, followed by Tukey's test. *$p<0.05$ represents statistical significance, comparing K-562 treated with imatinib mesylate for 24h (K-562 24h), 48h (K-562 48h) and imatinib-resistant K-562 (K-562R) cells to untreated K-562 (K-562).

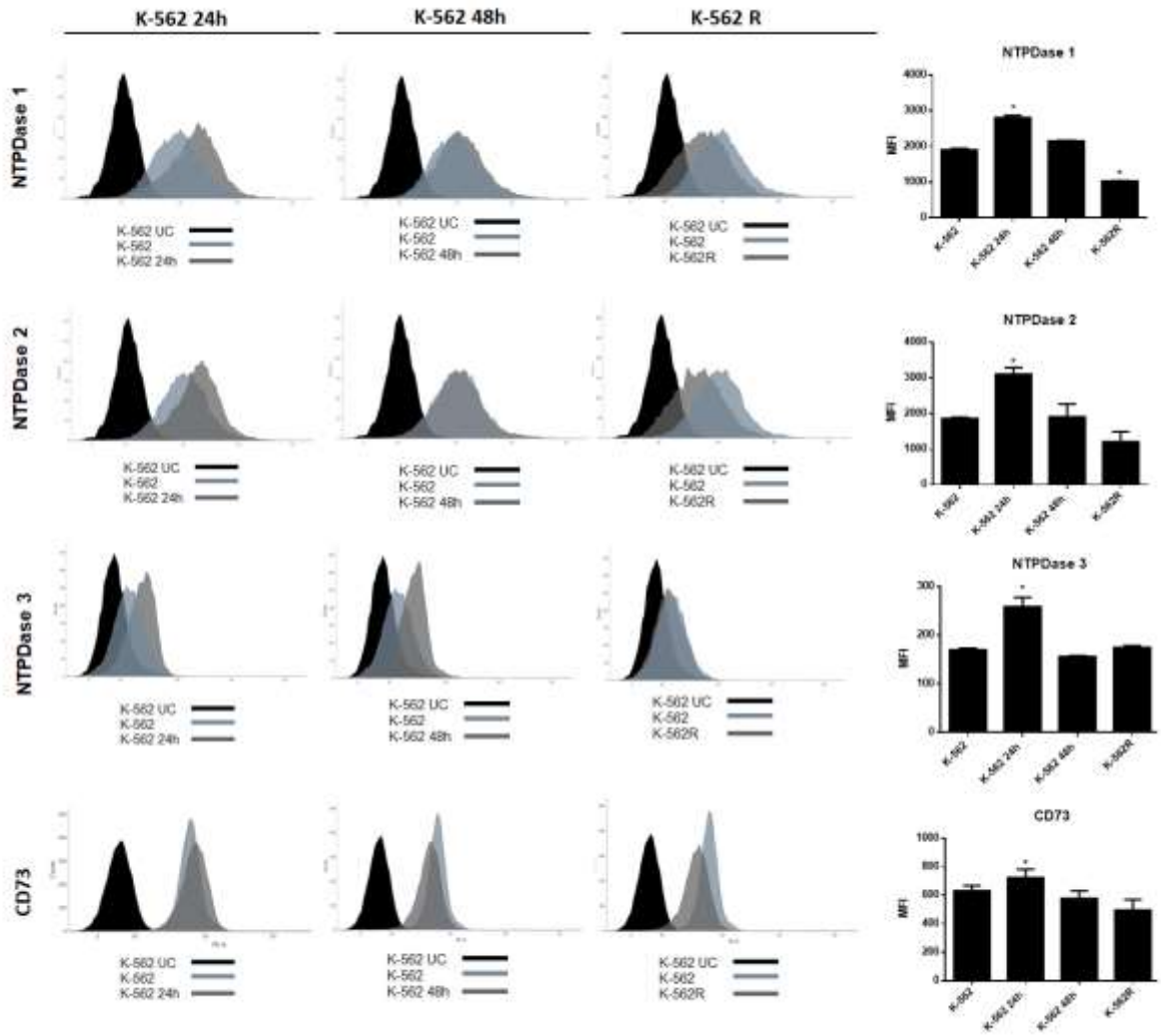

Fig.4 Determination of NTPDase1, -2, -3 and CD73 protein expression by flow cytometry. (a) Histograms demonstrating enzyme expressions in K-562 cells after treatment with imatinib mesylate for 24 hours (K-562 24h) and 48 hours (K-562 48h) and imatinib-resistant K-562 (K-562R) cells, compared to untreated K-562. (b) Mean fluorescence intensity (MFI) plot in the same groups. The experiment was performed twice (n=2) with samples in duplicate. Data were compared by ANOVA followed by Tukey's test. * $p < 0.05$ represents statistically significant difference from untreated K-562. K-562 UC; labeled control with secondary antibody or unlabeled control.

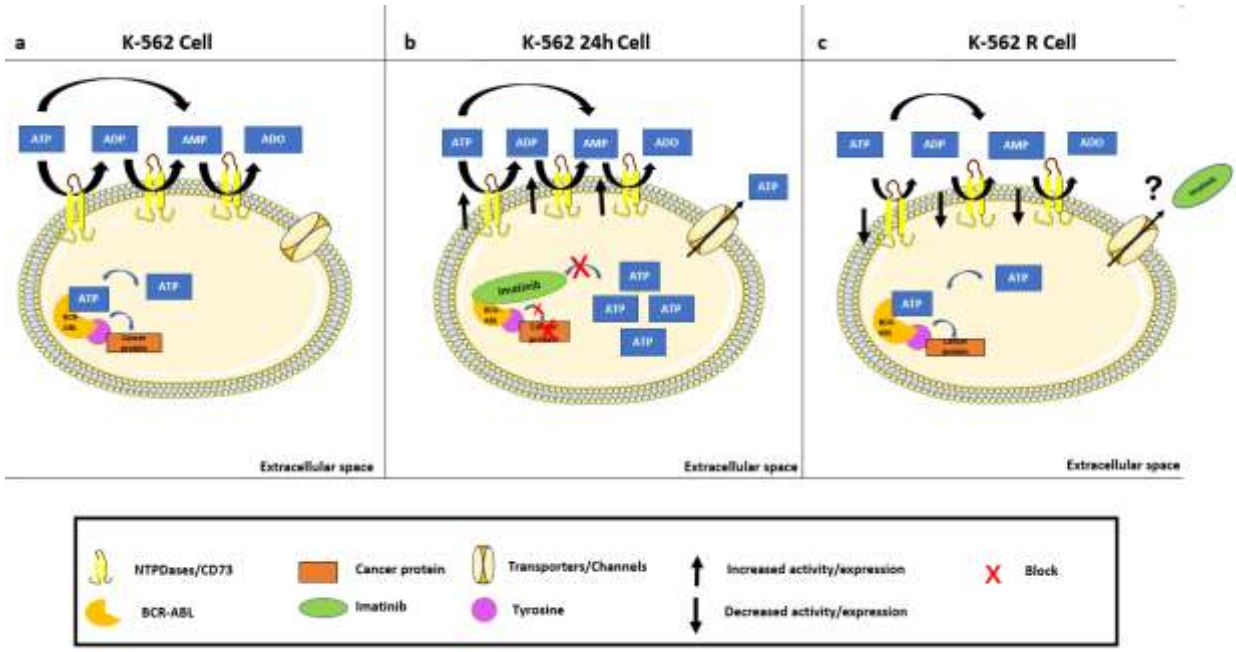

Fig. 5 Schematic illustration summarizing the influence of imatinib mesylate on the hydrolysis of nucleotides and the expression of ectonucleotidases in K-562 cells. (a) We emphasize that, regardless of the action of imatinib mesylate, enzymes participate in the pathology of chronic myeloid leukemia. (b) Twenty-four-hour treatment with imatinib mesylate blocks the ATP site of the BCR-ABL protein, causing intracellular ATP accumulation. ATP passes into the extracellular medium, stimulating increased ATP hydrolysis and increasing the expression of NTPDases. (c) In the imatinib-resistant line, this intracellular ATP accumulation does not occur, consequently there is a decrease in the hydrolysis and expression of the NTPDases.

Supplementary Figure

Table S1. Primer sequences, annealing temperatures (AT) and product sizes.

Gene	Primer sequence (5’ to 3’)	AT (°C)	Fragment size (bp)
CD44	F: AGAAGGTGTGGGCAGAAGAA R: AAATGCACCATTTCCTGAGA	60	116
CD133	F: TAAAGCTGGACCCATTGGCA R:CCTAAGATTACAGTTTCTGGCTTGT	60	156
Nanog	F: TGCAAGAACTCTCCAACATCCT R: AGTAAAGGCTGGGGTAGGTAGG	60	162
Sox-2	F: AGGATAAGTACACGCTGCCC R:TTCATGTGCGCGTAACTGTC	60	127
MDR1	F: TACAGTGGAATTGGTGCTGGG R: CCCAGTGAAAAAATGTTGCCA	56	259
ABCG2	F: GGTGGAGGCAAATCTTCGTTATTA R:GAGTGCCCATCACAACATCATCTT	62	154
ENTPD1	F: CTACCCCTTTGACTTCCA R: CTCCCCCAAGGTCCAAAG	62	176
ENTPD2	F: CTCCTACTGCTGTGCGTCC R: TGTCGTTCTCCTTGTCTGCC	60	206
ENTPD3	F: TACCGAACTCCAACCATCA R: CCTTGACTTTTTGCATACA	58	310
ENTPD5	F: CAGGTCAGCTGCATGGCCACA R: TCCAGGGCTCCCAGGGTTGC	62	226
ENTPD6	F: CAGCTGCAGACGGGCACGAG R:GCAGAAAGACCTGGCTTCAGTGCT	60	154
NT5E	F: CTCTTGCAACACCCATGTGC R: ACAGCTAATGCCGTGTGTCA	58	225
GAPDH	F: CAAAGTTGTCATGGATGACC R: CCATGGAGAAGGCTGGGG	60	195

Table S2. Specific activities for the hydrolysis of ATP, ADP, AMP and UDP in peripheral blood mononuclear cells (PBMNCs) after treatment with imatinib mesylate.

Cell line	**ATP**	**ADP**	**AMP**	**UDP**
PBMNCs	25 ± 4	21 ± 2	5 ± 3	12 ± 2
PBMNCs 24h	24 ± 1	21 ± 2	4 ± 0	12 ± 5
PBMNCs 48h	19 ± 4	19 ± 2	4 ± 0	14 ± 2

Specific activities are expressed as Means ± SD of three experiments (n=3) performed in triplicate (nmol of Pi liberated/min/mg of protein). Data were compared by ANOVA, followed by Tukey's test. *$p<0.05$ represents statistical significance, when comparing PBMNCs treated with imatinib mesylate for 24h (PBMNCs 24hs), and 48h (PBMNCs 48hs) to untreated PBMNCs (PBMNCs).

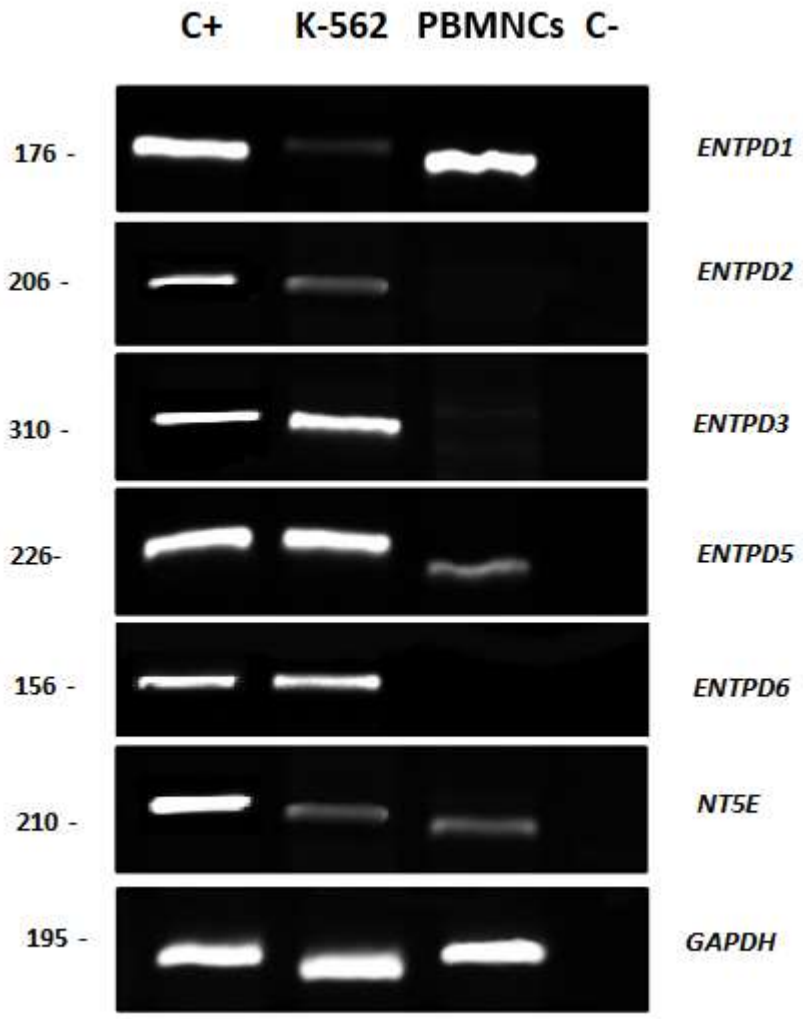

Figure S1. Expression of *ENTPDs* and *NT5E* in K-562 and peripheral blood mononuclear cells (PBMNCs). Total RNA was extracted and after cDNA synthesis the PCR reaction was performed using primer specifics as described in material and methods. Positive controls (C+) was used cell lines that already express these enzymes described in the literature and negative controls (C-) were performed with distilled water as template. The length (bp) of the PCR products obtained with each pair of primers is given in each figure.

IV. CAPÍTULO II

IV. CAPÍTULO 2 – Júlia Biz Willig, Nádia Miléo Garcês de Couto, Débora Renz Barreto Vianna, Camila da Silveira Mariot, Simone Cristina Baggio Gnoatto, Andréia Buffon, Diogo André Pilger. Betulinic acid derivative compound has a synergistic effect with imatinib mesylate in Chronic Myeloid Leukemia cell line modulating expression of proteins related to apoptosis and autophagy

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Betulinic acid derivative compound has a synergistic effect with imatinib mesylate in Chronic Myeloid Leukemia cell line modulating expression of proteins related to apoptosis and autophagy

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Abstract

Chronic myeloid leukemia (CML) is a myeloproliferative disease characterized by the formation of the BCR-ABL oncoprotein. Imatinib mesylate is the first line of treatment for CML, however many patients show therapeutic resistance. Currently, the search for new drugs based on natural or semisynthetic products has gained evidence in prospecting of new treatments. In this study, we investigate cytotoxic activity and possible mechanisms of action of the derivative compound of betulinic acid (BA) in CML BCR-ABL+ cell lines sensitive (K-562) and resistant (K-562R) to imatinib. In addition, we evaluate the drug combination of imatinib mesylate with this derivative compound in K-562 cells. The derivative compound was tested *in vitro* for apoptosis, cell cycle, autophagy and oxidative stress effects. The derivative compound showed cytotoxicity in K-562 (23.57μM ± 2.87) and K-562R (25.80μM ± 3.21) cells and when associated with imatinib mesylate presented a synergistic effect. Apoptosis was triggered by intrinsic pathway with activation of caspases 3 and 9, and cell cycle showed G0/G1 arrest. In addition, the compound lead to increased production of reactive oxygen species and induction of autophagy by also increasing the levels of proteins LC3II and Beclin-1. Based on these observations, we suggest that the derivative compound causes the death of the imatinib-sensitive and -resistant cell lines and is promising for development of new anticancer treatments against CML.

Keywords: betulinic acid derivative, imatinib mesylate, synergism, K-562, Chronic myeloid leukemia

1. Introduction

Inhibitors of tyrosine kinase (TKI) are the main form of treatment for chronic myeloid leukemia (CML), a myeloproliferative disease characterized by the formation of the BCR-ABL oncoprotein[1]. Imatinib mesylate, the first line of treatment for CML, is a first generation TKI and inhibits BCR-ABL tyrosine kinase activity through blockage of the ATP-binding site[2]. However, many patients show therapeutic resistance after some period of use or do not respond to treatment, even with the second and third generation of TKIs[3]. Currently, the search for new drugs based on natural or semisynthetic products has gained prominence in the development of new therapeutic options for haematological tumors.

Terpenes, polyphenols and alkaloids are secondary metabolites extracted from natural products and several studies in the literature present their antitumor activity[4]. Betulinic Acid (BA) is a pentacyclic triterpene found in many plants, fruits and vegetables, which has several therapeutic activities already demonstrated as anti-inflammatory, antiviral, antibacterial, antimalarial, immunomodulatory, antidepressive and antitumor [5–7]. Its antitumor activity is described in several tumor lines, like melanoma, neuroblastoma, glioblastoma, colon, hepatocellular, lung, prostate, breast, ovary, cervical carcinoma, and leukemia [8]. Its main mechanism of action is the induction of cell death for apoptosis, autophagy, modulation of kinase proteins and inhibition of DNA topoisomerases, among others [7, 8].

Nowadays, there are several strategies to try to reduce the therapeutic resistance to chemotherapy, one of which is to perform structural modifications in current molecules. Another is the search for molecules capable of promoting different mechanisms of cell death in order to achieve maximum effectiveness, reducing doses and the chance of acquiring resistance. Several studies have evaluated cytotoxic activity of BA derivatives. Triazole derivative of BA demonstrated moderate activity in HL-60 cells and induced extrinsic and intrinsic apoptosis[9]. Novel guanidine-functionalized triterpene acid derivative was synthesized and exhibited cell activity anticancer of Jurkat (T-lymphoblastic leukemia), K562 (chronic myeloid leukemia), U937 (histiocytic lymphoma), HEK 293 (embryonic kidney), and HeLa (cervical cancer)[10].

In this study we investigated the cytotoxic activity and possible mechanisms of action of the derivative compound of BA in CML BCR-ABL+ cell lines sensitive (K-562) and resistant (K-562R) to imatinib. In addition, we evaluated the drug combination of imatinib mesylate with this compound in K-562.

2. Material and Methods

2.1 Cell culture

The chronic myeloid leukemia cell line (K-562) was purchased from the Rio de Janeiro cell bank (Banco de Células do Rio de Janeiro (BCRJ), Rio de Janeiro, Brazil). Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum, 100U/mL pen-streptomycin and incubated at 37°C in 5% CO_2. The K-562R (imatinib-resistant cell line) was generated as described by Willig, et al. (unpublished data). Peripheral blood mononuclear cells (PBMNCs) were obtained from healthy donators through the Histopaque®-1077 (Sigma-Aldrich, USA) density gradient and used as control. The project was approved by Committee of Ethics of UFRGS (n.1.979.570).

2.2 Synthesis of derivative compound

Synthesis of the derivative compound of the triterpene BA was previously described by Couto, et al (unpublished data).

2.3 Cell counting

Cells were seeded in 96-well microplates ($8x10^3$/well) and then incubated at 37°C in 5% CO_2 for 24h. After, cells were treated with the derivative compound (0-75 µM) for further 48h. Cell counting was performed in a FACSVerse™ cytometer equipped with 488nm blue laser and flow sensor (BD Biosciences, San Jose, CA, EUA). Results were expressed as percentage of control. Dose-response curves were constructed and IC_{50} values were as determined by Graphpad Prism software (version 6.0).

2.4 Selectivity Index (SI) analysis

PBMNCs cells treatment was performed to assess the selectivity index (SI) in order to evaluate how selective is the compound to kill/damage cancer cells instead of normal cells. The degree of selectivity of derivative compound was expressed for each tumor cell line according to the equation SI = CC_{50}/ IC_{50} [7].

2.5 Analysis of the effects of drug combinations

K-562 cells were seeded into 96-well microplates ($8x10^3$/well) and after 24h were exposed to different concentrations the compound (5, 10, 20, 40, 80 µM), imatinib mesylate (0.17, 0.33, 0.67, 1.34, 2.68 µM) or in combination. After treatment for 48h, the cell counting was performed in a FACSVerse™ cytometer. CompuSyn software was used to calculate the combination index (CI) and isobologram to quantitatively determine the effect of drugs interactions. CI values less than 1, equal to 1, and greater than 1 represent synergism, additivity, and antagonism, respectively. The isobologram is formed by plotting the concentrations of each drug for 50% inhibition (EC50) on the x- and y-axis and connecting them with a line segment, which is ED50 isobologram. Combination points that fall on, below, and above the line segment represent additivity, synergism, and antagonism, respectively [11].

2.6 Evaluation of cell death

For evaluation of the mechanism of cell death K-562 and K-562R cells were treated with the concentration of the corresponding IC_{50} of the compound for 48 hours. Still, K-562 was treated with imatinib mesylate and combination with compound for 48 hours.

2.6.1 Annexin V–FITC/PI staining experiment

Phosphatidylserine (PS) externalization was determined by the annexin fluorescence signal of an annexin V–fluorescein isothiocyanate conjugate (Quatro G Pesquisa & Desenvolvimento, Porto Alegre, Brazil) according to the manufacturer's protocol. Briefly, cells were treated at the correspondent concentrations for 48h, and then harvested, centrifuged for 5 min at 1500 rpm, and the supernatants were discarded. Pellets obtained

were re-suspended with 150µL of annexin binding buffer. Cells were stained with 5µL annexin V and 5µL propidium iodide for 15min at room temperature in the dark. The apoptotic index was immediately determined on a FACSVerse™ flow cytometer (BD Biosciences).

2.6.2 Detection of morphological changes

To detect morphological changes that occurred during apoptosis process, nuclear staining was performed using DAPI. After 48h of treatment, cells were harvested and fixed in 4% paraformaldehyde for 20min. Subsequently, cells were stained with 4µg/mL DAPI (Sigma, St Louis, USA) for 30min at room temperature. After washing with PBS, samples were stored in the dark at 4°C and visualized on a fluorescence microscope (Olympus IX71 microscopy).

2.6.3 Cell Cycle Analysis

Cell lines were seeded in 6-well plates ($1x10^6$ cells/well) and incubated for 24h at 37°C in humidified incubator as previously described. Cells were treated using the correspondent concentrations of treatment and control cells were only maintained with RPMI 10% SFB. After a 48h period, cells were harvested and fixed in 70% ice-cold ethanol (v/v in PBS) for 24h. Then, cells were washed with PBS and a solution containing 12µg/mL propidium iodide, 0.1% Triton X-100, and 50µg/mL RNAse was added to each tube[12]. After 30min of incubation at room temperature, protected from light, cells were analyzed using a FACSVerse™ flow cytometer (BD Biosciences).

2.6.4 Autophagy Analysis

Autophagy analysis were performed in 24-wells plates where cells were seeded ($3x10^4$ cell per well) and maintained for 24 hours at 37ºC and 5% CO_2. After that, cells were treated for 48h. Posteriorly, cells were incubated with acridine orange (AO) (2.7µM) during 15min at room temperature as described previously. Assessment were made by FACS Verse flow cytometer (BD Biosciences, San Jose, CA, USA).

2.7 Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cells using TRIzol reagent method (Life Technologies, Carlsbad, CA, USA) and quantified in Nano-Drop® spectrophotometer (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized from 5µg of total RNA in total volume reactions (20µL) with M-MLV reverse transcriptase (Life Technologies, Carlsbad, CA, USA). Real-time polymerase chain reactions were performed in a Rotor-Gene Q (Qiagen, Maryland, Mam USA) using 1µL cDNA, specific primers (Table 1) and GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) according to manufacturer's instructions. The conditions applied were 95°C for 1 min, 40 cycles of 10s at 95°C, 15s at 60°C, and 20s at 72°C. The relative expression levels of the genes were determined by $2^{-\Delta\Delta CT}$ method and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* was used as an internal control.

2.8 Determination of ROS Generation

2,7-Dichlorofluorescin diacetate (DCFH-DA) was used to detect reactive oxygen species (ROS) generation. After treatment, cells were incubated with 10µM DHE for 30min. Fluorescence was the observed-on fluorescence microscope (Olympus IX71 microscopy) and FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA).

2.9 Statistical Analysis

Experiments were performed three times (n=3) with samples in triplicate and the results were analyzed by analysis of variance (ANOVA), followed by the Tukey test. The software used was GraphPad Prism (version 6.0). Significant values were considered for $p<0.05$.

3. Results

BA derivative compound inhibits the growth of human leukemia cells

Through cell counting we studied the cytotoxic effect of the derivative compound on K-562 and K-562R cell lines. As shown in figures 1a and 1b, the derivative induced a cytotoxic effect on K-562 and K-562R cells and the IC_{50} were 23.57µM ± 2.87 and 25.80µM ± 3.21, respectively. The same analysis in PBMNCs showed that these control cells didn't undergo the cytotoxic effect of the compound (Fig. 1c) and SI was obtained from the ratio

of CC_{50} values on PBMNCs cells and IC_{50} on cancer cell lines. The SI was 11.06 and 10.11 for K-562 and K-562R cells, respectively.

BA derivative compound and imatinib mesylate inhibited human leukemia cells growth through synergistic effect

Drug interaction was studied using CompuSyn software. Drug synergism was represented with the help of the combination index (Fig. 2a; Table 2) and isobologram (Fig. 2b). Co-treatment with the derivative and imatinib mesylate had a synergistic effect in all concentrations tested. However, treatment with 20μM of BA derivative e and 0.67μM of imatinib mesylate gave the lowest CI value (0.27), so this combination of concentrations was chosen to continue the experiments.

BA derivative compound induces apoptosis by increasing caspase-3 and caspase-9 expression in K-562 and K-562R cells

As revaled using flow cytometry, 48h treatment of K-562 and K-562R with the derivative showed positive effect on the quantity of early and late apoptotic cells (Fig.3). The apoptotic cell population significantly increased from 1.74% to 20.96% and 2.01% to 26.83% in K-562 and K-562R, respectively (Fig. 3c and 3f). Treatment with imatinib mesylate alone also increases the apoptotic cells from 1.74% to 20.72% (Fig. 3b). Co-treatment exhibited an even greater increase in the labeling of apoptotic cells from 1.74% to 78.35% (Fig. 3d). To confirm whether apoptosis induction would alter cell morphology, DAPI staining was used. In figure 4, we can see that all the treatments performed caused morphological changes, which were characterized by the formation of apoptotic bodies, condensation of the chromatin and the presence of fragmented nuclei with brighter blue fluorescence. In addition, we determined whether the apoptosis trigger was intrinsic or extrinsic through the expression of caspases 3, 8 or 9 by qRT-PCR (Fig.5). Our results demonstrate that the BA derivative treatment in K-562 and K-562R cells and co-treatment in K-562 cells increases the expression of caspase-3 and caspase-9, which are part of the intrinsic apoptosis pathway.

BA derivative compound causes alterations in cell cycle distribution of K-562 and K-562R cells

The results of the present study indicated that the derivative compound may have induced cell cycle arrest of K-562 and K-562R. Through flow cytometry, we first evaluated the cell cycle of K-562 and K-562R cells treated with the derivative (Fig. 6). When we compared the K-562 treated with the control, we found a higher sub-G0 peak which characterizes a cell death by apoptosis. There was also an increase in the proportion of cells in the G2/M phase increasing from 26.27% to 35.83% in K-562 cells and 22.00% to 36.11% in K-562R cells (Fig.6c and 6f, respectively). However, treatment with imatinib mesylate affects the G0/G1 cell cycle arrest in K-562 cells (Fig. 6b). Analyzing the co-treatment in K-562 cells, we can verify that increase in the proportion of cells in the sub-G0 and G0/G1 phase increasing from 13.54% to 18.67% and 32.95% to 45.75%, respectively when compared derivative compound treatment and co-treatment (Fig.6c and 6d, respectively).

BA derivative compound activates autophagy by increasing LC3-II and Beclin-1 expression in K-562 and K-562R cells

To evaluate death by autophagy, the acridine orange dye was used, which indicates the maturation of autophagosomes. Both imatinib mesylate and the derivative induce autophagy in K-562 cells (Fig. 7b and 7c). The co-treatment showed 77.25% of the cells labeled with AO (Fig. 7d). In K-562R cells there was a significant increase of 53.95% in AO positive cells after treatment with the derivative (Fig 7f). To confirm the mechanism by which the derivative compound, imatinib mesylate and the co-treatment induced autophagy in K-562 and K-562R cells, qRT-PCR expression of two proteins that play a key role in the autophagic pathway was performed. In figure 8, we show that all treatments increase the expression of autophagy markers LC3-II and Beclin-1 in K-562 and K-562R cells, as co-treatment in K-562 cells increased 32 ± 0.70 and 222 ± 1.41-fold when compared to control cells.

BA derivative compound induced intracellular ROS generation

To verify if the derivative compound was generating reactive oxygen species (ROS), the cells were exposed to an oxidation-sensitive fluorescent dye, DCFH-DA, and its fluorescence was measured using fluorescence microscopy and flow cytometry. In figure 9 we can see that the derivative, imatinib mesylate and co-treatment induced ROS generation. Through the medium intensity of fluorescence (MFI), we can verify that the co-treatment in K-562 cells showed the highest fluorescence detected (Fig. 9b). The derivative also increases the generation of ROS in K-562R cells (Fig. 9c).

Discussion

The discovery of tyrosine kinase inhibitors, with the introduction of imatinib mesylate in 2001, revolutionized the treatment of chronic myeloid leukemia [13]. Nevertheless, the appearance of adverse effects led to increased cases of discontinuation of treatment. The main adverse reactions involving imatinib mesylate are gastrointestinal disturbances and rashes, in addition to thrombocytopenia and neutropenia [14] . Still, discontinuation of treatment may lead to the development of therapeutic resistance. Resistance to imatinib is a complex process that still does not have an exact cause, however several studies demonstrate the participation of point mutations in the BCR-ABL protein, BCR-ABL overexpression and expression of drug efflux pumps [15, 16].

Natural compounds have been used in antineoplastic therapy not only as a high performance and high safety agent to induce cell death in leukemia cells but as a new therapeutic option in cases of resistance to classic chemotherapy, being used in combinations to try to avoid therapeutic resistance [4]. The main mechanism of action of chemotherapy is the induction of death via apoptosis; however, tumor cells may be resistant to this type of death [17]. In this sense, the search for new compounds capable of promoting multiple mechanisms of death, maximum efficacy, and few adverse effects has been studied.

In this work we demonstrate the cytotoxic activity of a betulinic acid (BA) derivative compound on imatinib-sensitive (K-562) and imatinib-resistant (K-562R) cells. At low concentrations the compound already exhibits a cytotoxic effect on K-562 cells, however

from the 25µM concentration the behavior is similar in the two cell lines. In addition, presented a selectivity index 10-fold higher to damage tumor cells than normal cells.

In K-562 cells it was also demonstrated the synergism of this compound with imatinib mesylate. The main advantage of the synergism is the possibility of dose reduction maintaining or increasing the therapeutic activity [11, 18]. Our results demonstrate that low concentrations of imatinib mesylate and compound have the same synergistic effect as high concentrations. This decrease in dose may lead to less therapeutic resistance and an improvement in treatment adherence due to the decrease in the adverse effects related to imatinib mesylate. Synergism with imatinib mesylate has already been reported with flavonoids, other class of natural product, in K-562 cells. Apigenin, luteolin and 5-desmethyl sinensetin had synergic effect with imatinib mesylate [19].

The antitumoral activity and molecular mechanisms of BA have been described in inhibit several studies [8]. BA presents results in combination with several chemotherapeutic drugs such as etoposide, doxorubicin and cisplatin to induce apoptosis and survival of tumor cells [20]. It is also demonstrated synergistic effect in tumor pancreatic cells with mithramycin A, inhibiting proliferation, invasion and angiogenesis [21].

Apoptosis is a mechanism of programmed cell death, which presents morphological changes and is characterized by the formation of apoptotic bodies, chromatin condensation, nuclear fragmentation, microtubular alterations or mitotic defects [17]. Activation of apoptosis can be initiated from two different pathways, extrinsic (cytoplasmic) or intrinsic (mitochondrial), in which the activity of different cysteine-dependent aspartate specific proteases (caspases) plays an important role [22]. In the present study, the derivative compound caused apoptosis in K-562 and K-562R cells, altering cell morphology and activating caspase-3 and caspase-9, which are part of the intrinsic pathway of apoptosis. Intrinsic apoptosis is mediated by mitochondrial outer membrane permeabilization, resulting in the activation of caspase-9, that directly activates caspase-3 and caspase-7 [17]. Our results corroborate with findings in the literature since BA promoted apoptosis in human hepatoblastoma, cervical cancer and leukemia cell lines

(HL-60 and K-562), altering the expression of caspases in both the intrinsic and/or extrinsic pathways [23–26]. BA inhibits the PI3K/AKT cell signaling pathway which is an important antitumor target especially for induction of mitochondrial apoptosis. This signaling pathway is responsible for several cellular functions including cell proliferation, tumor growth, as well as coordinating the cell cycle and cell migration, in addition to causing changes in the Bcl-2 and BAD family of proteins [17].

Our results still demonstrate a significant stop in the sub-G0 phase of the K-562 and K-562R cells cycles, also envying death by apoptosis. Cell cycle arrest is crucial in the mechanism of action of some drugs, altering replication, proliferation and cell division, and is already described as one of the mechanisms of BA. triterpenes induce accumulation of leukemia cells in the G0/G1 and G2/M phases [27]. In HeLa cell line, BA caused cell paresis in the G0/G1 phase after inhibition of the PI3K/AKT pathway [23].

Another way to activate apoptosis is by the unbalance of ROS, once in excess they can cause damage to proteins and DNA leading to cell death. Our work demonstrates that the derivative compound induces the production of reactive oxygen species (ROS), which could be a consequence of the activation of the caspase pathway and loss of the membrane potential in which ROS is generated [28]. In acute myeloid leukemia cells, BA combined with histone deacetylase inhibitor increased ROS generation with DNA damage, apoptosis and mitochondrial dysfunction [29]. Taxodice, another diterpene, induced apoptosis in K-562 cells and reduced the activities of mitochondrial respiratory chain complexes III and V which appeared to induce the production of ROS [30].

Autophagy is a physiological cellular process that leads to the degradation and recycling of damaged cellular components [17]. However, depending on the degree of activation it can lead to cell death [31]. Autophagic process can be monitored by the expression of related proteins such as LC3 I/II and Beclin-1. LC3 I is a cytosolic protein that is cleaved and converted to LC3 II after pro-autophagic stimulus. Beclin-1 is part of a type III phosphatidylinositol 3-kinase complex required for autophagic vesicle formation [12]. In cancer, autophagy exhibits a contradictory behavior, depending on the cell type it may be an important factor for induction of cell death or tumor progression [31]. It is now known that imatinib mesylate induces autophagy in K-562 cell line and in primary culture

of patients with CML, though this induction was not associated with BCR-ABL activity, but rather with endoplasmic reticulum stress [32]. Our results show that derivative compound causes increased autophagy death demonstrated with acridine orange incubation in the K-562 and K-562 R cell lines, in addition to demonstrating increased expression of LC3II and Beclin-1. Chemotherapies also cause autophagy as is the case of 5-fluorouracil in HCT116 cells, the human colon cancer cell line [33]. Other studies also report the induction of autophagy by BA via inhibition of the AKT-mTOR signaling pathway [34].Triterpene derivative, 3β-O-succinyl-lupeol, induced autophagosome formation in A549 cell, promoted the expression of autophagy-related proteins LC3-II and Beclin-1, beyond the increased ROS generation [35]. The search for molecules capable of presenting different mechanisms of action is increasingly evident in order to avoid therapeutic resistance, however, there is still no consensus among which proteins are connecting the cell death pathways [36].

In conclusion, our results in K-562 and K-562R cells demonstrated that the compound induces many molecular mechanisms. Our compound exhibits synergism with imatinib mesylate, inducing apoptosis via caspases-3 and 9, cell cycle arrest and autophagy death with increased expression of LC3II and Beclin-1. These results are promising and studies *in vivo* and with patient samples are needed to confirm the possibility for development of new anticancer treatments against CML.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures in this study were approved by Ethics Committee of UFRGS (CEP/UFRGS) of the Universidade Federal de Rio Grande do Sul, under protocol number 1.979.570.

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Tables

Table 1. Sequence, annealing temperature and products size of the primers used for qRT-PCR experiments.

Gene product	Primer sequences	Annealing temperature	Product size (bp)
Caspase-3	F-5'- ACATGGCGTGTCATAAAA -3' R-5'- CACAAAGCGACTGGAC -3'	60°C	120
Caspase-8	F-5'- CTGCTGGGGATGGCCACTGTG -3' R-5'- TCGCCTCGAGGACATCGCTC-3'	60°C	113
Caspase-9	F-5'- GAGTCAGGCTCTTCCTTTG -3' R-5'- CCTCAA ACTCTCAAGAGCAC-3'	60°C	241
LC3-II	F-5'-GAGAAGCAGCTTCCTGTTCTGG-3' R-5'-GTGTCCGTTCACCAACAGGAAG-3'	60°C	138
Beclin-1	F-5'-GGCTGAGAGACTGGATCAGG-3' R-5'-CTGCGTCTGGGCATAACG-3'	60°C	127
GAPDH	F: CAAAGTTGTCATGGATGACC R: CCATGGAGAAGGCTGGGG	60°C	195

Table 2. Derivative compound and imatinib concentration used in the different combinations evaluated and their respective combination index.

Drugs Combinations		**Combination Index (CI)**
Derivative compound (µM)	Imatinib (µM)	
5	0.17	0.59
10	0.33	0.37
20	0.67	0.27
40	1.34	0.44
80	2.68	0.38

Combination index values were generated by CompuSyn software using the formula $CI=(D)_1/(Dx)_1+(D)_2/(Dx)_2$, where $(Dx)_1$ or $(Dx)_2$ represents the concentration of drug 1 or 2 in a combination needed for achieving the same efficiency as that of the single drug 1 or 2 at D1 or D2, respectively. CI < 1 indicate drug synergism, CI > 1 show antagonism and CI = 1 represent additive effect.

Figures and Legends

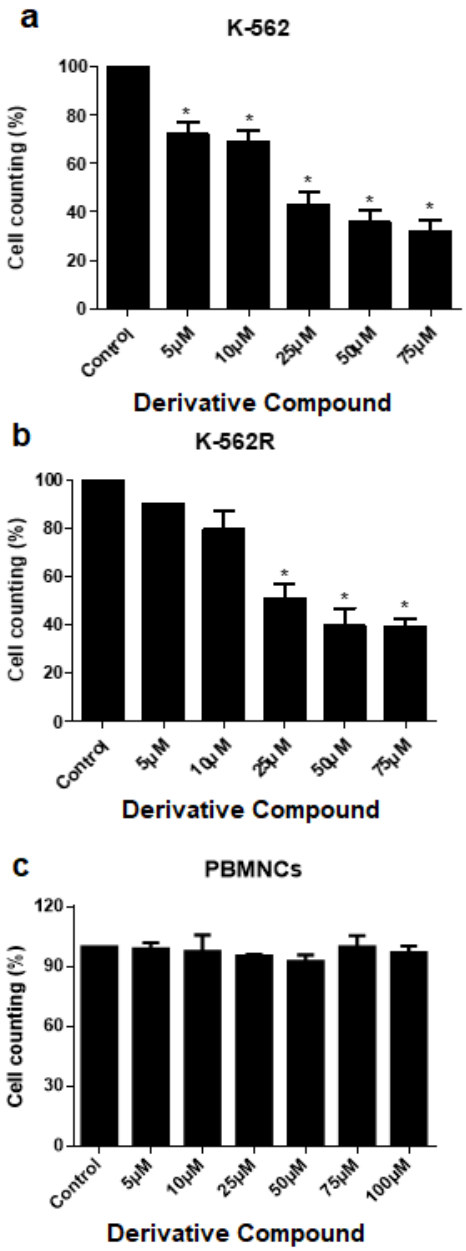

Figure 1. Inhibition of growth after 48h treatment with different concentrations of the derivative compound in (a) K-562 cells, (b) imatinib-resistant K-562 cells (K-562R) and (c) peripheral blood mononuclear cells (PBMNCs). Results are reported as mean ± standard deviation (SD) of three experiments (*$p<0.05$ relative to untreated control cells).

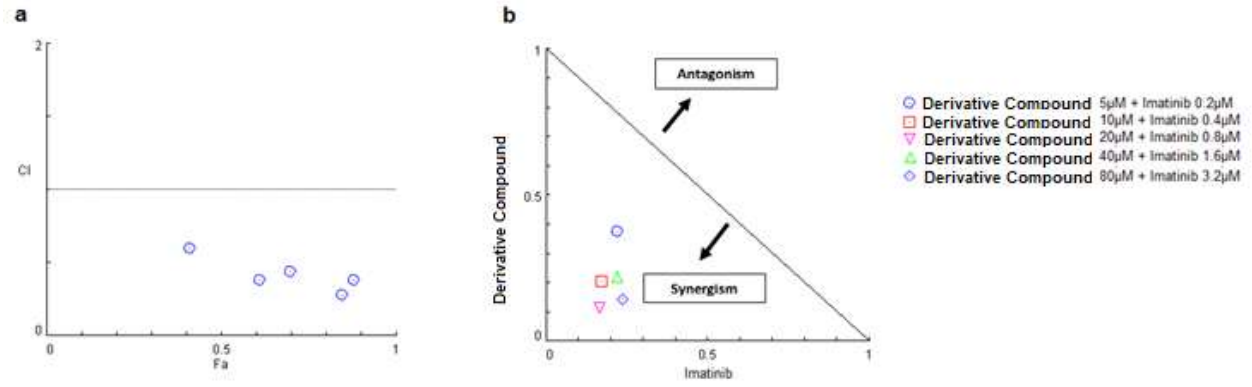

Figure 2. Combination of the derivative compound with imatinib mesylate synergistically inhibited growth of K-562 cell line after 48h treatment. (a) The CI values were calculated according to the Chou-Talalay' method by CompuSyn software and plotted with percent of cell growth inhibition as the fraction affected (Fa) cells. (b) Normalized isobologram for the combination. The symbols represent different combination ratio. Points that fall below the line are synergistic, above the line are antagonistic and on the line are additive.

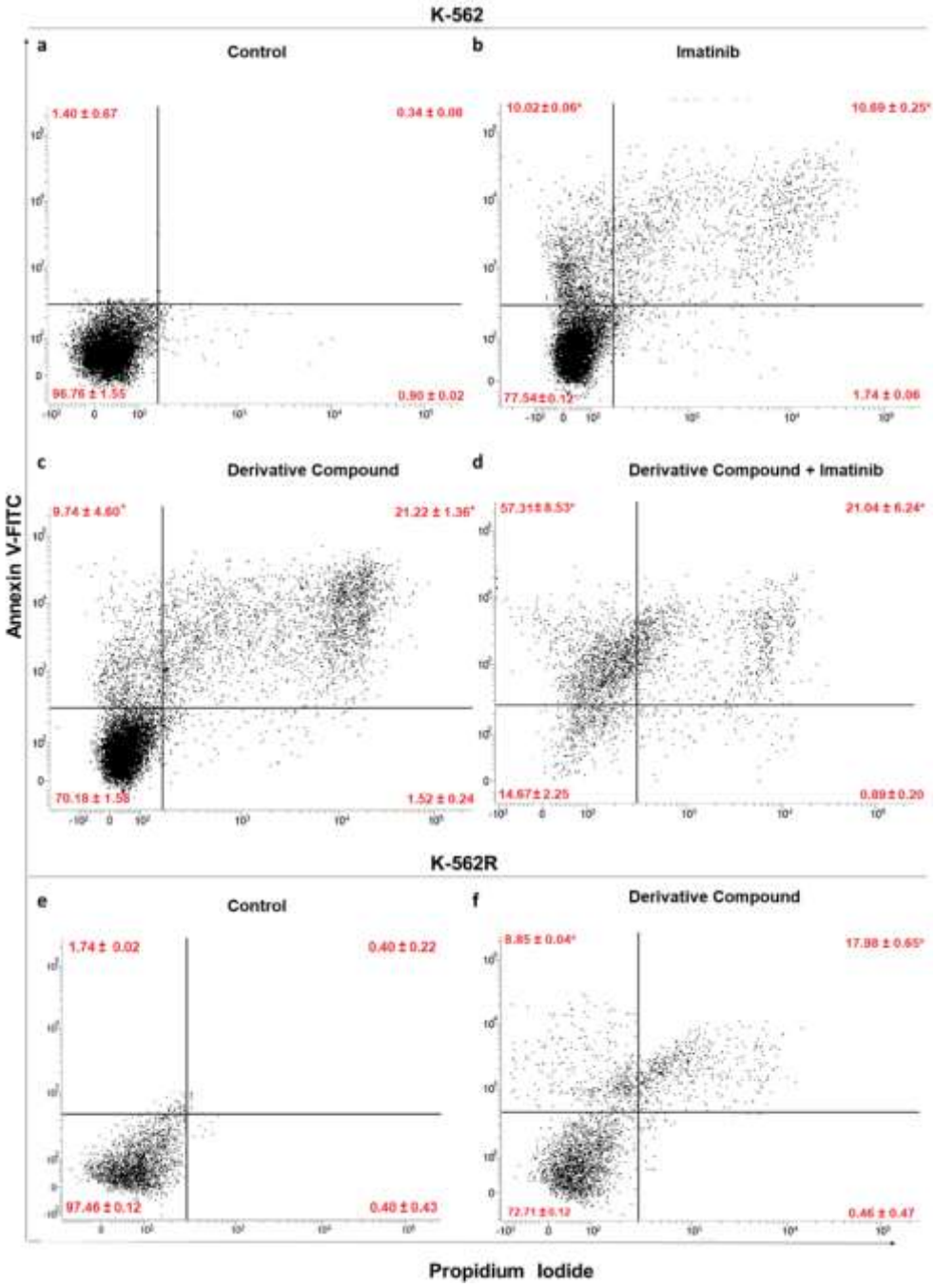

Figure 3. Analysis of apoptosis by flow cytometry after 48h of treatment of K-562 cells with (a) only RPMI-1640 medium, (b) imatinib mesylate, (c) derivative compound, (d) combination of derivative compound and imatinib mesylate, and of K-562R cells with (e) only RPMI 1640 medium, (f) derivative compound. Data are reported as mean ± standard deviation (SD) of three experiments (*$p<0.05$ relative to untreated control cells).

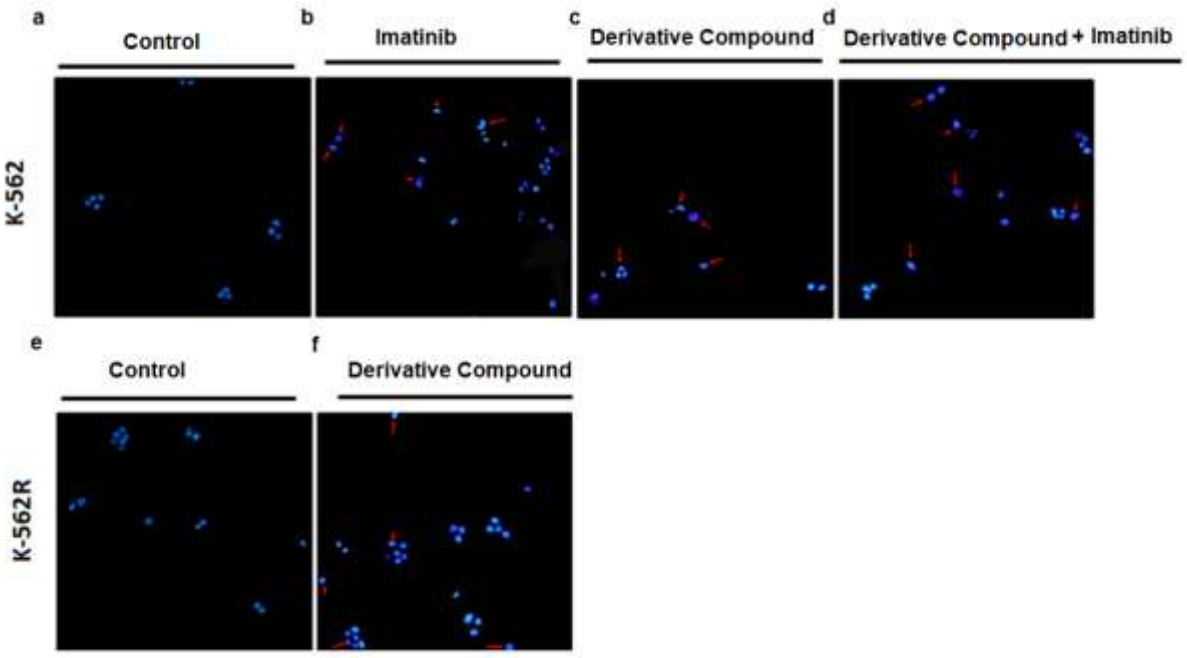

Figure 4. Apoptotic morphological changes observed by DAPI staining after 48h of treatment of K-562 cells with (a) only RPMI-1640 medium, (b) imatinib mesylate, (c) derivative compound, (d) combination of derivative compound and imatinib mesylate, and of K-562R cells with (e) only RPMI 1640 medium, (f) derivative compound. Classical apoptotic features such as cell shrinkage, blebbing and fragmented nuclei are highlighted (arrows).

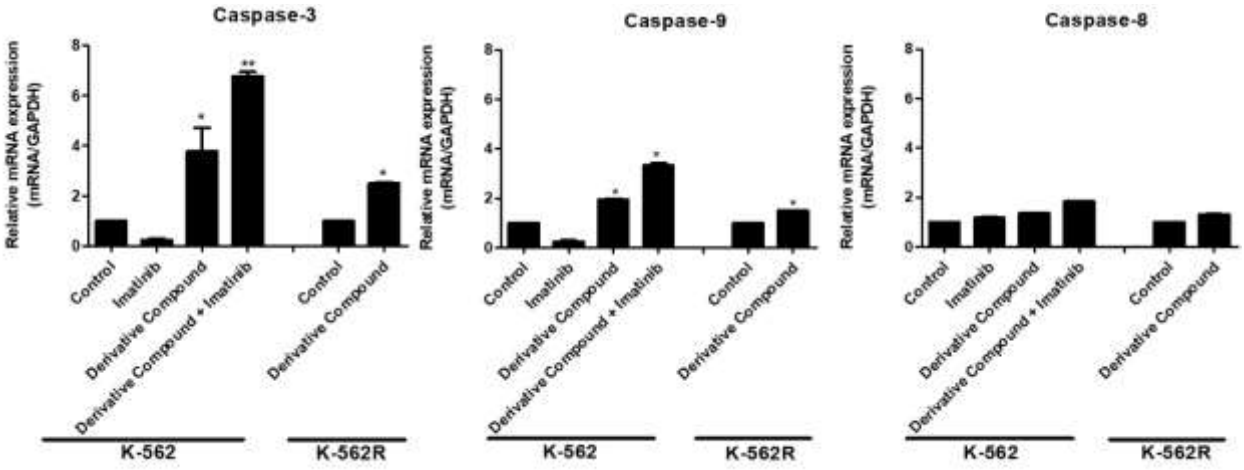

Figure 5. The mRNA expression levels of caspases 3, 9 and 8 were evaluated by qRT-PCR in K-562 cell line treated with imatinib mesylate, derivative compound and the combination of them, and in K-562R cells after treatment with the derivative compound. Treatments were performed with respective IC_{50} for 48h. Comparative analysis was performed with the $2^{-\Delta\Delta CT}$ method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control. The expression ratios were compared to the control. The values are representative of 3 different experiments. Data were compared by ANOVA followed by Tukey's test (*$p<0.05$ represents statistical significance).

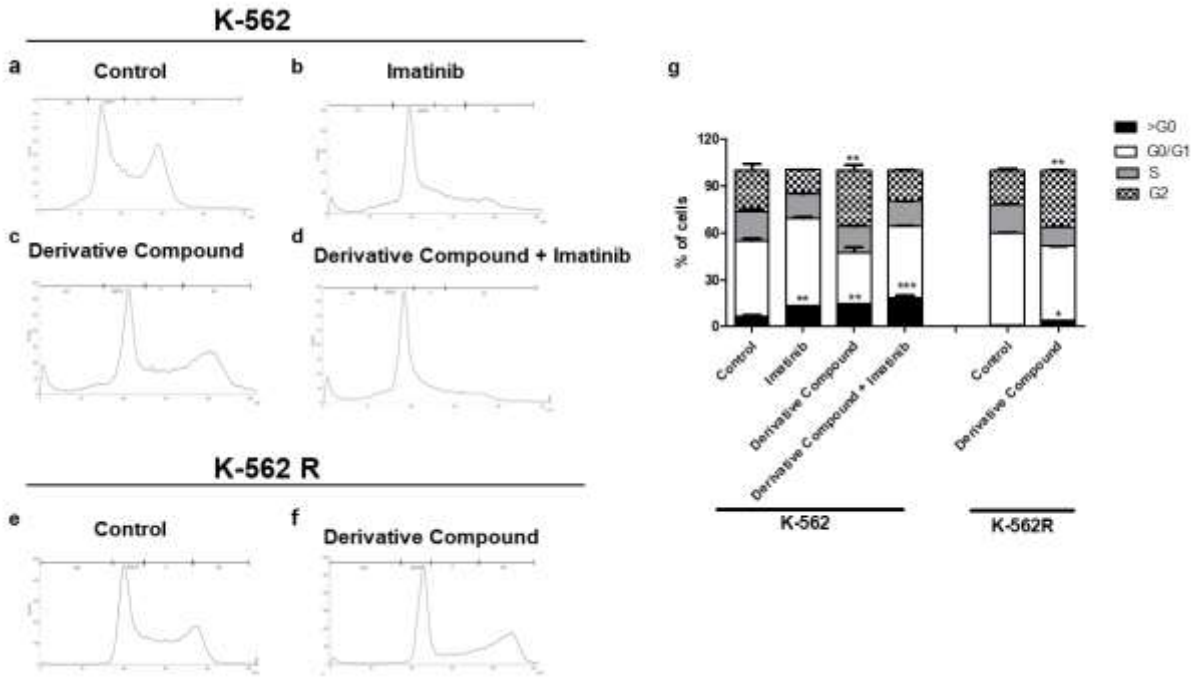

Figure 6. Cell cycle distribution after 48h of treatment of K-562 cells with (a) only RPMI-1640 medium, (b) imatinib mesylate, (c) derivative compound, (d) combination of derivative compound and imatinib, and of K-562R cells with (e) only RPMI 1640 medium, (f) derivative compound (g) Percentage of sub-G0, G0/G1, S and G2 are shown. Data are reported as mean ± standard deviation (SD) of three experiments (*p<0.05; **p<0.01; ***p<0.001 relative to untreated control cells).

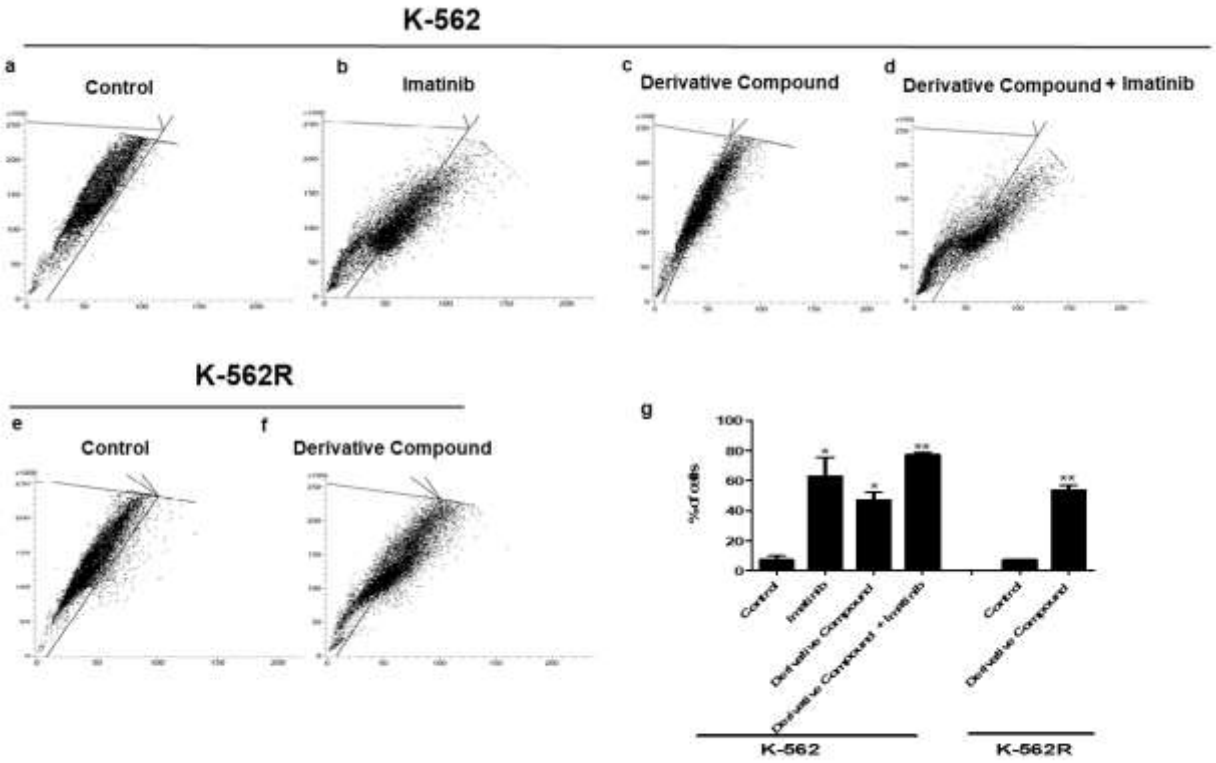

Figure 7. Autophagy observed with acridine orange (AO) staining by flow cytometry after 48 h of treatment of K-562 cells with (a) only RPMI-1640 medium, (b) imatinib mesylate, (c) derivative compound, (d) combination of derivative compound and imatinib, and of K-562R cells with (e) only RPMI 1640 medium, (f) derivative compound (g) Percentage of AO-positive cells are shown.

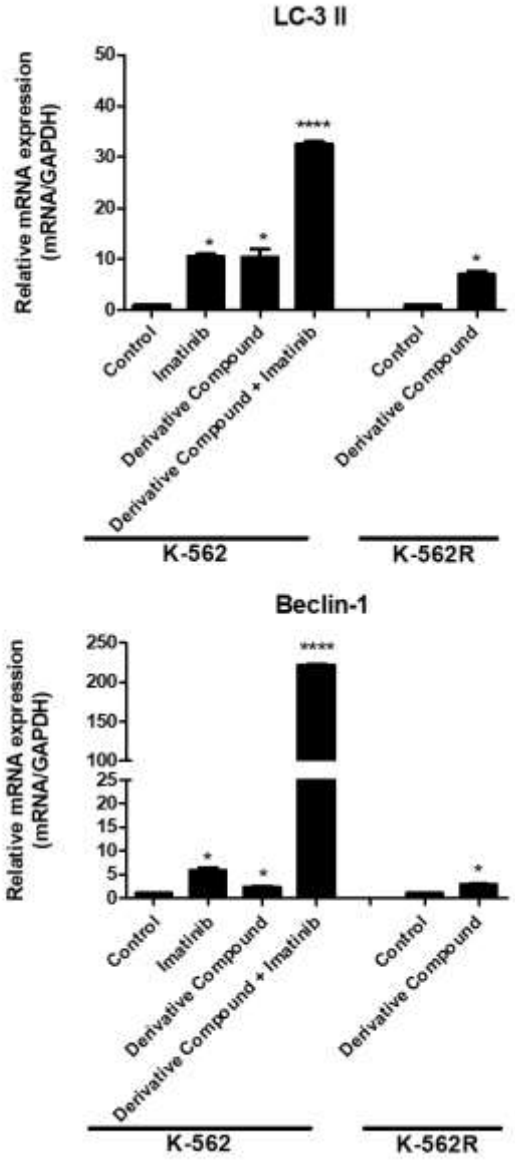

Figure 8. The mRNA expression levels of LC3-II and Beclin-1 were evaluated by qRT-PCR in K-562 cell line treated with imatinib mesylate, derivative compound and the combination of them, and in K-562R cells after treatment with the derivative compound. Treatments were performed with respective IC_{50} for 48h. Comparative analysis was performed with the $2^{-\Delta\Delta CT}$ method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control. The expression ratios were compared to the control. The values are representative of 3 different experiments. Data were compared by ANOVA followed by Tukey's test (*$p<0.05$ represents statistical significance).

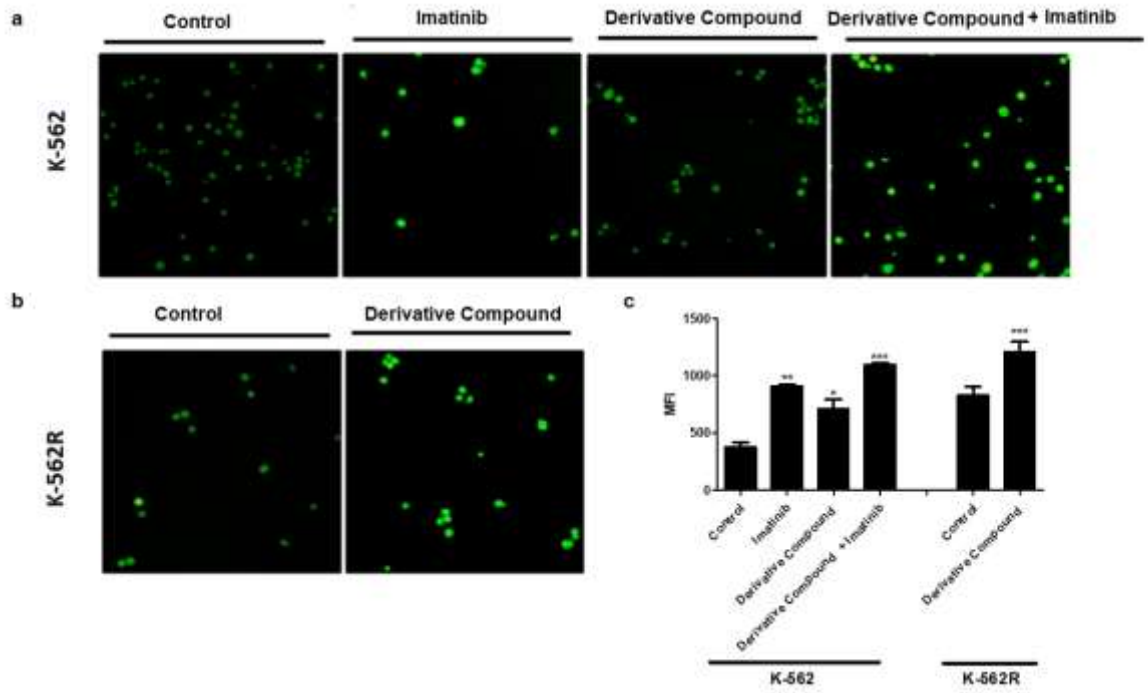

Figure 9. Influence of treatment with imatinib mesylate, derivative compound and their combination on reactive oxygen species (ROS). Cells were stained with 2,7-Dichlorofluorescin diacetate (DCFH-DA) and ROS level was observed by fluorescence microscopy in (a) K-562 cell and (b) K-562R, and (c) analyzed by flow cytometry.

V. DISCUSSÃO

As neoplasias podem ser divididas em neoplasias sólidas, as quais se caracterizam por um crescimento anormal de células de um tecido, ou neoplasias hematológicas, nas quais o aumento de células ocorre na medula óssea e/ou sangue periférico. Os tumores hematológicos mais comuns são as leucemias, as neoplasias mieloproliferativas, as linfoproliferativas e os linfomas. Dentre os cânceres sólidos, os mais frequentes são os de próstata, mama, colo de útero, pulmão, intestino, estômago e pele (SIEGEL; MILLER; JEMAL, 2017). As causas que levam ao desenvolvimento tumoral são variadas, podendo ser externas ou internas ao organismo, ou como frequentemente ocorre, de maneira combinada (AMERICAN CANCER SOCIETY, 2016). Os fatores externos podem estar relacionados com o ambiente, estilo e hábitos de vida dos indivíduos, principalmente alcoolismo e tabagismo (ABRALE, 2016). Já os fatores internos dizem respeito à própria biologia da doença, como fatores genéticos relacionados com alterações no DNA (INCA,2018).

O sucesso do tratamento do câncer depende muito do tipo tumoral e a fase do desenvolvimento em que se encontra a doença quando do seu diagnóstico. Quanto mais cedo for realizado, maiores são as taxas de resposta e as chances de cura. Por conseguinte, a elucidação de mecanismos envolvidos na fisiopatologia da doença, bem como a pesquisa de novos marcadores e alvos terapêuticos específicos, visam ampliar as possibilidades de intervenção precoce e redução da mortalidade da patologia.

É neste contexto que o sistema purinérgico tem sido relacionado através da sua conhecida influência na progressão tumoral, visto seu papel relacionado à proliferação, migração, diferenciação celular e secreção de fatores de crescimento e quimiocinas (DI VIRGILIO et al., 2018; DI VIRGILIO; ADINOLFI, 2017; LISA GIULIANI; CLARA SARTI; DI VIRGILIO, 2018). Diante dos poucos estudos que avaliam a relação do sistema purinérgico nas leucemias, no primeiro capítulo desse trabalho buscamos investigar o efeito do tratamento com imatinibe na expressão e funcionalidade das enzimas NTPDases em linhagem celular humana de LMC sensível e resistente ao imatinibe.

Inicialmente, para elucidar se a linhagem celular K562 (Ph+) de LMC apresentava diferença na atividade de hidrólise dos nucleotídeos de adenina em relação às células mononucleares de sangue periférico de indivíduos saudáveis (CMSP), foi realizada a

avaliação da atividade enzimática que indicou a presença de hidrólise dos nucleotídeos maior nas células leucêmicas. Posteriormente, foi realizada a identificação da expressão das *ENTPDs*, visto que a linhagem tumoral apresenta expressão diferenciada das enzimas em relação às células saudáveis. Esse resultado indicou que as enzimas NTPDases podem ter uma função diferencial na LMC em comparação com células de indivíduos saudáveis. Essa diferença de atividade enzimática em células tumorais já foi relatada em tumor endometrial, o qual apresentava expressão de CD39 e CD73 superior em relação ao tecido normal pela análise de imunohistoquímica. (ALIAGAS et al., 2014). Em pacientes com câncer de próstata, a taxa de hidrólise dos nucleotídeos de adenina foi elevada quando comparada a pacientes normais e o nível de expressão de CD73 foi relacionada à progressão tumoral (GARDANNI F et al., 2019).

A próxima etapa do trabalho consistiu na avaliação da influência do imatinibe sobre a sinalização purinérica. Para isso, primeiramente foi desenvolvido no nosso laboratório uma adaptação e validação da indução da resistência ao imatinibe nas células K-562 (Ph+). Para isso, as células foram tratadas com concentrações crescentes de imatinibe, na qual após 6 meses de tratamento a resistência final foi avaliada através da curva dose-resposta para determinação do IC_{50} (WANG et al., 2015). A resistência final foi determinada através do quociente do IC_{50} das células resistentes sobre IC_{50} das células sensíveis, na qual observamos que células K-562R apresentaram resistência ao imatinibe 64,08 vezes superior à das células K-562 (Ph+). Nosso resultado está de acordo com o encontrado por Wang e colaboradores que obtiveram um quociente de resistência final de 20,75 vezes superior nas células resistentes ao imatinibe (WANG et al., 2015). Observou-se que com o aumento da concentração de imatinibe nas células K-562 (Ph+), houve uma mudança fenotípica nas células, apresentando uma morfologia esferoide, assim como aumento de marcadores de pluripotência, como SOX-2 e Nanog e marcadores de adesão, CD44 e CD133 (BONO; DELLO SBARBA; LULLI, 2018; CAI et al., 2018; CURTARELLI et al., 2018; ZHANG et al., 2014). Sabe-se que a exposição a longo prazo de imatinibe induz a regulação positiva dos marcadores de adesão e alta expressão de Nanog e que este achado está diretamente relacionado com a quimioresistência. Além disso, a indução da resistência ao imatinibe está relacionada

com aumento da expressão de bombas de efluxo de drogas como ABCB1/MDR1 e ABCG2. São mecanismos independentes de BCR/ABL, nos quais as células tumorais não respondem ao tratamento ou a proteína oncogênica não está inibida de forma eficaz (BEGICEVIC; FALASCA, 2017; GOTTESMAN; FOJO; BATES, 2002; KOSZTYU et al., 2014).

Nossos resultados demonstraram que o efeito agudo do imatinibe (tratamento de 24 horas) aumentou a atividade de hidrólise dos nucleotídeos ATP, ADP, AMP e UDP, bem como a expressão das enzimas *ENTPD1,-2,-3, -5* e *NT5E.* Esse resultado não foi observado em 48 horas de tratamento, nos indicando como hipóteses de que a degradação do imatinibe já tenha ocorrido devido ao tempo de meia vida do fármaco, além da baixa estabilidade do ATP no meio extracelular. Outra possibilidade seria que ao longo do efeito crônico do tratamento até o desenvolvimento da resistência ao fármaco, possa ocorrer a diminuição da taxa de hidrólise e expressão das enzimas como também foi demonstrado nos nossos resultados. O mecanismo de ação do imatinibe ocorre no sítio de ligação do ATP da proteína quinase BCR-ABL, a qual bloqueia a ligação do ATP e consequentemente a inibição da atividade quinase (MITCHELL et al., 2012). Quando as células K-562 (Ph+) são tratadas com imatinibe, o mesmo interage com a proteína fazendo com que o ATP acumule. Sabemos que esse achado é reforçado pelo fato de que quando a fração de CMSP foi tratada com imatinibe não houve diferença na atividade enzimática dos nucleotídeos testados. Esse acúmulo intracelular de ATP poderia ser levado ao espaço extracelular através das vesículas secretoras (exocitose), microvesículas derivadas da membrana plasmática, por transportadores (família ABC), ou por canais (como panexina-1 e conexinas) (DI VIRGILIO; ADINOLFI, 2017). No meio extracelular o ATP poderia interagir com receptores ou ser degradado pelas enzimas NTPDases (ZIMMERMANN, 2001).

Como mencionado anteriormente, o papel do ATP e das NTPDases na LMC ainda é pouco investigado. Entretanto, em outros tipos de leucemias, como cultura primária e linhagens de LMA, o ATP exerce efeito inibitório na proliferação celular (DULPHY et al., 2014). Na LLC a expressão de CD39 está principalmente relacionada com estágios avançados da doença e desfecho clínico negativo (SCHETINGER et al., 2007). As

NTPDase1 e -2 apresentam relação principalmente com o sistema imunológico, além de apresentarem, funções no equilibro da hidrólise dos nucleotídeos principalmente de ATP e ADP. A NTPDase5 apresenta relação com importantes vias de sinalização celular como PI3K/PTEN e AKT as quais encontram-se alteradas na LMC, como por exemplo, na diminuição da apoptose via STAT5, hiperativação de moléculas antiapoptoticas Bcl-x, inativação de moléculas pró-apoptoticas BAD via AKT e além de desregulação da citoadesão celular (BRACCO; BERTONI; WINK, 2014; ROSSARI; MINUTOLO; ORCIUOLO, 2018). A diminuição do gene *ENTPD6* está relacionado com a resistência de células testiculares ao tratamento da cisplatina (TADA et al., 2011).

Diversos estudos investigaram a participação de receptores no desenvolvimento de resistência terapêutica. A análise da expressão do receptor P2X7 A e B foi avaliada em pacientes com LMA, na qual a subunidade P2X7 B foi relacionada com a resistência a dauronorrubicina e propensão a recaída no tratamento. Além disso, o cotratamento com antagonistas do P2X7 reduziu de forma mais eficaz o crescimento das células tumorais em modelo xenoenxerto de LMA (ORIOLI, E. et al.2019). Outro estudo também avaliou a participação dos receptores P2Y2, P2Y1, P2Y6 no mecanismo de resistência à inibição da ALK, uma proteína aberrante encontrada em um grupo de pacientes com câncer de pulmão de não pequenas células. Essa proteína tem como primeira linha de tratamento o medicamento crizotinibe, o qual também é um inibidor competitivo de ATP da tirosina quinase da proteína ALK (BURGESS et al., 2016). Esses receptores P2Y medeiam a resistência, pelo menos em parte, através da ativação da proteína quinase C (PKC) (DI VIRGILIO; ADINOLFI, 2017). A superexpressão de PKC também ocorre na resistência ao imatinibe na LMC levando à ativação da sinalização de RAF e MEK/ERK (MA et al., 2015). Em nosso trabalho, a investigação dos receptores aparece como uma perspectiva necessária para aprofundar o entendimento do sistema purinérgico e sua eventual participação no desenvolvimento da resistência aos ITQ.

Atualmente, uma nova abordagem para o sistema purinérgico está sendo investigada. Apesar da peculiaridade de cada tipo celular, o sistema purinérgico (nucleotídeos, receptores e enzimas) está surgindo com um fator prognóstico e alvo terapêutico no tratamento do câncer. Por exemplo, as enzimas CD39 e CD73 estão sendo

propostas como inibidores de *check-in point* na imunoterapia contra diferentes tipos de câncer (ALLARD; CHROBAK; STAGG, 2015). A utilização de inibidores de CD39 tem demonstrado importância no desenvolvimento tumoral de linfoma folicular, inibição da proliferação celular em células de melanoma e adenoma de colón (CAI et al., 2016a; MÜLLER; MURAKAMI; ROBSON, 2011; OKULICZ et al., 2016), além da participação de anti-CD73 em linhagem celular de câncer de mama, câncer hepático entre outros (MÜLLER; MURAKAMI; ROBSON, 2011).

Os compostos semissintéticos também são utilizados para inibição das principais enzimas que fazem parte do sistema purinérgico. Kanwal e colaboradores avaliaram o efeito de compostos de bases de Schiff derivados de triptamina através da relação estrutura atividade e *Docking* molecular com as enzimas NTPDases (KANWAL et al., 2019).

Apesar da grande descoberta dos ITQ, eles apresentam diversas reações adversas que fazem com que ocorra intolerância, descontinuidade ou interrupção do tratamento. As principais reações adversas relatadas pelos pacientes são distúrbios gastrointestinais, erupções cutâneas, além de trombocitopenia e neutropenia. Segundo dados do estudo SIMPLICITY, realizado na Europa e Estados Unidos, 27,8% dos pacientes com imatinibe descontinuaram o tratamento principalmente nos 3 primeiros meses (HEHLMANN et al., 2018).

Diante disso, os compostos naturais vêm sendo investigados como uma alternativa terapêutica para diversos tumores. Os terpenos apresentam atividade antitumoral em diversas linhagens celulares como câncer de pâncreas, próstata, mama, gástrico, pulmão, cervical, ovários e nas leucemias (FULDA, 2009). O principal mecanismo de ação dos quimioterápicos é a indução da morte via apoptose; todavia, as células tumorais podem apresentar resistência a esse tipo de morte (GALLUZZI et al., 2018). Nesse sentido, a busca de novos compostos capazes de promover múltiplos mecanismos de morte, máxima eficácia e poucos efeitos adversos vêm sendo estudado.

Estudos têm demonstrado que alterações nas posições C-3 e C-28 da estrutura triterpênica do ácido Betulínico (AB) podem levar a produção de derivados semissintéticos potencialmente mais ativos e mais seletivos que os produtos de origem

(NEDOPEKINA et al., 2017; WAECHTER et al., 2017). No segundo capítulo desse trabalho investigamos a ação citotóxica de um composto derivado do AB, no qual foi adicionado um anel hidroxietil-2-hidroxi-fenólico na posição C-28 através de uma ligação éster, além de demonstrar seu efeito sinérgico com imatinibe. Esse composto foi escolhido por apresentar resultados promissores nas linhagens tumorais de câncer cervical e mama (Couto NM., e colaboradores, dados não publicados).

Um dos efeitos esperados é o sinergismo, que tem como uma das vantagens retardar o mecanismo de resistência tumoral e a possibilidade de utilização de doses menores dos agentes quimioterápicos, possibilitando a redução do número de administrações e, como consequência, redução dos efeitos adversos associados com um aumento da adesão ao tratamento (CHOU, 2016; CHOU; MARTIN, 2005). Para a análise do sinergismo entre o composto derivado e o imatinibe utilizou-se o software CompuSyn (Cambridge, MA). O efeito dessa combinação foi expresso como índice de combinação (CI).

O CI é um método usado para quantificar o tipo de interação causada pelo efeito da combinação de diferentes fármacos. Esse método é baseado na Lei da Ação das Massas e o princípio do efeito-médio derivado de modelos enzimáticos desenvolvidos por Chou and Talalay, o qual é amplamente utilizado em terapias antineoplásicas. Sendo assim, por meio desse método, um CI igual a 1,0 indica um efeito aditivo, um CI maior que 1,0 indica antagonismo, enquanto que um CI menor que 1,0 indica sinergismo (CHOU; MARTIN, 2005). O isobolograma é uma representação gráfica na qual também se avalia sinergismo ou antagonismo em combinações de fármacos (CHOU, 2016). A partir da avaliação de citotoxicidade dos compostos isolados é possível calcular as concentrações em combinação utilizadas. Nesse estudo, a combinação entre composto derivado e imatinibe resultou em doses sinérgicas em todas as concentrações testadas na linhagem K-562 (Ph+). E a combinação com o menor CI (maior sinergismo) foi considerada para os ensaios subsequentes.

Danisman Kalindemirtas e colaboradores investigaram a combinação de produtos naturais com imatinibe na linhagem celular K-562 (Ph+), no qual luteolina, apigenina e sinensetina apresentaram efeito sinérgico quando combinado com imatinibe e

aumentaram a proporção de células apoptóticas (DANIŞMAN KALINDEMIRTAŞ et al., 2018). Em modelo xenoenxerto em camundongos de câncer de pâncreas, a combinação entre AB e mitomicina produziu atividade antitumoral sinérgica, inibindo a proliferação, invasão e angiogênese (GAO et al., 2011). O AB e doxorubicina, cisplatina ou actinomicina B apresentaram efeito sinérgico em diferentes linhagens tumorais como meduloblastoma, glioblastoma, melanoma e em cultura primária de carcinoma renal induzindo apoptose e superexpressão de Bcl-2 (FULDA; DEBATIN, 2005).

Outro aspecto investigado em nosso trabalho foi o possível mecanismo de ação do composto derivado nas células K-562 (Ph+) e K-562R. Sabe-se que a apoptose e autofagia desempenham papéis fisiológicos e patológicos em diversas doenças, incluindo as neoplasias, e fazem parte de uma sequência de eventos que causam alterações na estrutura celular e, eventualmente, resultam em morte celular (GALLUZZI et al., 2018). Entretanto, ainda é controversa a relação entre os próprios mecanismos de apoptose e autofagia. Existem duas maneiras pelas quais a autofagia e apoptose podem estar diretamente ligadas (GUMP; THORBURN, 2013). O processo da autofagia poderia controlar a apoptose tornando-o mais ou menos provável e, também, a apoptose poderia controlar a autofagia através da ativação da via das caspases (MAIURI et al., 2007). De acordo com nossos resultados, o composto derivado apresentou efeito citotóxico nas células K-562 (Ph+) e K-562R, demostrando mecanismo de morte celular através da indução da apoptose pela via intrínseca e alteração da autofagia com aumento da expressão de LC3II e Beclin-1. Estes resultados foram corroborados pela detecção de alterações no ciclo celular e indução do aumento de espécies reativas de oxigênio (ROS).

Wang e colaboradores demonstram que ácido gambógico inibe a fosforilação da via AKT em células de câncer de pâncreas e essa inibição ativa Beclin-1, regulador chave no processo da autofagia. Também demonstrou que a supressão da expressão Bcl-2 aumenta a expressão de Beclin-1. A proteína Bcl-2 é um fator importante na autofagia, já que se liga à Beclin-1 inibindo a autofagia e impedindo esta última proteína de desempenhar seu papel central na autofagia (WANG et al., 2019). Em células K-562 (Ph+), o tratamento com fungo medicinal *Ganoderma* induziu apoptose mitocondrial na qual foi correlacionada com a fragmentação do DNA, ativação de caspases-3/8/9 e

desregulação da via BAX/Bcl-2. A autofagia foi investigada como atividade citoprotetora e, apesar do aumento do acúmulo de LC3-II, foi evidenciada desregulação Beclin-1/Bcl-2 e formação de vesículas autofágicas. Já o pré-tratamento com inibidores da autofagia 3-MA e CQ aumentaram a apoptose, além de induzir o aumento de ROS independente da via apoptose (HSEU et al., 2018).

Estudos evidenciam o dano gerado no DNA pelo excesso de oxigênio reativo como consequência à indução da apoptose e autofagia. Em células de câncer cervical, BA induziu a parada no ciclo celular na fase G0/G1 na qual foi associada à apoptose pela expressão aumentada de Bad e Caspase-9 e aumento de ROS. O aumento de ROS foi inibido pela utilização de um antioxidante, glutationa, que bloqueou o processo apoptótico (XU et al., 2017).

Tendo em vista todos os resultados apresentados anteriormente, o potencial demonstrado contra células leucêmicas sensíveis e resistentes ao imatinibe e o mecanismo pelo qual o composto derivado age, nosso trabalho fornece evidências de que ele poderia ser utilizado como adjuvante na terapia antineoplásica na LMC.

I. CONCLUSÕES

A partir dos resultados obtidos nesse estudo, puderam-se obter as seguintes conclusões:

a) Foi demonstrado, de forma inédita, que a linhagem K-562 (Ph+) apresenta expressão das *ENTPDs* e *NT5E.*
b) A hidrólise dos nucleotídeos foi padronizada na linhagem tumoral, a qual apresenta um aumento de atividade em relação às células normais;
c) O tratamento de 24 horas com mesilato de imatinibe aumentou a expressão das E*NTPD*1,-2,-3,-5, além de aumentar a hidrólise dos nucleotídeos de adenina e UDP;
d) Através da técnica do HPLC conseguimos avaliar a degradação do ATP ao longo do tempo;
e) A resistência ao imatinibe diminui a expressão e atividades das enzimas, principalmente a E*NTPD*1 e -5;
f) O composto derivado demonstrou um potencial antitumoral em linhagem celular K-562 (Ph+) e K-562R;
g) O composto derivado apresentou efeito sinérgico quando tratado com mesilato de imatinibe nas células K-562 (Ph+);
h) O composto derivado apresentou mecanismo de morte celular através da indução da apoptose via caspase intrínseca, alteração da autofagia com aumento da expressão de LC3II e Beclin-1. Além disso, foi observada alteração no ciclo celular e indução do aumento de espécies reativas de oxigênio (ROS).

VII. PERSPECTIVAS

Diante dos objetivos inicialmente propostos para avaliação do sistema purinérgico em modelo celular de LMC e a utilização de um novo composto como adjuvante ao tratamento, surgem algumas perspectivas:

a) Avaliação da participação dos receptores purinérgicos no desenvolvimento da LMC e sua relação com o tratamento do mesilato de imatinibe;
b) Avaliação da expressão da NTPDase 5 por análise de citometria de fluxo;
c) Avaliação do efeito do tratamento com composto derivado nas vias de sinalização celular por western blot e em modelos *in vivo;*
d) Reavaliação de todos os modelos experimentais em células com sítios de resistência dependentes de BCR-ABL como mutações de ponto (em especial T315I);
e) Utilização de modelos de cultura primária a partir de células de pacientes sensíveis e resistentes ao imatinibe.

VIII. REFERÊNCIAS BIBLIOGRAFICAS

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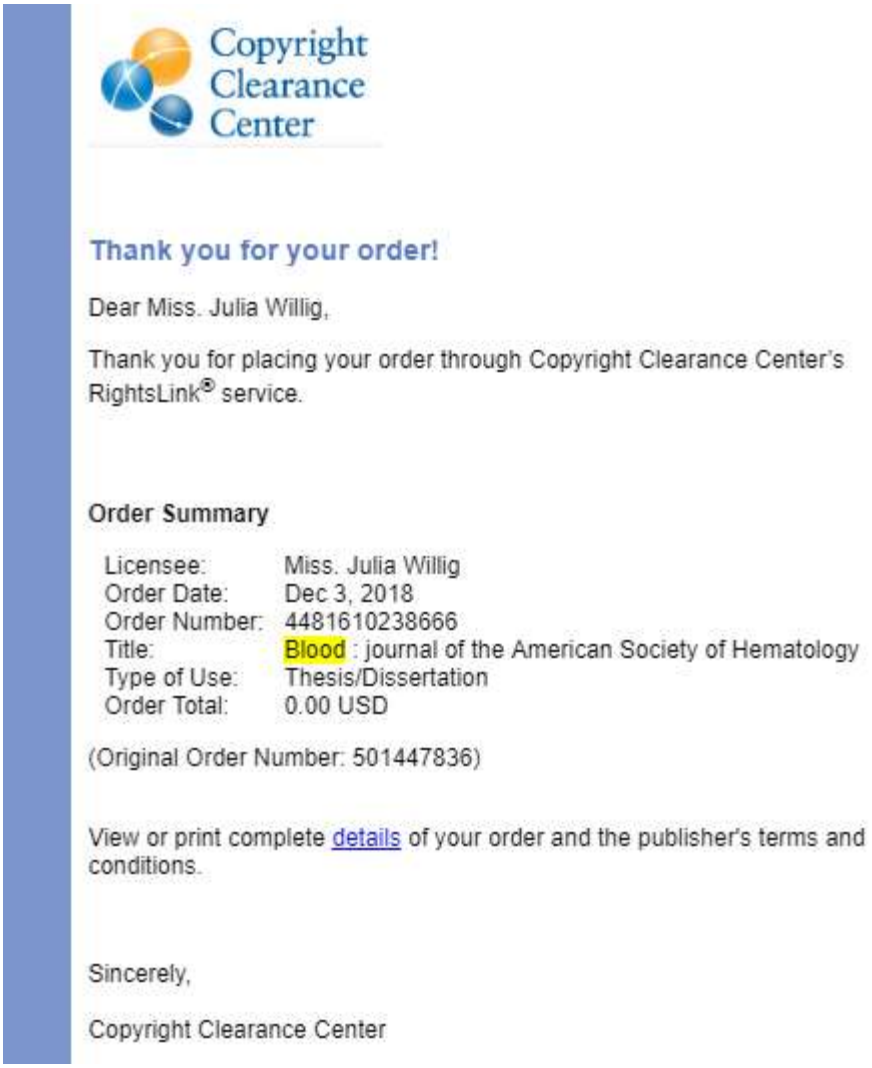
IX. ANEXO

IX.1 Autorização para uso de imagem como figura 3 da seção Introdução



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