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"Definição de alvos moleculares em diferenciação, morte e resistência de células tumorais"

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas para obtenção do Título de Doutor em Biologia Funcional e Molecular na área de Bioquímica.

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RESUMO

A eficiência do tratamento do câncer sob vários aspectos, mesmo com os avanços "farmacotecnológicos", ainda permanece como desafio para a medicina. Diante desse fato, novos agentes que atuem de forma alvo-específico, apresentem poucos efeitos colaterais e possam impedir o "escape" das células tumorais à indução de morte, são extremamente desejáveis. No presente trabalho, foram abordados 3 aspectos da atividade antitumoral da riboflavina: indução da apoptose das células de câncer prostático, indução da diferenciação de células leucêmicas e aumento da biodisponibilidade intracelular do quimioterápico mitoxantrona. Sob esses 3 aspectos, através do estudo de vias de sinalização, identificamos mediadores moleculares responsáveis pela ação da riboflavina como antitumoral. A atuação da riboflavina irradiada em células de câncer de próstata foi dependente da inibição da PI3K. De forma interessante, observamos uma potencial ação inibitória da metástase, evidenciada pela inibição das metaloproteinases 2 e 9 e da angiogênese pela diminuição da expressão do VEGF. Em relação à diferenciação das células leucêmicas evidenciamos o envolvimento do receptor TNFR1, bem como das proteínas ciclina D, JNK, Src quinase e das proteínas tirosinas fosfatases SHP2 e PTPα. Portanto, proteínas com diferentes localizações celulares foram afetadas, culminando com a diminuição da proliferação, manutenção da sobrevivência celular, interrupção da progressão do ciclo celular e reorganização do citoesqueleto, efeitos metabólicos essenciais para a ocorrência da diferenciação. Esse trabalho de tese também demonstrou a aplicabilidade da técnica do Pepchip para a identificação de diferenças metabólicas entre duas linhagens das células da leucemia eritroblástica, K562 e Lucena. Outra abordagem interessante nesse trabalho foi o uso da riboflavina com a finalidade de aumentar a biodisponibilidade celular de quimioterápicos tradicionais e a utilização de inibidores de proteínas fosfatases como estratégia para reverter a resistência de células tumorais. De acordo com os resultados, esse trabalho aponta de forma inédita uma nova função da vitamina B2 como antitumoral.

ABSTRACT

Cancer therapy efficiency, under several aspects, even with the progress of “pharmacotechnology”, remains as a challenge for the medicine. According to this factor, new agents that act on specific target, present low side-effects and prevent cancer cells “escaping” from death induction, are extremely desirable. In this work, 3 aspects of antitumoral property of riboflavin were evaluated: apoptosis induction of prostate cancer cells, leukaemic cells differentiation and increase of intracellular bioavailability of chemotherapic (mitoxantrone). Under these 3 aspects, and by signal transduction approach, we identified molecular mediators responsible for antitumoral activity of riboflavin. The action of irradiated riboflavin on prostate cancer cells was dependent on PI3K inhibition. Interestingly, we also observed a potential inhibitory action of metastasis, as demonstrated by the inhibition of metalloproteinases 2 and 9 and decreasing of angiogenesis by downregulation of VEGF. In relation to leukaemic cells differentiation we demonstrated the involvement of TNFR1, as well as cyclin D, JNK, Src kinase and protein tyrosine phosphatases SHP2 and PTPα. Therefore, proteins with different cellular localizations were affected culminating in decreasing of cell proliferation, maintaining cell survival, cell cycle arrest and cytoskeleton rearrangement, crucial metabolic effects for the occurrence of differentiation process. This work also demonstrated the applicability of Pepchip technique for identifying the differences between 2 erytroblastic leukaemia cell lines, K562 and Lucena. Other interesting approach, in this work, was the use of riboflavin for improving chemotherapic cellular bioavailability and the strategical use of protein phosphatase inhibitors for reverting tumor cells resistance. According to our findings, this work spotlights the novel function of the vitamin B2 as an antitumoral agent.

ÍNDICE

ABREVIATURAS

AIF ("apoptosis-inducing factor"): fator de indução de apoptose

Bax ("Bcl-2 associated x protein"): proteína x associada a Bcl-2

Bcl-2 ("B-cell lymphoma protein 2"): proteína inibidora de apoptose detectada primeiramente em linfomas de células B

Bid ("BH3 interacting domain death agonist"): agonista de domínio de morte associado a BH3

CD45: tirosina fosfatase hematopoiética célula-específica

Cdc(s) ("cell division cycle"): proteína(s) reguladora(s) da divisão celular

DISC ("death-inducing signaling complex"): complexo intracelular de indução de morte

ERKs ("extracellular signal-regulated kinases"): quinases reguladas por sinal extracelular

FAD: flavina adenina dinucleotídeo

FADD ("Fas-associated death domain protein"): proteína com domínio de morte associada a Fas

Fas / Apo1 / CD95: membro da superfamília de receptores TNF

FasL ("Fas-ligand"): proteína ligante de receptor Faz

FAK ("Focal Adesion Kinase"): Proteína Quinase de Adesão Focal

FMN: flavina mononucleotídeo

IAP ("inhibitory apoptosis protein type 1"): proteína inibidora de apoptose

IKKα ("I Kappa B kinase alpha"): quinase α de IκB

IκB: proteína inibidora de NFκB

JNKs ("c-Jun NH_2 terminal protein kinases"): proteínas quinases NH_2 - terminal de c-Jun

MAPKAPKs: proteínas quinases ativadas por MAPKs

MAPKK: MAPK quinase

MAPKKK: MAPKK quinase

MAPKPs / MKPs: fosfatases de MAPKs

MAPKs ("mitogen-activated protein kinases"): proteínas quinases ativadas por mitógenos

MDR ("multidrug resistance"): fenótipo celular caracterizado pela resistência a múltiplas drogas

MEK (MAPK ERK kinase): proteína quinase de ERK

MMP-2 ("matrix metalloprotease 2"): metaloproteinase de matriz tipo 2

MMP-9 ("matrix metalloprotease 9"): metaloproteinase de matriz tipo 9

MNKs ("MAPK-interacting kinases"): proteínas de interação a MAPKs

MSKs ("mitogen and stress ativated kinases"): quinases ativadas por estresse e mitógenos

NFκB: fator nuclear kappa B

p21: proteína inibidora de quinases dependentes de ciclinas

PARP ("poli ADP-ribose polymerase"): poli (ADP-ribose) polimerase

PI3K ("phosphatidylinositol 3-kinase"): fosfatidilinositol-3 quinase

PKA ("protein kinase A"): proteína quinase dependente de AMP cíclico

PKB / Akt ("protein kinase B"): proteína quinase B

PKC ("protein kinase C"): proteína quinase C

PP2A: proteína fosfatase 2A

PPPs: fosfoproteínas fosfatases

PTEN ("phosphatase and tensin homolog"): proteína tirosina fosfatase supressora de tumor

PTPs: proteínas tirosina fosfatases

PTPα ("Protein Tyrosine Phosphatase alpha"): Proteína Tirosina Fosfatase alpha

LMW-PTP ("Low Molecular Weight Protein Tyrosine Phosphatase"): Proteína Tirosina Fosfatase de Baixa Massa Molecular.

Raf: serina-treonina quinase implicada na sinalização de crescimento e sobrevivência celular.

Ras ("rat sarcoma viral oncogene"): família de proteínas GTPásicas reguladoras de diversas vias de transdução de sinais associadas ao crescimento, diferenciação e apoptose celular.

SHP2 ("SH2 containing protein tyrosine phosphatase 2"): proteína tirosina fosfatase com domínio SH2

Smac/DIABLO ("second mitochondria-derived activator of caspase / direct IAP binding protein with low PI"): proteína mitochondrial inibidora de IAPs

STATs ("signal transducers and activators of transcription"): moléculas transdutoras de sinal e ativadoras de transcrição

TNF ("tumor necrosis factor"): fator de necrose tumoral

TNFR ("tumor necrosis factor receptor"): receptor do fator de necrose tumoral

TRADD ("TNF receptor associated death domain"): domínio de morte associado a TNFR

TRAF ("TNF receptor-associated factor"): fator associado ao receptor TNF

TRAIL / Apo 2L ("TNF receptor apoptosis-inducing ligand"): membro da superfamília de receptores

VEGF ("Vascular endothelial growht factor"): fator de crescimento do endotélio vascular

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1. Introdução

1.Introdução

1.1. Câncer

O Câncer é originado por um processo evolucionário pelo qual células somáticas mutadas escapam do rigoroso controle exercido por mecanismos celulares no sentido de evitar a divisão descontrolada. Muitos mecanismos têm sido propostos e definidos na tentativa de explicar o surgimento, progressão, manutenção tumoral, e mais recentemente fatores relacionados com a aquisição de resistência a terapias vêm sendo intensamente estudados e definidos (Lowe *et al*., 2004). Consequentemente, muitos aspectos relacionados com o desenvolvimento do câncer foram exaustivamente estudados e muitos fatores atualmente são definidos como promotores desta doença, tais como: predisposição genética, estilo de vida, fatores ambientais (exposição à radiação ou carcinógenos). Acrescentado a estes, outros importantes mecanismos que permitem a progressão tumoral em sítios secundários, mais conhecido como metástase, e também aqueles responsáveis pela aquisição de resistência, que permitem a manutenção da célula tumoral mesmo depois de terapias agressivas, têm sido alvos de investigação.

Apesar dos avanços no desenvolvimento de fármacos para alvos específicos, a quimioterapia tradicional ainda se mantém como opção preferencial de escolha nos protocolos terapêuticos. No entanto, a eficácia desses protocolos ainda se encontra abaixo do esperado.

Tendo em vista que os quimioterápicos, além de não serem muitas vezes alvo-específicos, quando usados em monoterapia parecem não ser tão eficientes. Portanto, alguns especialistas têm cogitado que este fator poderia favorecer o desenvolvimento da resistência (Stebbing *et al*, 2007). Assim, mais do que a definição de inibidores para alvos específicos, é preciso levar em consideração que em uma célula tumoral vários mediadores biológicos podem estar alterados. Portanto, estes mediadores biológicos são alvos potenciais para

uma terapia combinada e teoricamente mais eficiente e consequentemente, tornando o tratamento melhor tolerado pelo paciente.

1.1.2. Leucemias

A carcinogênese é um processo de múltiplos passos envolvendo anormalidades na expressão ou função de proteínas codificadas por uma variedade de genes pertencentes a uma mesma célula. Tais anormalidades afetam o balanço entre proliferação celular, apoptose e diferenciação e nas malignidades hematopoiéticas levam a uma expansão anormal de clones de células tronco (Alison *et al*., 2004). Observa-se que a total transformação celular para a malignidade exige mudança numa gama de genes e apenas anormalidades que desencadeiam proliferação anormal não são suficientes para a gênese da célula maligna. De fato, a transformação para a malignidade exige a alteração na expressão de um conjunto de genes o que, geralmente, leva ao desenvolvimento de um fenótipo de resistência à morte celular apoptótica. Logo, anormalidades em vários genes das células tronco são necessárias para desregular a proliferação celular e, também, desviar os controles que poderiam normalmente desencadear apoptose, diferenciação ou senescência.

O quadro de leucemia mielóide aguda (LMA) caracteriza-se por um grupo de desordens em clones de células tronco hematopoiéticas levando ao acúmulo de mais de 30% de blastos na medula óssea. Esse acúmulo leva a substituição das células normais mielocíticas, megacariócitos e células eritrocíticas resultando em alteração no funcionamento da medula óssea e complicações associadas tais como cegueira, anemia e infecções (Alison *et al*., 2004; McKenzie, 2005). LMA são heterogêneas e 40% dos pacientes possuem anormalidades genéticas como inversões e translocações cromossomais que levam a fusão de genes e síntese de proteínas com funções alteradas. Essas translocações geralmente envolvem fatores de transcrição e outras moléculas relacionadas ao aparato transcricional e resultam em proliferação anormal de células precursoras mielóides, diminuição na taxa de morte apoptótica e interrupção do ciclo celular e processo de diferenciação (Alison *et al*., 2004;

Bernasconi *et al*., 2004; McKenzie, 2005). A incidência de LMA aumenta com a idade sendo mais comum na sexta década de vida (McKenzie, 2005). A doença também atinge crianças e jovens, porém adultos mais velhos quando comparados com pacientes jovens, têm um pior prognóstico (Stone *et al*., 2004).

Durante os últimos 20 anos as terapias básicas usadas no tratamento da LMA mudaram muito pouco. Os avanços no entendimento da fisiopatologia da doença ainda não levaram a uma melhora significativa na sobrevida de adultos e somente um terço destes pacientes acaba conseguindo a cura. A sobrevivência livre da doença é rara e os efeitos adversos do tratamento acabam sendo pouco tolerados por pessoas mais velhas (Stone *et al*., 2004).

Atualmente o desenvolvimento de novos tratamentos para a LMA baseia-se em terapias mais direcionadas enfocando alvos específicos, a exemplo do fármaco Imatinib (Gleevec®) usada com sucesso significativo no tratamento de leucemia mielóide crônica (LMC). Modulação de resistência, estratégias anti-angiogênicas, imunoterapias e agentes moduladores de transdução de sinais são algumas das novas terapias em desenvolvimento para o tratamento da LMA. Neste contexto, o trióxido de arsênio (As_2O_3) tem se mostrado um fármaco promissor, de sucesso significativo no tratamento de LMA reincidentes principalmente quando usado em associação com o ácido transretinóico (ATRA), tratamento que tem levado a um índice de cura de 70 a 85% dos casos de leucemia promielocítica (Stone *et al*., 2004; Tallman, 2004). Adicionalmente, avanços têm sido documentados no desenvolvimento de inibidores de baixa massa molecular relativa, os quais geralmente têm como alvos componentes de vias de sinalização como no caso da cascata Ras-ERK, freqüentemente ativada por mutações (Levis, 2005).

1.1.2.1. Câncer e Leucemias no Brasil

Segundo dados da literatura (Parkin *et al*., 2001) a previsão para o ano 2000 seria de mais de 10 milhões de novos casos de câncer no mundo, dentre os quais 53% ocorreriam em países em desenvolvimento. No Brasil, segundo o

Instituto Nacional do Câncer (INCA), as estimativas para o ano 2006 apontaram uma média de 472.050 novos casos de câncer, acompanhando a mesma magnitude observada no mundo, sendo que os tipos mais incidentes, à exceção de pele não melanoma, foram os de próstata e pulmão no sexo masculino e mama e colo do útero para o sexo feminino.

As estimativas apontaram, também, aumento no número de casos de leucemias sendo esperado um total de aproximadamente 9.550 casos novos no Brasil, dos quais 5.330 afetaram indivíduos do sexo masculino e 4.220 indivíduos do sexo feminino**.**

Ainda tomando como base as estimativas do INCA, as leucemias contribuirão com significativa parcela de novos casos de câncer no Brasil, atingindo principalmente as populações dos maiores centros urbanos reconhecidamente detentores de precários sistemas de saúde e recursos econômicos insuficientes para o adequado atendimento do grande número de usuários. Desta maneira, terapias mais eficientes no tratamento da leucemia reduziriam não somente o número de óbitos, mas também o tempo e recursos gastos durante o tratamento de pacientes. Os tratamentos químio e radioterápicos são bastante custosos para o sistema público de saúde devido ao relativo alto custo dos fármacos e equipamentos utilizados e, também, a mão-de-obra especializada necessária ao acompanhamento do paciente durante os períodos de internação. Uma terapia mais efetiva que proporcionasse melhor prognóstico para os pacientes e menos gastos para o Estado seria de grande interesse e vantagem para o Brasil, fato que justificaria maiores investimentos na área de pesquisas básicas e desenvolvimento de medicamentos.

1.1.2. Cancer de Próstata

O câncer de próstata continua sendo uma das causas mais comuns de adenocarcinomas nos homens, perdendo apenas para o câncer de pulmão. Como o câncer de próstata raramente é sintomático, cerca de 38% a 51% dos pacientes apresentam metástase quando diagnosticados (Papatsoris and

Papavassilou, 2001). O tecido alvo da metástase relacionado com o câncer de próstata é o osso, sendo a metástase óssea uma das principais causas de mortalidade. Acredita-se que fatores produzidos pelas células do câncer estimulam os osteoclastos, células ósseas responsáveis pela reabsorção da matriz (Inoue *et al*., 2005).

Mesmo diante de tantas informações a respeito do câncer de próstata, pouco progresso tem sido alcançado no tratamento desta doença. Atualmente, várias estratégias têm sido definidas como promissoras para o tratamento, incluindo a terapia gênica (Figueiredo *et al*, 2007; Freytag *et al*, 2007).

1.1.2.1. Processo de Invasão Celular

A metástase é a capacidade que um tumor maligno tem de estabelecer sítios secundários em tecidos funcionalmente não relacionados com aquele que o originou. O tumor sofre mudanças progressivas no seu fenótipo de forma a evoluir, conseguindo vias de disseminação (linfática e hematogênica) e atingir diversos órgãos. A metástase ocorre quando as células neoplásicas desprendidas do tumor primário conseguem se adaptar ao micro ambiente do novo tecido a ser colonizado. A formação de um sítio secundário tumoral dar-se a partir do desprendimento da célula do tumor primário e sua migração/invasão para outro tecido onde o microambiente favoreça sua permanência (Podgorski *et al*, 2007). Microscopicamente foi determinado que o processo de invasão tumoral inclui adesão, proteólise e migração através da lâmina basal e a chegada da célula ao tecido alvo da invasão. Do ponto de vista molecular a função de diferentes genes e fatores está envolvida nestes processos: incluindo moléculas de adesão célula-célula, moléculas de adesão célula-matriz, proteases, e também proteínas envolvidas na regulação dos filamentos de actina (Crnic and Christofori, 2004; Ye *et al*, 2007).

1.1.2.2. Metaloproteinases de Matriz

O processo de migração celular é orquestrado por uma série de enzimas, entre elas, as proteolíticas (proteases) as quais são capazes de degradar componentes da Matriz Extracelular (MEC). Dentre as proteases envolvidas neste processo, as metaloproteinases (MMPs) apresentam papel crucial haja visto que são capazes de degradar vários componentes proteicos da MEC. Portanto, as MMPs são alvos de muitos estudos de migração, invasão e metástase celular. Estas enzimas permitem que, aliado à migração celular, haja também uma proliferação de células endoteliais (angiogênese), a qual permite a irrigação sanguínea do local, favorecendo a nutrição e oxigenação dessas células em proliferação (Deryugina and Quigley, 2006, Sodek *et al*, 2007).

Do ponto de vista molecular a migração celular envolve alterações dinâmicas do citoesqueleto, interações entre célula/MEC, proteólise localizada e contrações de filamentos de actina e miosina (Friedl and Wolf, 2003). Em tais eventos estão envolvidas GTPases como Rho, cdc42 e Rac, além da organização e ou/desorganização de adesões pontuais contendo integrinas (Liotta and Kohn; 2001). Diante desse fato, diversos estudos têm relacionado proteínas componentes do citoesqueleto/adesão focal (FAK, Src, paxilina, etc.) com a progressão metastática.

1.2. Morte Celular

A proliferação e morte celular não compartilham muito de suas maquinarias efetoras em dirigir a célula a morte ou permitir que esta prolifere. Entretanto, estes processos estão acoplados em vários níveis através da ação de moléculas responsáveis por orquestrar a expansão celular. Estas moléculas por sua vez são geralmente alvos de mutações oncogênicas, mutações estas que cooperam com a proliferação celular, de forma a desacoplar este processo da apoptose durante a transformação e tumorigênese.

A morte celular programada é um importante processo no desenvolvimento e na doença. O papel da morte celular na embriogênese ou

patogênese, os sinais que controlam este processo e os mecanismos que definem o destino da célula têm despertado grande interesse. A modulação deste evento oferece a oportunidade de controlar processos como o câncer (Guimarães and Linden 2004).

Inicialmente Kerr e colaboradores (1972) definiram pelo menos dois tipos diferentes de morte celular, necrose e apoptose, termo usado como sinônimo de morte celular programada. Entretanto, atualmente é sabido que os processos e os mecanismos que decidem o destino celular são muitos e muitas nuances são definidas dentro de um mesmo processo, como por exemplo, a apoptose (Bursch *et al*, 2000; Guimarães and Linden 2004).

A avaliação da morte celular em diferentes sistemas fornece suporte à hipótese que a variedade de programas de morte celular é disparada em situações distintas. Vários tipos de morte celular programada com objetivos, maquinaria celulares e também morfologicamente diferentes, têm sido descritos. Alguns exemplos de morte celular programada atualmente estudados e descritos são: a apoptose dependente de caspases e suas variações, necrose programada, autofagia e catástrofe mitótica (Castedo *et al*, 2004, Gozuacik and Kimchi, 2007).

A definição molecular destes eventos e conhecimento detalhado dos participantes dos mesmos, permitem a modulação terapêutica das vias de morte celular em condições patológicas.

1.2.1. Vias Clássicas da Apoptose

Pelo menos duas vias podem disparar a apoptose, sendo que estas são finamente reguladas. A via intrínseca é o programa primário de resposta a sinais de sobrevivência, stress celular e injúria. O fator central nesta via é a mitocôndria, pois é no espaço intermembranas que são sequestrados uma variedade de efetores pro-apoptóticos, que quando liberados, induzem a entrada da célula em apoptose. A permeabilidade mitocondrial é determinada por um balanço entre as proteínas pró-apoptóticas Bax/Bak e anti-apoptóticas

Bcl2/BclXL. A apoptose é favorecida quando ocorre a dominância de fatores pró-apoptóticos Bax/Bak que são responsáveis pela permeabilização mitocondrial permitindo assim a liberação de proteínas pró-apoptóticas. Uma delas, o citocromo c, age em conjunto com o adaptador de morte celular Apaf-1 ativando a caspase 9, que por sua vez ativa a caspase 3 e a caspase 7. Uma vez ativadas, as caspases (cisteíno proteases) clivam proteínas importantes, orquestrando dessa forma, a morte celular. Esta via é regulada em muitos níveis, incluindo o nível transcricional e pós-transcricional de membros da família Bcl2, através ainda da expressão de componentes efetores de morte e de inibidores de caspases, denominadas proteínas inibidoras da apoptose (IAPs).

A via extrínseca de indução de morte celular é iniciada à partir da ativação de receptores de superfícies celular, tais como: Fas/CD95, TNFR e o DF-5, por seus respectivos ligantes FasL, TNF e TRAIL (Peter and Krammer, 2004). Uma vez ligados a estes receptores forma-se o complexo de sinalização de indução de morte (DISC), o qual ativa a caspase 8. Em alguns tipos celulares, este único evento é capaz de levar a célula a apoptose. Entretanto, em outros sistemas a apoptose via receptor também requer a participação da via mitocondrial através da ativação da proteína Bid que tem sua ativação mediada pela caspase 8 (Scaffidi *et al.*, 1998; Wilkinson *et al*., 2003; Peter and Krammer, 2004).

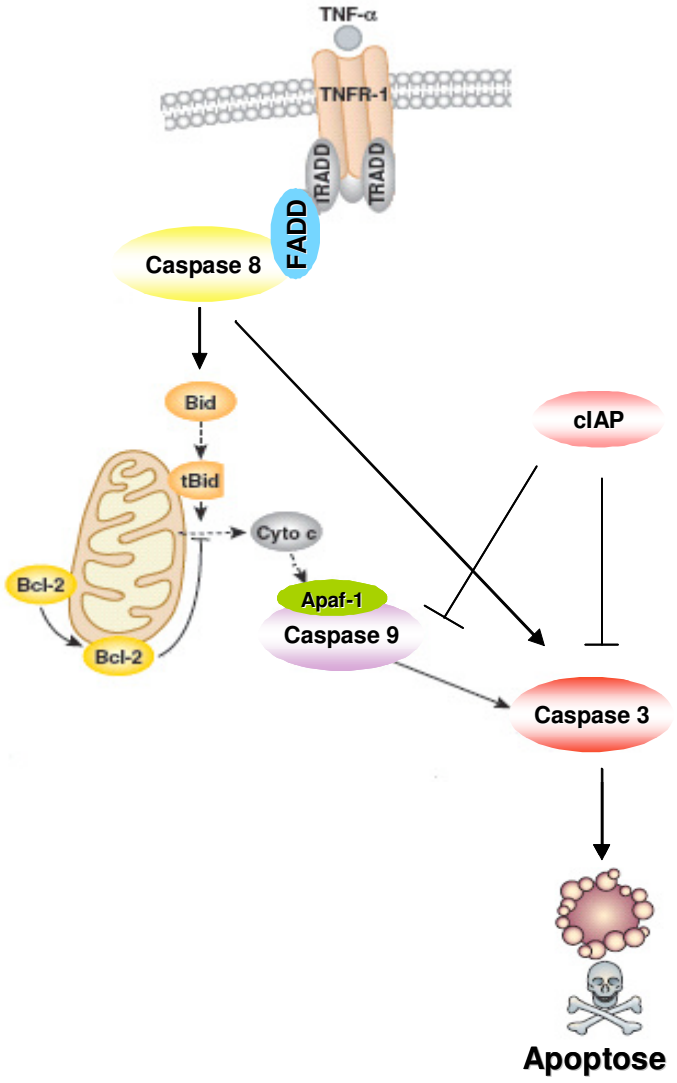


Figura 1 – Via extríseca (receptor) e intrínseca (mitocondrial) da apoptose.

1.3. Proteínas Fosfatases X Papel Biológico

O balanço entre fosforilação e desfosforilação de proteínas é a base para o controle de diversos eventos biológicos disparados por efetores extracelulares tais como: hormônios, mitógenos, carcinógenos, citocinas, neurotransmissores e substâncias ou metabólitos tóxicos (Harrison *et al*., 1999; Ostman and Bohmer, 2001; Ferreira *et al*, 2006). Em particular, a fosforilação e desfosforilação de resíduos de treonina, serina e tirosina em proteínas têm emergido como eventos chaves na regulação da divisão, diferenciação, desenvolvimento celular e apoptose, regulação do metabolismo e expressão gênica, contração, transporte, locomoção celular, aprendizado e memória (Trowbridge, 1991; Johnson and Barford, 1993; Denu *et al*., 1996; Zhang, 1997; Cohen, 1998; Harrison *et al.,* 1999; Krebs and Graves, 2000; Aoyama *et al.,* 2003; Chiarugi *et al.,* 2004).

1.3.1. Proteínas Fosfatases e Apoptose

Em eucariotos a atividade de pelo menos 30% das proteínas pode ser regulada por fosforilação, principal mecanismo que regula uma grande variedade de processos celulares como sobrevivência, diferenciação e morte celular (Garcia *et al*., 2003).

Com base na função, estrutura, seqüência, especificidade, sensibilidade a ativadores e inibidores, as proteínas fosfatases podem ser divididas em dois grandes grupos: serina/treonina fosfatases e tirosina fosfatases (PTPs) - (Jia, 1997; Aoyama *et al*, 2003).

As proteínas tirosina fosfatases (PTP), uma família estrutural e funcionalmente diversificada de enzimas sinalizadoras, juntamente com proteínas tirosina quinases, modulam os níveis celulares de fosforilação em tirosina (Zhang, 2003). Os membros desta família apresentam um domínio catalítico altamente conservado, caracterizado pela presença da seqüência consenso $C(X)_5R(S/T)$ (Zhang, 2003). A presença de cisteína no sítio ativo é responsável pela característica comum das PTPs de serem inibidas por

pervanadato, p-cloromercuribenzoato e outros agentes oxidantes (Aoyama *et al*, 2003).

As PTPs podem ser classificadas em quatro classes de acordo com o aminoácido presente no sítio catalítico. As PTPs de classe I possuem um mecanismo catalítico baseado na presença da cisteína, a qual confere as PTPs suscetibilidade a ação de agentes oxidantes, sendo estes capazes de inibir a ação destas fosfatases por promoverem oxidação da cisteína catalítica. Os membros constituintes da classe I são as chamadas PTPs "clássicas" ou específicas e as proteínas fosfatases de "especificidade dual" ambas evoluíram de um mesmo ancestral. As PTPs de classe II possuem um mecanismo catalítico também dependente da presença de cisteína, entretanto, estas são evolutivamente mais antigas, sendo comumente encontradas em bactérias. Em humanos esta classe é representada por uma PTP de baixa massa molecular (LMWPTP), que catalisa especificamente a desfosforilação de resíduos de tirosina. Estas fosfatases não compartilham homologia de seqüência com qualquer outra PTP, exceto pelo "motivo assinatura" no sítio catalítico e o mecanismo de catálise (Zhang, 2003). Possuem importante papel na regulação da proliferação celular uma vez que a alta expressão destas enzimas inibe a proliferação de células normais e transformadas (Aoyama *et al.*, 2003).

As PTPs de classe III, que também possuem uma cisteína catalítica, desfosforilam resíduos de Tirosina/Treonina. Em humanos esta classe é representada pelo grupo das fosfatases Cdc25: Cdc25A, Cdc25B e Cdc25C (Zhang *et al.,* 2002; Zhang, 2003). Estas são três importantes reguladoras do ciclo celular por desfosforilarem as quinases dependentes de ciclinas (Cdks) no sítio inibitório da porção N-terminal, reação requerida para ativar estas quinases e promover a progressão do ciclo celular. A quarta classe de PTPs apresenta catálise baseada num resíduo de aspartato e é dependente de cátion (Ferreira *et al.,* 2006).

As PTPs específicas são divididas em fosfatases tipo receptor e enzimas citosólicas. As PTPs tipo receptor (CD45, PTPα, PTPγ) são enzimas modulares consistindo de um segmento extracelular, envolvido na comunicação célula-célula e transdução de sinais, seguido por um único segmento transmembrana com um ou dois domínios PTPs citoplasmáticos (Alexander, 2000; Zhang, 2001; Aoyama *et al.*, 2003). As PTPs citosólicas (PTP1B, PTP2, VH1 e SHP) apresentam um único sítio catalítico e várias extensões amino e carboxi-terminais contendo domínios extracatalíticos, tais como domínios SH2, envolvidos diretamente na regulação da atividade catalítica ou endereçamento e reconhecimento de substratos, estratégia que aumenta a capacidade catalítica da enzima (Zhang *et al.*, 2002; Aoyama *et al.*, 2003).

PTPs de especificidade dual, atuam tanto em resíduos de fostirosina quanto fosfoserina e fosfotreonina, possuindo maior afinidade por fosfotirosina. Alguns componentes do grupo agem também sobre outros substratos além de fosfoproteínas sendo que, em geral, as enzimas do grupo participam da regulação de sinais mitogênicos e desempenham importante papel no controle do ciclo celular (Wu *et al.*, 1999; Zhang, 2001). Fazem parte deste grupo fosfatases de proteínas quinases ativadas por mitógenos (MAPKPs) e fosfatase supressora de tumor (PTEN), entre outras (Zhang *et al.*, 2002; Aoyama *et al.*, 2003).

Em contraste às proteínas tirosina quinases (PTKs) que apresentam potencial em promover o crescimento, as PTPs podem agir como supressoras de tumor e inibir o crescimento celular. PTPs também têm sido implicadas na ativação de linfócitos B e T e na sinalização por insulina, o que faz destas enzimas alvos atrativos para o desenvolvimento de fármacos usados numa variedade de doenças como câncer, inflamação, diabetes e obesidade (Pils and Schultz, 2004; Haugh *et al.*, 2004).

A família de proteínas serina/treonina fosfatases compreende duas subfamílias de enzimas conhecidas como fosfoproteínas fosfatases (PPP) e proteínas fosfatases dependentes de magnésio (PPM), as quais apresentam em

comum similaridade de estrutura primária (Van Hoof, Goris, 2003; Garcia *et al.*, 2003).

A proteína fosfatase tipo 2 (PP2A) é a principal proteína serina/treonina fosfatase envolvida na regulação de vários processos celulares incluindo diferentes vias de transdução de sinais, progressão do ciclo celular, replicação do DNA, transcrição gênica e síntese de proteínas (Van Hoof and Goris, 2003; Yu *et al.*, 2004; Janssens, 2005). Tal diversidade de funções é explicada em parte pela sua complexidade estrutural e de regulação. A estrutura central da enzima é composta por uma subunidade estrutural (A) e uma subunidade catalítica (C) que pode encontrar-se em forma livre ou associar a uma terceira unidade denominada subunidade regulatória (B) formando o complexo trimérico da holoenzima. Atualmente têm sido descritas três classes de subunidades regulatórias B sendo que cada uma delas existe na forma de pelo menos duas isoformas diferentes. A existência de múltiplas classes e isoformas das subunidades regulatórias B é consistente com a hipótese de que diferentes complexos de PP2A executem específicas e distintas funções fisiológicas graças à especificidade de substrato e localização celular conferidas pelas diferentes subunidades B (Hahn, 2004).

Fosforilação de proteínas intracelulares geralmente ocorre em resíduos de serina e treonina e a PP2A é responsável por significante fração da atividade serina/treonina fosfatase na maioria das células, sendo inclusive responsável pelo controle de pelo menos 50 proteínas quinases (Van Hoof and Goris, 2003; Yu *et al.*, 2004; Janssens *et al*, 2005).

PP2A está envolvida tanto na regulação quanto na resposta celular à apoptose, agindo tanto “upstream” quanto “downstream” de caspases efetoras (Van Hoof and Goris, 2003). A função pró-apoptótica de PP2A é consistente com seu papel na progressão do ciclo celular, crescimento e sobrevivência celulares, inibindo direta ou indiretamente a quinase cdc2, MAPKs e PKB/Akt, respectivamente. Além de regular negativamente o crescimento celular, a atividade de PP2A também contribui para a natureza transiente das ativações de

proteínas quinases ativadas por mitógenos (MAPKs) por fatores de crescimento, o que evita uma ativação constitutiva suficiente para causar transformação tumorigênica (Schontal, 2001).

A relação da PP2A com a regulação do processo apoptótico envolve, entre outras ações, a desfosforilação de proteínas da família Bcl-2. Bcl-2 e Bcl-Xl quando fosforiladas associam-se a membros pró-apoptóticos do grupo neutralizando a ação de seus antagonistas e evitando o extravasamento de fatores apoptóticos mitocondriais. A proteína Bad, ao contrário de Bcl-2 e Bcl-Xl, é membro pró-apoptótico da família Bcl-2 sendo sua função também altamente regulada por fosforilação reversível. A fosforilação de Bad, principalmente no resíduo de Ser-136, resulta em sua associação com proteínas 14-3-3 e retenção no citosol, o que impede que se associe e inative Bcl-2 e Bcl-Xl mitocondriais.

A ação de PP2A na regulação do processo apoptótico também ocorre através da regulação negativa de vias de sinalização relacionadas a estímulos de sobrevivência. A via de sinalização da MAPK ERK está relacionada a estímulos de proliferação e sobrevivência celulares sendo, também, conhecida sua ação inibitória de apoptose mediada por citocinas como Fas, TNFα, e TRAIL (Chiang et al., 2003).

1.4. Proteínas Quinases Ativadas por Mitógenos (MAPKs)

As MAPKs (serina/treonina quinases) fazem parte da grande família de proteínas serina/treonina quinases da qual também são membros a proteína quinase dependente de AMP cíclico (PKA), proteína quinase B ou Akt (PKB/Akt) e a proteína quinase C (PKC) (Cano and Mahadevan, 1995; Cross et al., 2000). Estas conectam receptores de superfície celular a alvos regulatórios dentro das células. Através de uma via de sinalização constituída por receptores com atividade tirosina quinase intrínseca e receptores acoplados a proteína G, proteínas adaptadoras e fosforilações seqüenciais de proteínas quinases, as MAPKs respondem a uma variedade de estímulos ambientais tais como fatores de crescimento ou proliferação, citocinas e estresse químico ou físico,

controlando, portanto eventos tão distintos quanto sobrevivência, morte, transformação ou adaptações celulares (Haneda et al., 1999; Cross et al., 2000; Nebreda and Porras, 2000; Chang and Karin, 2001; Wada and Penninger, 2004).

A ampla variedade de funções desempenhadas por MAPKs são mediadas através da fosforilação de vários substratos incluindo fosfolipases, fatores de transcrição e tradução, moléculas de ciclo celular, proteínas adaptadoras e proteínas de citoesqueleto, entre outras. MAPKs também catalisam a fosforilação e ativação de várias proteínas quinases denominadas "proteínas quinases ativadas por MAPKs" (MAPKAPKs ou MKs) entre as quais podemos destacar RSKs, MSKs (mitogen and stress ativated kinases), MNKs (MAPK-interacting kinases) e MAKPAPKs 2, 3 e 5 (Wada and Penninger, 2004; Roux and Blenis, 2004).

Dentro da grande família das MAPKs três grupos têm sido caracterizados em mamíferos: a família das "extracellular signal-regulated kinases" (ERKs), "c-Jun NH_2 terminal kinases" (JNKs) e a família das p38 MAPKs (Johnson *et al.*, 2002; Roux and Blenis, 2004; Wada and Penninger, 2004).

Cada família de proteínas MAPKs opera através de cascatas de sinalização caracterizadas por alta especificidade e funcionamento paralelo (Schenk and Snaar-Jagalska, 1999). A via de sinalização envolvendo MAPKs é composta por um conjunto de três quinases conservadas evolutivamente e ativadas em reações seqüenciais. A MAPK, uma serina/treonina quinase, é ativada por uma MAPK quinase (MAPKK) de especificidade dual que fosforila tanto em serina/treonina quanto em tirosina em um motivo Thr-X-Tyr (onde X é glutamato, prolina ou glicina para ERK, JNK e p38, respectivamente) da MAPK alvo. As MAPKKs, por sua vez, são fosforiladas e ativadas por MAPK quinase quinases (MAPKKKs) que recebem sinais de receptores da superfície celular ativados por um estímulo ou através de interações com proteínas de ligação a GTP da família de Ras ou Rho e/ou outras quinases (Cowan and Storey, 2003; Torres, 2003) (figura 2). Na ausência de sinais, os constituintes das cascatas de

MAPKs retornam ao estado desfosforilado e inativo, sugerindo papel essencial de proteínas fosfatases na regulação de MAPKs. Serina/treonina fosfatases, tirosina fosfatases e serina/treonina/tirosina fosfatases de especificidade dual têm sido implicadas na regulação dessas vias, destacando-se o papel de MAPKs fosfatases (MAPKPs) como MAPKP-1, MAPKP-2, MAPKP-3 e serina treonina fosfatases como PP1 e PP2A (Haneda *et al.*, 1999; Tamura *et al.*, 2002).

A via das ERKs geralmente está relacionada ao crescimento celular, diferenciação e sinais de sobrevivência enquanto as MAPKs das famílias JNK e p38 relacionam-se primariamente com sinais pró-apoptóticos e inibidores do crescimento, também participando de respostas pró-inflamatórias. Contudo, a ativação das vias de p38 MAPKs e JNKs também desempenha papel anti-apoptótico, de proliferação e sobrevivência e o que determinará o destino celular serão suas próprias condições inerentes ao tipo e ao contexto celular (Liu and Lin, 2005, Zarubin and Han, 2005).

As diversas funções das MAPKs regulam a trajetória do desenvolvimento celular e, como mostrado, podem tanto controlar a sobrevivência, transformação ou morte celular. Quando o delicado balanço entre ciclo celular, proliferação, diferenciação e apoptose é desregulado por fatores ambientais e/ou genéticos podem ocorrer transformações malignas onde geralmente as cascatas sinalizadoras de MAPKs estão desreguladas. Desta maneira, as vias de sinalização de MAPKs são alvos potenciais para o desenvolvimento de novas estratégias de combate ao câncer (Platanias, 2003).

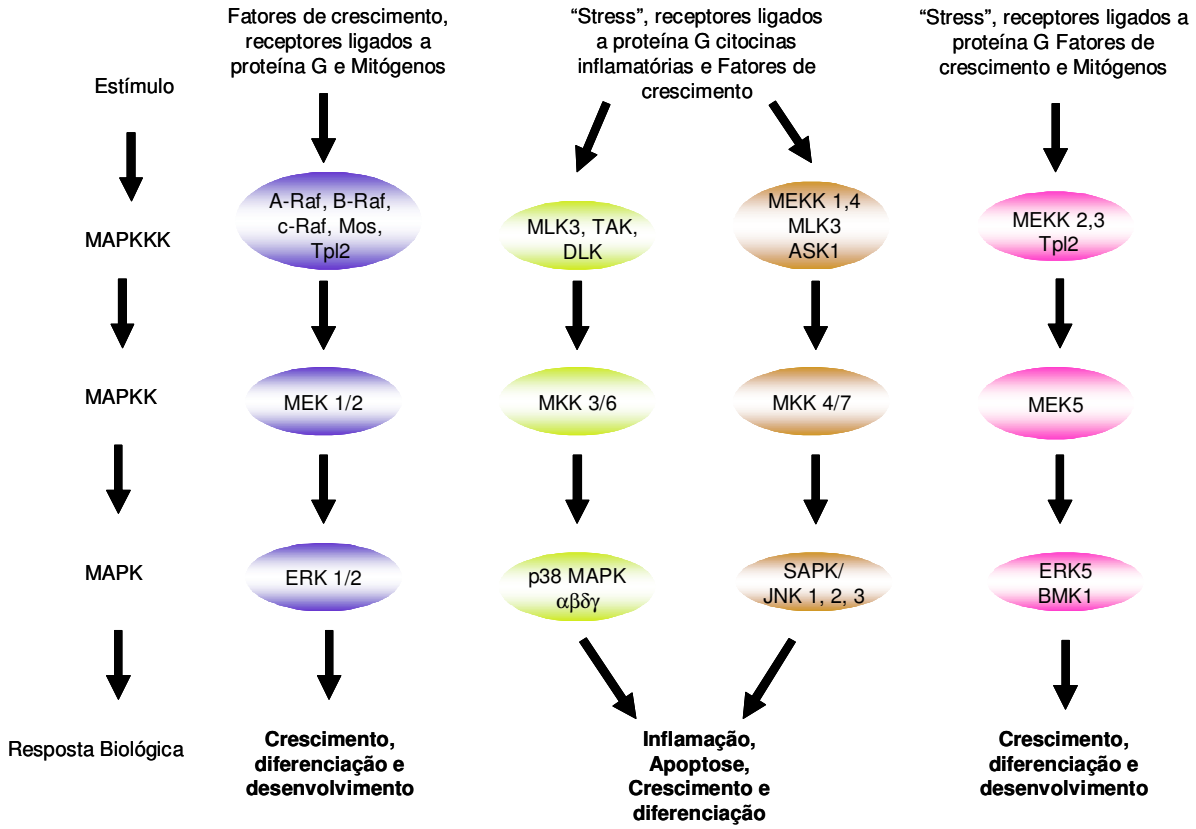


Figura 2 - Cascata de sinalização de proteínas quinases ativadas por mitógenos (MAPKs).

1.5. Diferenciação Celular

A diferenciação celular é o processo em que as células se especializam e "ganham" dessa forma, funções definidas. Portanto, na hematopoese, o bloqueio desse processo culmina com um acúmulo de células mielóides imaturas caracterizando a leucemia mielóide aguda (LMA). Desta forma, LMAs normalmente apresentam alguns sinais morfológicos de diferenciação que permitem uma classificação dentro de diferentes subgrupos FAB (grupo cooperativo Franco-Americano-Britânico). As LMAs são classificadas e diagnosticadas baseado em características morfológicas, marcadores imunológicos e/ou citogenéticos. O tratamento de LMAs inclui duas etapas principais, a indução e a consolidação. A primeira visa a remissão hematológica e a segunda objetiva evitar a recidiva ou recaída do paciente (Bruserud and Gjertsen, 2000).

Atualmente a terapia da diferenciação é considerada como importante parte do tratamento em alguns subgrupos de leucemias. Especificamente para a leucemia promielocítica aguda (LPA), a qual é caracterizada pelo bloqueio da diferenciação das células mielóides no estado de promielócito, a indução de diferenciação com o ácido trans-retinóico (ATRA) combinado com a terapia quimioterápica é considerada condição essencial para o tratamento da LPA. A indução de diferenciação enquanto opção terapêutica é interessante porque diminui a morbi-mortalidade relacionada com o tratamento quimioterápico, sendo atualmente considerada no tratamento de outras LMAs. Dois grandes estudos randomizados compararam a terapia da indução de diferenciação seguida por quimioterapia com o tratamento quimioterápico apenas, e ambos demonstraram um aumento do tempo de vida livre da doença em pacientes com LPA que receberam a terapia combinada (Tallman et al, 1997). Um estudo mostrou que a terapia combinada é ainda mais eficiente se feita ao mesmo tempo, em vez de sequencialmente (Fenaux et al, 1999).

Recentemente diferentes compostos provenientes de diferentes classes químicas têm sido considerados como agentes indutores de diferenciação, tais como: Vitamina D3 e derivados, derivados do arsênio, citocinas e combinações das mesmas, assim como alguns agentes quimioterápicos que apresentam essa habilidade em doses mais baixas do que aquelas usadas na terapia citotóxica. Estudos são ainda necessários na tentativa de prever a utilidade dos tratamentos combinados com indutores de diferenciação no tratamento de outras leucemias.

O uso de indutores de diferenciação não está limitado ao tratamento das leucemias, tem sido sugerido atualmente o uso destes fármacos no tratamento de tumores sólidos. Isto se deve principalmente ao fato desses agentes promoverem inevitavelmente a parada da proliferação celular. Principalmente, as vitaminas A e D, que também são componentes da dieta, têm sido extensivamente estudadas como potenciais agentes na prevenção e terapia do câncer. Estes agentes têm habilidade de regular processos chaves da transformação neoplásica, tais como: proliferação e diferenciação. Devido a isto, estas vitaminas têm sido citadas como interessantes agentes na prevenção e controle da progressão tumoral, assim como tem sido mostrado que as mesmas podem interferir em processos como a neovascularização tumoral (Enciso and Hirschi, 2007).

1.6. Resistência Celular ao Tratamento Quimioterápico

A terapia da diferenciação tem sido também sugerida como uma forma de controlar a expressão de bombas de efluxo, como por exemplo, a P-glicoproteína e as MRP1, as quais estão envolvidas com resistência celular a quimioterápicos. Isto se deve ao fato de agentes indutores de diferenciação modularem de alguma forma a expressão destas bombas ao mesmo tempo que induzem diferenciação. Ou seja, as células mais maduras têm uma tendência de apresentar níveis de expressão diminuídos destas proteínas.

Resistência múltipla às drogas (MDR) é descrita como resistência à drogas citotóxicas estruturalmente não relacionadas, o que acarreta uma ineficiência do tratamento quimioterápico. MDR tem sido associada com o decréscimo da retenção celular da droga como resultado do efluxo mediado por transportadores de membrana dependente de ATP ou compartimentalização da droga nos lisossomos e complexo de Golgi (Campone et al 2001; Gong et al 2003).

A maioria dos agentes antineoplásicos tem como alvo molecular o DNA ou enzimas nucleares, com a ocorrência do sequestro do fármaco em organelas citoplasmáticas ou a extrusão via P-glicoproteína. Com isto, há uma diminuição da interação fármaco-alvo, e consequentemente, diminuição do efeito terapêutico destes agentes. Outro evento importante que pode favorecer a resistência é a alcalinização do meio intracelular. A presença de diferentes mecanismos provavelmente é coexistente em vários casos de resistência a fármacos. (Larsen et al, 2000)

Vários compostos parecem modular a atividade das bombas de efluxo, isto se pode dar pela inibição direta destas ou pela regulação dos níveis de expressão das mesmas. A definição de vias de sinalização envolvidas na aquisição de resistência torna-se neste momento de extrema importância para o desenvolvimento de terapias mais eficazes no que diz respeito à prevenção ou modulação mais efetiva dos fatores relacionados com resistência a quimioterápicos (Sandor *et al*, 1998; O'Connor, 2007).

1.7. Importância da Definição do Perfil Quinômico Celular

Classicamente, a atividade quinásica e a fosforilação de proteínas era estudada usando ensaios de quinase no gel ou por técnicas como “western blotting”. Por essa razão o estudo de um grande número de quinases em um único experimento não era uma tarefa simples, pois as técnicas citadas anteriormente requerem uma quantidade relativamente grande de amostra, o que as torna pobremente adaptadas à prática clínica. Sob esse aspecto, a

situação foi melhorada na década de 90 quando foram criados os primeiros anticorpos para sítios específicos de fosforilação. Usando estes anticorpos foi possível detectar e determinar os níveis de ativação de algumas quinases pelo clássico "western blotting", mas também por outras técnicas como por exemplo, o ELISA ("enzyme linked immuno sorbent assay"). Entretanto, uma limitação das técnicas citadas anteriormente é que as mesmas se prestam a analisar apenas um tipo de fosforilação por experimento. Considerando-se que o ambiente intracelular é extremamente complexo, o desenvolvimento de técnicas aptas a avaliar a atividade quinásica total, poderia fornecer potencialmente informações importantes do estado de fosforilação celular em condições específicas (Diks and Peppelenbosch, 2004).

Devido aos eventos de fosforilação e desfosforilação definirem o destino celular, como citado anteriormente, o estabelecimento de um quadro geral do estado quinásico celular pela tecnologia de "arrays" tornou-se muito importante no estudo do quinoma celular. Assim, em um único experimento consegue-se retratar a condição celular em determinada situação, sendo possível traçar as principais vias de sinalização envolvidas com determinados processos, descobrir novos substratos, ou ainda definir as principais vias moduladas por um fármaco. No momento em que as quinases e fosfatases continuam sendo interessantes alvos para o desenvolvimento de terapias mais específicas, as tecnologias de microarranjos podem ser utilizadas como importantes ferramentas.

1.8. Riboflavina

Flavinas são compostos químicos versáteis envolvidos em uma variedade de fenômenos biológicos (Souza *et al.*, 2005). Destacam-se no papel central que desempenham no metabolismo aeróbico através da habilidade de catálise de reações de desidrogenação envolvendo dois elétrons e, participação em reações de transferência de um elétron da cadeia respiratória. Flavoproteínas têm demonstrado exercer importantes papéis na transdução de sinais envolvidas na morte celular programada, regulação de relógios biológicos e estão envolvidas

em vários processos dependentes de luz, tais como, fotossíntese e reparo de danos no material genético (Edwards *et al*.,1999a; Massey, 2000; Powers, 2003).

A riboflavina **(Figura 1)**, 7,8-dimetil-10-ribitil-isoaloxazina, é um dos componentes do complexo vitamínico B2 e também desempenha importante papel como sensibilizador fotoquímico. Tem sido alvo de numerosos estudos na área de Terapia Fotodinâmica, uma forma de fotoquimioterapia onde um composto fotosensível é acumulado em um tecido tumoral, submetido posteriormente à iluminação, em um processo que culmina com indução de morte celular (Edwards *et al*.,1999b).

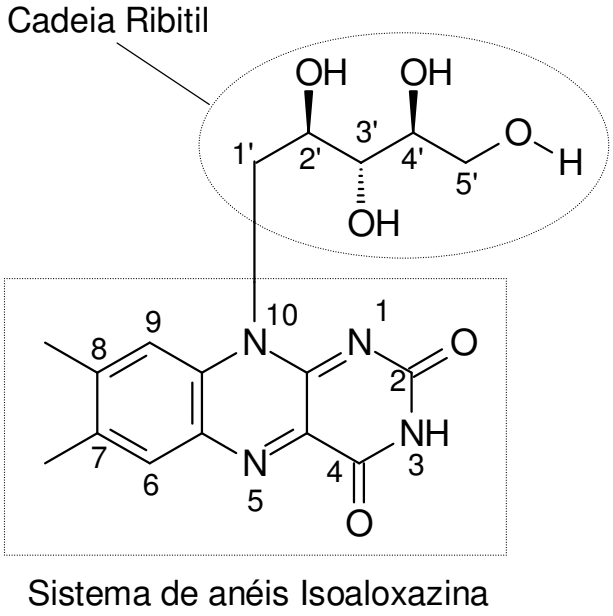
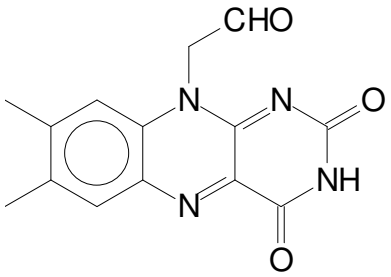


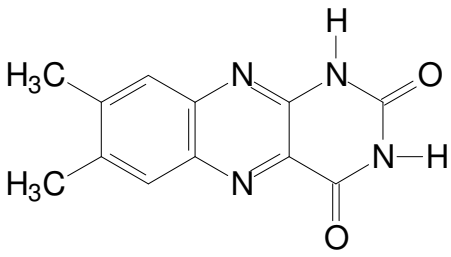
Figura 1. Estrutura da riboflavina. Em destaque, a cadeia ribitil e o sistema de anéis isoaloxazina que apresenta o sistema π conjugado.

A riboflavina é sensível à luz UV e visível, em cuja presença gera oxigênio singlete (mecanismo tipo II) ou espécies radicalares (mecanismo tipo I), incluindo espécies reativas de oxigénio, tais como, íon superóxido, radical hidroxil e peróxido de hidrogênio. Embora o potencial de redução da riboflavina

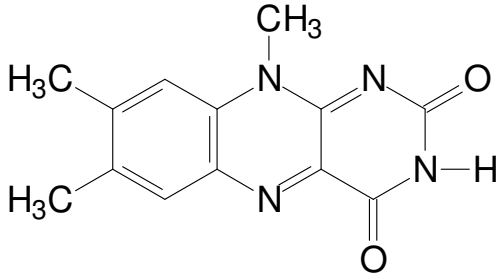
seja -0,3V, em pH 7,0 (Meisel and Neta, 1975), o potencial redox do seu estado triplete ativado muda para 1,7V, valor superior ao potencial redox de importantes biomoléculas, tais como, aminoácidos, proteínas, lipídios e ácidos nucléicos, os quais podem sofrer fotodegradação na presença da riboflavina. Trabalhos recentes (Ahmad *et al.*, 2004a; Ahmad *et al.*, 2004b; Holzer, *et al.*, 2005) têm revisto os aspectos fotoquímicos da riboflavina e os resultados mostram que essa flavina é degradada através de uma variedade de reações, incluindo fotólise (fotoredução intramolecular) e fotoadição (fotoadição intramolecular), como principais reações (Ahmad *et al.*, 2004a; Ahmad *et al.*, 2004b). A fotodegradação da riboflavina em soluções aquosas resulta na formação de numerosos produtos entre os quais destacam-se 7,8-dimetil-10-(formilmetil)-isoaloxazina, lumicromo e lumiflavina **(Figura 2)**.



7,8-dimetil-10-(formilmetil)-isoaloxazina



Lumicromo



Lumiflavina

Figura 2. Estrutura química dos principais fotoprodutos da riboflavina.

A riboflavina irradiada com luz UV ou visível provoca ainda efeitos letais em células de mamíferos em cultura e essa citotoxicidade tem sido atribuída à fotooxidação da riboflavina com triptofano e/ou tirosina resultando numa complexa mistura de agregados (fotoadutos) indólicos, flavínicos e indólico-flavínicos (Silva *et al*., 1994; Edwards and Silva, 2001).

As considerações acima mencionadas nos levaram a investigar, no presente trabalho, os efeitos dos fotoprodutos, resultantes da irradiação da riboflavina, sobre células de leucemia mielóide humana (HL60). Esta linhagem celular é geralmente aceita como um modelo válido para o estudo da biologia da leucemia mielóide (Treigyté *et al*., 2000; Ferreira, *et al*., 2004) e avaliação de fotosensibilizadores (Korbelik *et al*., 1993).

A atividade anti-leucêmica da riboflavina irradiada já fora anteriormente demonstrada por Edwards e Silva (2001), autores que reportaram mudanças morfológicas similares as de células apoptóticas em HL60 tratadas com riboflavina irradiada. Esses resultados foram confirmados por nosso grupo e adicionalmente, descrevemos, pela primeira vez, o mecanismo molecular responsável pela indução da apoptose das células HL60 tratadas com a riboflavina irradiada. Basicamente, o processo de indução apoptótica está relacionado ao aumento da expressão de Fas e FasL o qual ativa cascatas apoptóticas principalmente através da ativação da caspase 8. Pôde-se também constatar que as atividades de JNK e proteínas fosfatases são significativamente afetadas pelos fotoprodutos gerados pela irradiação da riboflavina. Cabe aqui ressaltar que os efeitos citotóxicos da riboflavina estão relacionados diretamente à formação desses fotoprodutos estáveis e não a uma possível ação de espécies reativas de oxigénio, geradas durante o processo de irradiação, visto que a ação citotóxica dos fotoprodutos da riboflavina pode ser observada mesmo depois de longo período após a irradiação do composto (Souza *et al*., 2006).

OBJETIVOS

A atividade antileucêmica dos fotoprodutos da riboflavina levou-nos a investigar a atividade antitumoral em modelo de tumor sólido e a indução da diferenciação de células leucêmicas. Além disso, avaliou-se também a capacidade da riboflavina atuar como adjuvante no “drug delivery” e de inibidores de proteínas fosfatases como alternativa para reverter a resistência tumoral a quimioterápicos.

2. Resultados

2. Resultados

Os resultados estão dispostos na forma de artigos (publicado/submetidos):

Artigo 1 – A possible anti-proliferative and anti-metastatic effect of irradiated riboflavin in solid tumours.

Artigo 2 – Signal transduction pathways involved in the differentiation process of leukaemia cells triggered by irradiated riboflavin.

Artigo 3 – Biochemical differences between erythroblast leukemia cell and its counterpart with resistance phenotype: kinases and phosphatases profiles.

Os resultados também permitiram a produção de duas patentes:

Patente 1 – (PI0505220-3) - Ferreira, C.V.; Souza, A.C.S.; Aoyama, H.; Queiroz, K.C.S.; Peppelenbosch, M.P. Potencialização da Ação de Antineoplásicos pela associação com Riboflavina. 2005.

Patente 2 – (PI0604691-6) - Ferreira, C.V.; Aoyama, H.; Queiroz, K.C.S.; Peppelenbosch, M.P.; Silva, M.A.S.; Jucá, M.B. Novo uso médico, composições farmacêuticas e método de reversão da resistência a quimioterápicos utilizando inibidores de proteínas fosfatases. 2006.

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A possible anti-proliferative and anti-metastatic effect of irradiated riboflavin in solid tumours

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Abstract

Riboflavin is a potent photosensitizer as well as part of the vitamin B complex. Recently we demonstrated that the products generated by irradiation of riboflavin have potential as anti-leukaemic therapy. The possible action, however, of the riboflavin photoproducts in solid cancers has not been addressed. Hence, we investigated the effects of irradiated riboflavin on androgen-independent human prostate cancer cells (PC3), a known model for solid tumour cells with an exceptional resistance to therapy. Our results show that riboflavin photoproducts are cytotoxic to these cells in a FasL–Fas-dependent manner. Furthermore, irradiated riboflavin inhibited matrix-degrading proteases, caused downregulation of VEGF and upregulation of TIMP1 suggesting anti-metastatic potential. Together, these results show that the anti-neoplastic action of riboflavin photoproducts is not limited to haematological malignancies, warranting clinical studies in solid tumours.

Keywords: Prostate cancer; Riboflavin; Apoptosis

1. Introduction

In recent years, vitamins and their derivatives are gaining prominence in anti-cancer strategies, especially in haematological cancers. For example all-trans retinoic acid (vitamin A) is now widely used for the treatment of myeloid leukaemia [1,2] and vitamin D is in clinical trial for this disease as well [3]. Very recently, we provided evidence that photoderivatives of riboflavin, a constituent of the vitamin B complex (vitamin B2), has strong activity in haematological malignancy as well [4]. The potential of such vitamin-derived products in solid

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tumours has, however, received much less attention, with the possible exception of vitamin D, which is highly promising in pancreatic cancer [5].

We showed earlier that in leukaemia, the photoproducts of riboflavin act through the activation of FasL–Fas-dependent signalling, which together with diminished protein kinase B-dependent survival signalling seems responsible for its anti-leukaemic action [4]. As FasL–Fas signalling should, in principle, also be anti-neoplastic in solid tumours we felt that these results prompted investigation into the possible usefulness of riboflavin photoproducts (Fig. 1a) in solid tumours. Hence, we decided to study the action of irradiated riboflavin in the PC3 prostate cancer cell culture system, which is generally considered to be a good experimental model for highly therapy resistant solid tumour cells [6]. The results show that irradiated riboflavin inhibits cell growth in this androgen-independent prostate cancer, apparently dependent on the induction of caspase-mediated cell death through activation of the FasL–Fas system. Furthermore, concomitantly activity of collagen matrix-degrading proteases was reduced, suggesting anti-metastatic potential. Together these results provide important support for commencing clinical testing of riboflavin photoproducts in terminal solid tumour cancer.

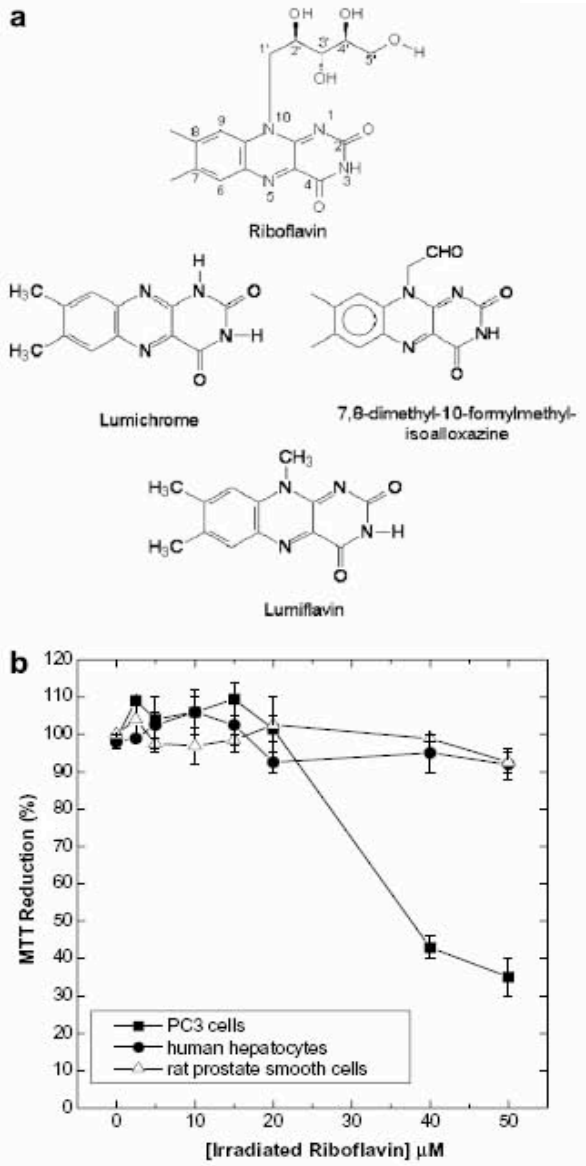

Fig. 1. Irradiated riboflavin presents cell specific toxic effect. (a) Chemical structure of riboflavin and its photoproducts. (b) Cells were treated with different concentrations of irradiated riboflavin. The effect of photoproducts was evaluated by MTT reduction after treating the cells for 24 h. In the absence of irradiated riboflavin, cell viability was considered as 100%. The experiment was performed in a 24 wells plate. Results represent the means ± standard error of three experiments run in triplicate ($p < 0.05$).

2. Material and methods

2.1. Reagents

Polyclonal antibodies against anti-phospho-p38 mitogen-activated protein kinase (MAPK p38), anti-phospho-c-jun-NH_2-terminal protein kinase 1/2 (JNK 1/2), PKB/Akt, phosphatidylinositol-3 kinase (PI3K), AIF, anti-rabbit, anti-goat and anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against phospho-PP2A, TNF receptor 1 (TNRF1), Fas-associated death domain protein (FADD), Bcl2, Bax, c-IAP1, NFκBp65, IKKα, TIMP-1, VEGF and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fas neutralising antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Riboflavin and Fumonisin B1 were from Sigma Chemical Co. (St. Louis, MO). Caspase 3 Colorimetric Assay Kit was obtained from R&D systems (Minneapolis).

2.2. Cell lines and culture

PC3 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD). PC3 and normal rat prostate smooth cells were routinely grown in RPMI 1640 medium (Sigma Chemical Co.) containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified incubator with 5% CO_2 in air. Dexamethasone (1 μM) and human insulin (1 pM) were added to normal prostate smooth cell medium.

Human hepatocyte line (IHH) was a kind gift from Professor Didier Trono and Dr. Tuan Nguyen. IHH cells were maintained at 37 °C and 5% CO_2 in phenol red-free DMEM/F-12 medium (Gibco-BRL) containing 1 μM dexamethasone, 1 pM human insulin (Humalog, Lilly) and antibiotics (100 IU/mL penicillin, 100 mg/mL streptomycin) with 10% fetal bovine serum.

2.3. Riboflavin irradiation

Solution of 250 μM riboflavin in RPMI 1640 medium at the pH 7.4 (15 mL) was placed in a Petri dish and irradiated with UV light (253.5 nm) for 30 min; the lamp was placed 40 cm from the riboflavin solution. The RPMI 1640 medium was irradiated in the same conditions and used as a control. After irradiation the percentage of riboflavin and its photoproducts was the following, as determined by mass spectrometry: 79% of riboflavin, 6.2% of lumichrome, and 14.8% composed of formylmethylflavin, lumiflavine, 2-ketoriboflavin and 4-ketoriboflavin.

2.4. Treatment of cells with irradiated riboflavin

Cells were incubated in 24-well plates until reach the semiconfluence and then treated with different concentrations of irradiated riboflavin (5–50 μM final concentrations) for 24 h. In order to investigate the mechanisms of riboflavin cytotoxicity, PC3 cells were also pre-treated with 5 μM of Fumonisin B1 or anti-Fas (10 μg/mL) for 30 min. Cell viability was assessed by the MTT reduction assay.

2.5. MTT reduction assay

The medium containing irradiated riboflavin was removed and 1 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (0.5 mg/mL of culture medium) was added to each well. After incubation for 4 h at 37 °C, the medium was removed and the formazan crystals solubilized in 1 mL of ethanol. The plate was shaken for 5 min on a plate shaker and the absorbance was measured at 570 nm [7].

2.6. Western blotting

Cells (3×10^7) were lysed in 200 μL cell lysis buffer (50 mM Tris [tris(hydroxymethyl)aminomethane]–HCl [pH 7.4], 1% Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA (ethylene glycol tetraacetic acid), 1 mM *O*-vanadate, 1 mM NaF and protease inhibitors [1 μg/mL aprotinin, 10 μg/mL leupeptin and 1 mM 4-(2-amino-ethyl)-benzolsulfonyl-fluoride-hydrochloride]) for 2 h on ice. Protein extracts were cleared by centrifugation, and the protein concentration was determined using the Lowry method [8]. An equal volume of 2× sodium dodecyl sulfide (SDS) gel loading buffer (100 mM Tris–HCl [pH 6.8], 200 mM dithiothreitol [DTT], 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added to samples and boiled for 10 min. Cell extracts, corresponding to 3×10^5 cells, were resolved by SDS–polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (2%) in Tris-buffered saline (TBS)–Tween 20 (0.05%) and incubated overnight at 4 °C with appropriate primary antibody at 1:1000 dilution. After washing in TBS–Tween 20 (0.05%), membranes were incubated with anti-rabbit, anti-goat or anti-mouse horseradish peroxidase-conjugated secondary antibodies at 1:2000 dilutions (in all Western blotting assays) in blocking buffer for 1 h. Detection was performed by using enhanced chemiluminescence (ECL).

2.7. Caspase 3 activity assay

Caspase 3 activity was determined by the measurement of *p*-nitroaniline (pNA) released from the cleavage of caspase 3 substrate (Ac-DEVD-pNA) at 405 nm.

2.8. Zymographic analysis

The proteolytic activity of MMP-2 and MMP-9 was assayed by gelatin zymography as described by de Souza et al. [9]. After the treatment the viable cell number was determined, the culture medium was collected and stored at −20 °C in the presence of 1 mM PMSF (phenyl-methylsulphonyl fluoride-serine-protease enzyme inhibitor). Samples were diluted in non-reducing buffer (0.1 M Tris–HCl, pH 6.8, 20% glycerol, 1% SDS and 0.001% bromophenol blue). Volume of the samples loaded was proportional of the viable cells and resolved by SDS–polyacrylamide gel (10%) and 4% gelatin. Protein renaturation was performed in 2% Triton X-100 for 1 h followed by incubation with 50 mM Tris–HCl and 10 mM $CaCl_2$ (pH 7.4) at 37 °C for 18 h. Gels were stained with 0.5% Coomassie blue G 250 for 30 min and then washed in a 30% methanol and 10% glacial acetic acid solution.

2.9. Statistical evaluation

Western blots represent three independent experiments. Quantitative analysis of the proteins was performed by volume densitometry after scanning the film (data are presented as the protein to β-actin ratio). Cell viability data were expressed as the means ± standard errors of three independent experiments carried out in triplicates. Data from each assay were analyzed statistically by ANOVA. Multiple comparisons among group mean differences were checked with the Tukey test. Differences were considered significant when the p value was less than 0.05.

3. Results

3.1. Irradiated riboflavin displays differential effects on cell viability

PC3 cells represent a fast growing cell type remarkably resistant to chemotherapeutic intervention. Also in our hands, cultures of PC3 cells expanded exponentially when grown in fetal calf serum containing medium. Importantly, however, irradiated riboflavin dose-dependently stopped PC3 growth and was even cytotoxic with an apparent IC_{50} of 20–30 μM (Fig. 1b). The toxic effect was strictly dependent on the presence of riboflavin photoproducts, since non-irradiated riboflavin had not influence on PC3 cell growth. Thus, the anti-neoplastic effect of riboflavin seems not limited to haematological malignancies, but also extends to therapy-resistant solid tumour cells, at least as judged from the PC3 model system. Importantly, normal cells such as human hepatocytes and rat prostate smooth cells were not sensitive to irradiated riboflavin (Fig. 1b).

3.2. Fas neutralising prevents the toxic effect of riboflavin photoproducts

In leukaemia, the anti-neoplastic effect of riboflavin seems dependent on the induction of FasL–Fas signalling. In order to test whether a similar situation also holds true for the PC3 model system, cultures were tested for riboflavin photoproducts cytotoxicity in the presence of a Fas-neutralising antibody or solvent control. It appeared that whereas control cultures responded to irradiated riboflavin with a strong inhibition of cell growth, Fas-neutralising pre-treated cultures were not affected by riboflavin photoproducts (Fig. 2a). Furthermore, concomitant with irradiated riboflavin-induced cytotoxicity, FADD levels were increased, in apparent agreement with the upregulation of the FasL–Fas signalling system as a consequence of the treatment with riboflavin. The latter responses to riboflavin photoproducts were specific to the FasL–Fas signalling system. TNFR1 as well as NFκB were down-regulated which is in agreement with the upregulation of

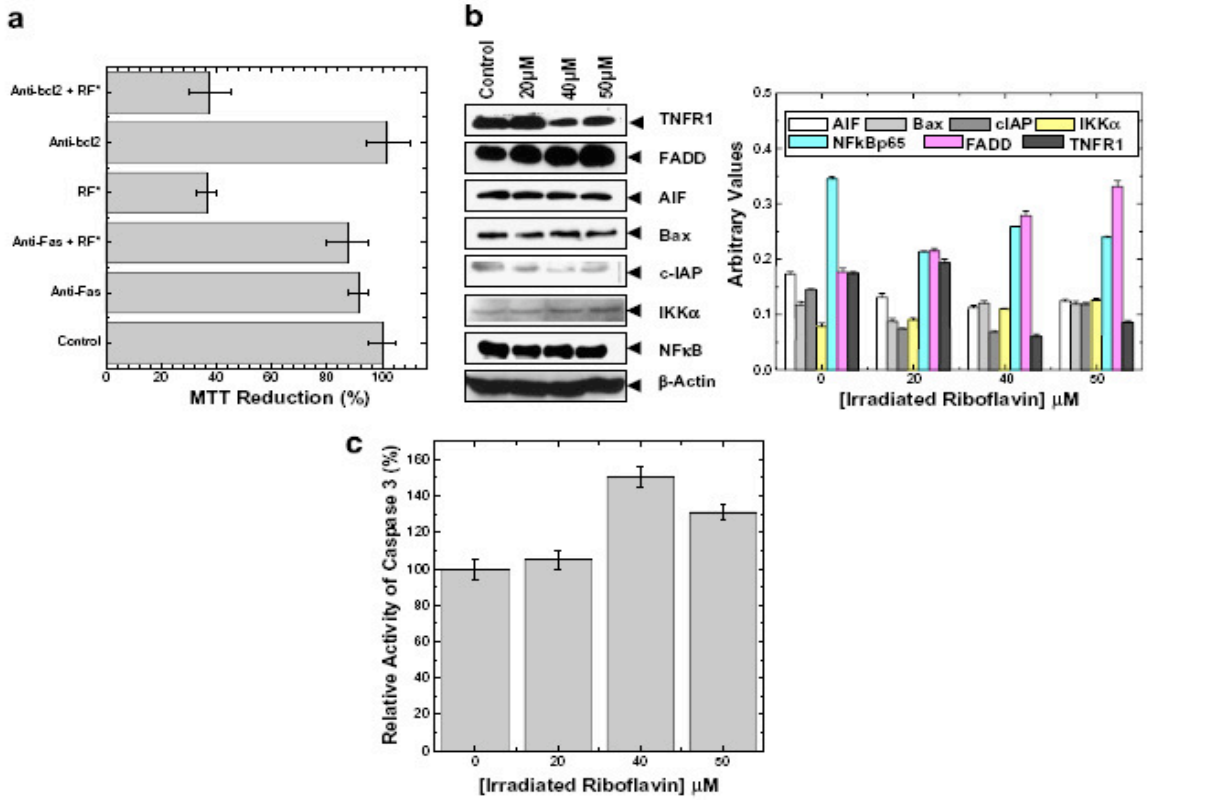

Fig. 2. Apoptosis induction in prostate cancer cells by irradiated riboflavin. (a) Cells were incubated with Fas neutralising antibody (anti-mouse) or anti-Bcl2 (10 μg/mL) for 30 min and afterwards treated with 50 μM irradiated riboflavin (RF*) for 24 h. Viability was determined by the MTT reduction assay. (b) The expression of TNFR1 and FADD was evaluated by immunoblotting using extracts obtained from cells treated with irradiated riboflavin. (c) Colorimetric assay was performed after 24 h of treatment to determine caspase 3 activity. Results represent the means ± standard error of three experiments run in triplicate ($p < 0.05$). Anti-Bcl2 (anti-mouse) was used as control.

IKKα (Fig. 2b). The expression of other pro-apoptotic proteins was checked; the expression of AIF as well as Bax displayed a slight decrease after irradiated riboflavin treatment. Additionally, cIAP1 level dropped when 40 and 50 μM irradiated riboflavin were used. Finally we controlled for the expression of other proteins involved in the Fas-induced cytotoxic response; their presence was readily detected. Furthermore, activation of caspases, a hallmark of Fas-induced cytotoxicity, was readily detected after stimulation of PC3 cells with irradiated riboflavin (Fig. 2c). We conclude that the cytotoxic response to riboflavin photoproducts in the PC3 model system is reminiscent to that in leukaemia cell, this action of riboflavin being specifically mediated via the induction of FasL–Fas signalling.

3.3. Riboflavin photoproducts action in the PC3 model system is highly similar to that in leukaemia

In leukaemic cells, treatment with irradiated riboflavin is associated with a downregulation of survival signals through the PI3K/AKT signalling pathway. When the activity of this signalling system was tested in the PC3 model, we observed strong inhibition of AKT/PKB and of p110 PI3K protein expression levels, showing that as in leukaemic cells, irradiated riboflavin targets this signalling pathway. MAPKp38 was also inhibited by irradiated riboflavin treatment, while in regard to JNK1/2 was observed an activation at 40 μM irradiated riboflavin. Additionally, the protein serine/threonine phosphatase (PP2A) was inhibited by irradiated riboflavin, an effect seen in leukaemia as well (Fig. 3a). Further evidence that the effects induced by irradiated riboflavin in PC3 cells are similar to those seen in leukaemic cells was obtained in experiments using Fumonisin B1, a known inhibitor of the enzyme ceramide synthase involved in *de novo* synthesis of ceramide in the endoplasmic reticulum and mitochondria. This compound was previously shown to prevent the cytotoxic response to riboflavin photoproducts in HL60 cells, a model system for leukaemia. When tested in the PC3 system, Fumonisin B1 potently prevented the toxic effect of irradiated riboflavin (Fig. 3b). Thus multiple aspects of irradiated riboflavin signalling in leukaemia are reflected in the PC3 model system as well.

3.4. Metalloproteinases activities, VEGF and TIMP1 expression are affected by irradiated riboflavin

An important difference between haematological malignancies and solid tumour cancers is that the latter depend on their capacity to degrade matrix proteins for successful dissemination through the body (metastasis), whereas in haematological malignancy this much less of an issue. Hence, we were interested to analyse the capacity of riboflavin photoproducts to influence the activity of enzymes capable of breaking down matrix components. PC3 are reported to be highly metastatic and accordingly zymographic analysis revealed substantial activity of the metalloproteinases 2 and 9 with regard to a collagen substrate. Interestingly, treatment of PC3 cultures with riboflavin photoproducts substantially decreased the activity of these enzymes with concomitant upregulation of TIMP-1 (Fig. 4). In addition, we observed donwregulation of VEGF. Apparently, the induction of cytotoxicity is accompanied by a reduction of matrix degrading potential as well as inhibition of angiogenesis, suggesting that treatment with irradiated riboflavin might reduce metastatic potential in solid tumours.

4. Discussion

The clinical management of prostate cancer remains highly challenging and novel adjuvant strategies are urgently called for. In the present study, we provide compelling evidence that the anti-neoplastic effects of riboflavin photoproducts as observed in leukaemia cells are mirrored in the PC3 model system, not only with respect to a strong cytotoxic effect of this compound but also with respect to the underlying molecular mechanism, the induction of FasL–Fas signalling. The PC3 model system is well known to be unusually resistant to pharmacological intervention, thus these results agree well for possible expansion of clinical trials with irradiated riboflavin to solid tumour types [6]. The results obtained support the candidacy of these photoproducts as adjuvant agents in prostate cancer therapy, as toxic effects of the photoproducts on the cancer cells were observed at concentrations lower than 40 μM. This concentration is assumed to be non-toxic for non-transformed cells. Despite the property of riboflavin as a photosensitizer is documented in the literature, the effect of its photoproducts has not been addressed in detail, neither *in vivo* nor *in vitro*. Wollensak and coauthors have reported that riboflavin/UVA treatment leads to a dose-dependent keratinocyte damage [10]. In addition, the applicability of irradiated riboflavin has already been suggested as an inactivator of pathogens of blood components [11,12].

The specificity of apoptosis being involved rather than necrosis or other type of cell death was highlighted by the activation of caspase 3, which effect was maximum when 40 μM irradiated riboflavin was used. This result was in agreement with the low level of c-IAP1 observed at 40 and 50 μM of irradiated riboflavin. However, there is a possibility

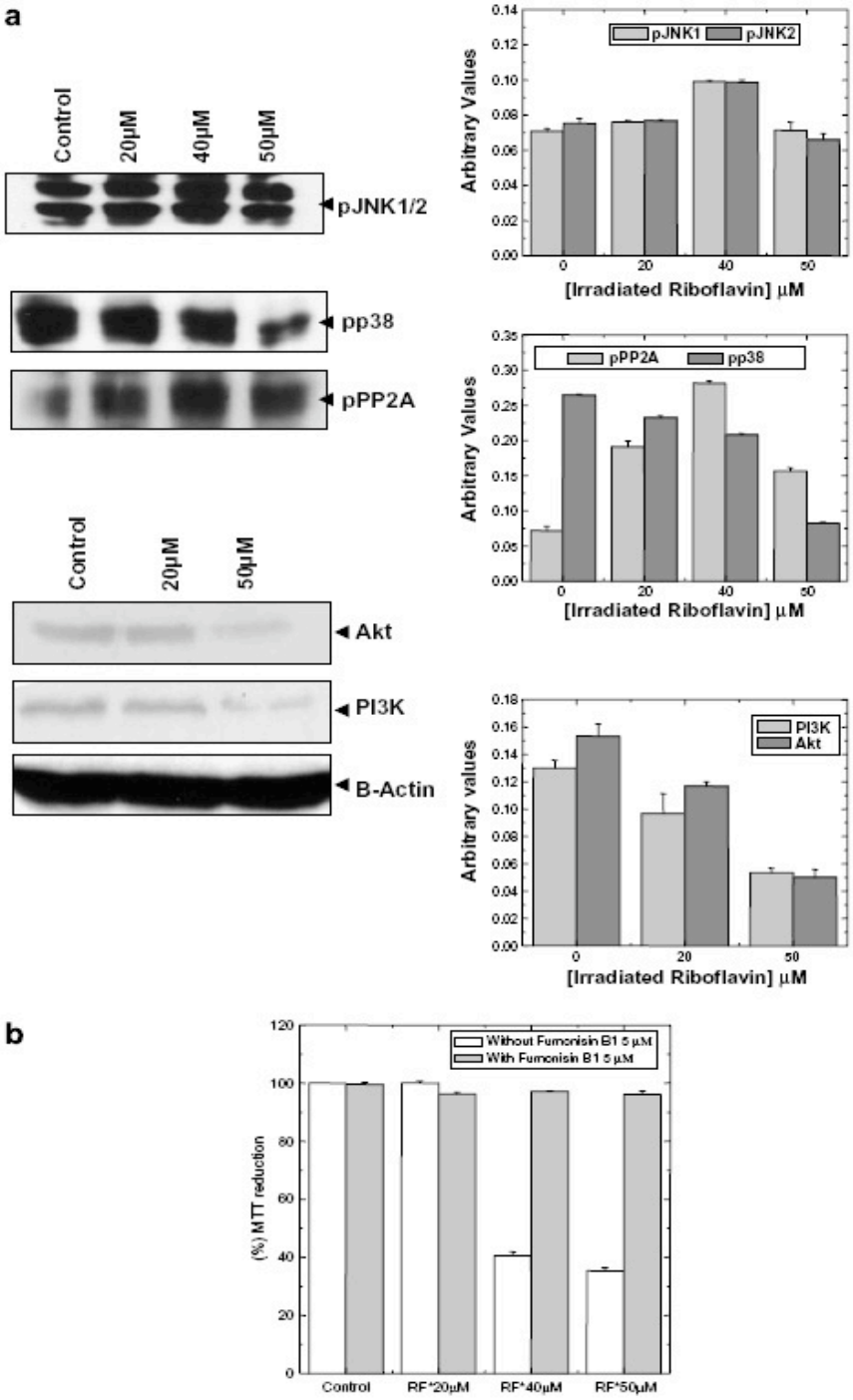

Fig. 3. Irradiated riboflavin causes differential effect on the expression of proteins involved in cancer cell survival and death. (a) Cells were treated with irradiated riboflavin and immunoblotting analysis was performed to evaluate the expression levels of key proteins involved in cell survival and death. (b) Cells were incubated with 5 μM of Fumonisin B1 for 30 min and afterwards treated with irradiated riboflavin for 24 h. Viability was determined by the MTT reduction assay. RF*: irradiated riboflavin.

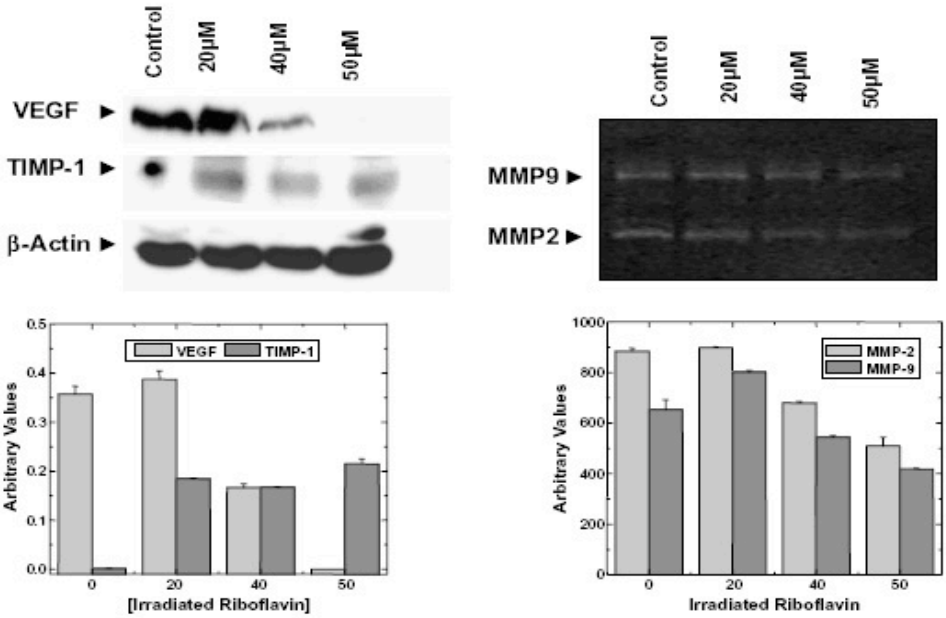

Fig. 4. Effect of irradiated riboflavin in metalloproteinases 2 and 9 activities and expression of VEGF and TIMP1. The proteolytic activity of MMP-2 and MMP-9 was assayed by gelatin zymography in the culture medium of prostate cancer cells treated with different concentrations of irradiated riboflavin for 24 h. The loading medium volume on the gel was normalized by the viable cell number after treatment (control −250,000; 20 μM RF* −181,500; 40 μM RF* −118,750; 50 μM RF* −82,500 cell/mL, loading volume: 4.95, 6.8, 10.4 and 15 μL, respectively). VEGF and TIMP1 expressions were determined by Western blotting.

of occurrence of other type of cell death at the highest concentration of irradiated riboflavin used. Interestingly, AIF did not seem to be involved in the death mechanism triggered by irradiated riboflavin, since the level of this protein remained unchanged.

This remarkable action of these photoproducts may well be explained by our observations that a multitude of relatively unlinked death pathways is targeted, which together may all be necessary to drive apoptosis in this system (inhibition of e.g. Fas or ceramide signalling is already sufficient to blunt the apoptotic process). Pre-treatment with anti-Fas neutralising antibody resulted in complete inhibition of cell death, indicating that the extrinsic pathway is a major signalling response involved in the apoptotic process induced by riboflavin. This result is consistent with the upregulation of FADD observed in PC3 cells treated with riboflavin photoproducts. Despite the coexpression of both cell-surface proteins Fas and FasL and the constitutive secretion of biologically active soluble FasL, prostate cancer cells are resistant to Fas-mediated apoptosis [13–15]. However, there are some reports in the literature that demonstrated the reactivation of the apoptotic potential of autocrine Fas signal in these cells when PI3K is inhibited [15]. Our findings also indicate that inhibition of PI3K represents a pivotal role in the riboflavin-mediated PC3 death. Therefore, our data imply that PI3K inhibition induced in prostate cancer cells occurs through the action of FasL secreted by the cell, acting in an autocrine or paracrine fashion, and/or through the action of Fas/FasL interactions occurring on the cell surface.

Intriguingly, our results showed that the inhibition of ceramide synthase by Fumonisin B1 was able to counteract cell death provoked by irradiated riboflavin at all concentrations tested, suggesting that riboflavin photoproducts-induced PC3 cell death is associated to the activation of Fas receptor induced via a ceramide-dependent pathway or vice-versa. In this respect, previous data from the literature have shown that ceramide as a second lipid messenger is involved in the reorganization of lipid rafts into ceramide-enriched platforms responsible for increasing Fas clustering and activation [7,16–22]. In contrast, ceramide release after Fas ligation has also been described [22]. In fact, an increase in ceramide synthesis in the mitochondria, which is under the control of ceramide synthase and cerami-

dase activities, seems to occur prior to the execution phase of apoptosis, and recent evidence suggests that this event is due, at least in part, to the ceramide ability to form protein-permeable channels in the outer mitochondrial membrane, thus favouring the release of cytochrome *c* and apoptogenic proteins such as Smac/DIABLO and HtrA2/Omi among others [16]. The fact that cells treated with irradiated riboflavin presented downregulation of TNFR1 as well as NFκB, suggests that this vitamin and its photoproducts display anti-inflammatory property. Recently, Bertollo and coworkers demonstrated that riboflavin inhibits early and prolonged inflammatory responses towards LPS by unknown mechanism [23].

The reversible phosphorylation of proteins, regulated by protein kinases and protein phosphatases, influences virtually all cellular functions and it is an essential mechanism in the control of cell proliferation, differentiation and transformation [24–26]. Riboflavin photoproducts induced a decrease in the phosphorylation level of both serine/threonine and tyrosine residues. The inhibition of the serine/treonine phosphatase PP2A observed after treatment is in agreement with other works showing an induction of apoptosis by PP2A inhibitors [4].

Especially encouraging is the observation that riboflavin photoproducts might suppress the metastatic potential of solid tumours which is supported by the following statements: (a) inhibition of metalloproteinase-mediated degradation of the matrix component collagen as demonstrated by decreasing MMPs activities and upregulation of TIMP-1; (b) downregulation of VEGF; (c) inhibition of MAPK p38. Because of MMPs extracellular matrix-degradation activities, certain aspects of these enzymes involvement in tumour metastasis, such as tumour-induced angiogenesis, tumour invasion, and establishment of metastatic foci at secondary site, have received extensive attention that resulted in an overwhelming amount of information in favour of critical roles of MMPs in these processes. In this context, physiological MMPs inhibitors, TIMPs, and natural products have been considered as important negative modulators of cancer cells invasion [27–29]. MAPK p38 plays a multitude of cellular functions, and recently Ye and Yuan reported that inhibition of p38 impaired the angiogenesis process [30]. Based on this observation, we can hypothesize that the inhibition of p38 by irradiated riboflavin could be also important to prevent PC3 cells metastasis.

The ultimate molecular basis for the strong effects of riboflavin photoproducts on various kinds of cancer cells remains somewhat enigmatic. The products generated are markedly more hydrophobic as compared to the parent compound riboflavin, due to the loss of the ribityl moiety of the molecule during the photodegradation. Thus, it is highly likely that they will either end up in the plasma membrane or enter the cytosol without the need for active take up by the cancer cell. Further studies, employing purification of interacting proteins using radio labeled parent compound may well be required to answer this question. In any case the remarkably similar biochemical changes observed in PC3 and leukaemia cells suggest that a common upstream mediator is responsible for death in both cell types. Alternatively, specific interference with the build of specialized lipid plasma membrane domains, like lipid rafts, is a possibility as well. The present study has shown that riboflavin photoproducts are highly promising candidates for solid tumour cancer therapy and provide strong support for commencing small scale human phase I/II studies in terminal solid tumour cancer employing these compounds. Lastly, it is important to mention that our findings about the effect of irradiated riboflavin in tumour cells as well as others related to intact riboflavin pointed out the broad application of this vitamin in the cancer therapy field. For instance, there are some reports on the literature about the augment of tamoxifen efficacy against breast cancer cells, when this chemotherapic was combined with riboflavin. This property is based on the capacity of riboflavin in maintaining the cellular antioxidant response closer to normal level [31].

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Signal transduction pathways involved in the differentiation process of leukaemia cells triggered by irradiated riboflavin

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Running Title: Differentiation induction by irradiated riboflavin

Abstract

One of the challenges of the cancer therapy is to combine efficacy with low side effects and consequently improving the life quality of the patient. Leukaemia represents a spectrum of diseases in which the cost of cure may be substantial with short- and long-term side effects. Therefore, new agents are needed to extend survival, improve cure rates, and avoid undesirable treatment-related toxicities. In this scenario, there are at least two aims a) to provide therapeutic agents with very specific target and b) to discover agents, which present differential action mechanism in comparison with the traditional chemotherapy, e.g. differentiation promotors. Herein, we demonstrated by using traditional technique combined with kinome profile that lower dose of irradiated riboflavin (0.5/5 μM) is able to alter myeloid leukaemia cells metabolism which culminates with differentiation. In this process, we spotlighted at least three cascades with their respective mediators TNFR1 activation and in turn, increasing of NFκB level, PTPalpha/Raf/Src/SHP2/Stat3 cascade and JNK activation. Importantly, cytoskeleton rearrangement was also observed. Our findings provide strong evidence that irradiated riboflavin is an interesting and promising antileukaemic agent, mainly due to present some characteristics which might causes less side-effects when compared with the traditional chemotherapics used as differentiation inducers.

Key words: Irradiated riboflavin, HL60 cells, differentiation, signal transduction

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1. Introduction

Acute leukaemia in adults and children represents a spectrum of diseases ranging from those with a very poor prognosis, such as AML in older patients, to disease such as childhood

ALL where the cure rates are high. However, even for those subtypes with high cure rates, the cost of cure may be substantial with short- and long-term side effects. Therefore, new agents are needed to extend survival, improve cure rates, and avoid undesirable treatment-related toxicities (Tallman et al., 2005; Tallman, 2007). In other words, drugs must be both safe and effective, as also has been adviced by US Food and Drug Administration. In this scenario, there are at least two challenges a) to provide therapeutic agents with very specific target and b) to discover agents, which present differential action mechanism in comparison with the traditional chemotherapy, e.g. differentiation promoters (Tallman, 2007).

In recent years, vitamins and their derivatives are gaining prominence in anti-cancer strategies, especially in haematological cancers. For example all-trans retinoic acid (vitamin A) is now widely used for the treatment of myeloid leukaemia (Hisatake et al., 2001; Tsuzuki et al., 2004) and vitamin D is in clinical trial for this disease as well (Trump et al., 2006). Very recently, we provided evidence that photoproducts of riboflavin, a constituent of the vitamin B complex (vitamin B2), can induce apoptosis of both haematological and solid malignancies (Souza et al., 2006; Queiroz et al., 2007). In both cellular models the molecular mechanism responsible for irradiated riboflavin, in a concentration range of 10-30μM, inducing cell death was dependent on Fas receptor activation together with diminished protein kinase B-dependent survival signalling. In addition, irradiated riboflavin displayed desirable and interesting properties as antitumoral agent, inhibiting MMPs and VEGF expression (Queiroz et al., 2007).

Herein, we demonstrated, by using traditional technique combined with pepchip, that lower dose of irradiated riboflavin (0.5/5 μM) is able to alter myeloid leukaemia cells metabolism which culminates with differentiation. In light of signal transduction pathways, our findings reveal that irradiated riboflavin action is dependent on very well organized process activation. We spotlighted at least three cascades with their respective mediators which culminate with leukaemia cells differentiation: TNFR1 activation and in turn increase of NFκB level, PTPα activation and activation of Raf/Src/SHP2/Stat3 cascade and activation of JNK. Importantly, cytoskeleton rearrangement was also observed, which process is required for cell morphology changing during differentiaton.

2. Materials and methods

Cell line and reagents - HL60 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Polyclonal antibodies against, antiphospho-p38 mitogen-ativated protein kinase p38, antiphospho-p42/44 (ERK1/2), antiphospho-c-jun-NH_2-terminal protein kinase (JNK), antiphospho-MAPK/ERK Kinase 1/2 (MEK1/2), antiphospho-cRaf, antiphospho-pan PKC, antiphospho-STAT3, antiphospho-PTEN, antiphospho-PKB, antiphospho-SHP2, antiphospho-PTPα, antiphospho-cdc2, p21, FADD, AIF, pan-actin, antirabbit and antimouse peroxidase-conjugated antibodies were purchased from Cell Signalling Technology (Beverly, MA). Antibodies against phospho-PP2A, phosphotyrosine, phosphothreonine, NFκB p65, receptor-associated factor 2 (TRAF2), caspase 8, Bcl2, Bax, TNF receptor 1 (TNFR1), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Riboflavin and anti-cyclin D1 were from Sigma.

Cell culture - HL60 cells were routinely grown in suspension in RPMI 1640 medium supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal bovine serum, at 37°C in a 5% CO_2 humidified atmosphere. In all experiments, 5×10^5 and 6×10^5 cells/ml were treated for 24 and 48 h with irradiated riboflavin, respectively.

Irradiation of riboflavin and treatment of HL60 cells - a solution of 250 µM riboflavin was prepared in RPMI 1640 culture medium and irradiated with UV light for 15 min. Afterwards, cells (5 or 6×10^5 cells/ml) were treated with 0.5 and 5 µM of irradiated riboflavin.

MTT reduction assay - medium containing irradiated riboflavin was removed and 1.0 ml of MTT solution (0.5 mg MTT/ml of culture medium) was added to each well. After incubation for 4 h at 37°C, the medium was removed and the formazan was released by solubilisation in 1.0 ml of ethanol. The plate was shaken for 5 min on a plate shaker and the absorbance measured at 570 nm (Mosmann, 1983).

NBT reduction assay - after treatment of the HL60 cells with irradiated riboflavin (0.5 and 5 µM), for 24 and 48 h, the cell number was estimated by trypan blue exclusion. For all concentrations tested, the cell number utilized was the same (1×10^6 cells/ml). After treatment, the cells were harvested by centrifugation and suspended in 100 µl of NBT solution (4 mg/ml). After the addition of 100 µl of TPA (2 µg/ml), the cell suspension was incubated at 37° C for 20min and 200µl of 1N HCl were added at 4 C to terminate the reaction. Then, the cells were centrifuged and 600 µl of DMSO were added to the cell pellets to solubilize the formazan deposits. The amount of formazan was measured at 560 nm (Freire et al., 2003).

Western bloting analysis - cells (3×10^7) were lysed in 200 µL of lysis buffer [50 mM Tris-HCl (pH 7.4), 1% Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF

and protease inhibitors (1 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl-fluoride-hydrochloride)] for 2 h in ice. Protein extracts were cleared by centrifugation and protein concentrations were determined using the Lowry method (Hartree, 1972). An equal volume of 2X sodium dodecyl sulfate (SDS) gel loading buffer [100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.1% bromophenol blue and 20% glycerol] was added to samples which were subsequently boiled for 10 min. Cell extracts, corresponding to 3×10^5 cells, were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to PVDF membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (2%) in Tris-buffered saline (TBS)-Tween 20 (0.05%) and incubated overnight at 4°C with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with antirabbit or antimouse horseradish peroxidase-conjugated secondary antibodies, at 1:2000 dilutions (in all Western blotting assays), in blocking buffer for 1 h. The detection was made using enhanced chemiluminescence (ECL).

NFκB translocation - briefly, 2 x 10^7 cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 0.2 mL ice-cold cell extract buffer (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]–KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KC1, 0.5 mM DTT, and 0.2 mM phenylmethysulfonyl fluoride [PMSF]). The cells were kept on ice for 10 minutes to allow them to swell, mixed by vortex for 10 seconds, and microfuged at 4° C at 14000g for 30 seconds. The supernatant was discarded, and the pellet was resuspended in 30 µL nuclear extraction buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgC1_2$, 0.2 mM EDTA(ethylenediaminetetraacetic acid), 0.5 mM DTT, and 0.2 mM PMSF), placed on ice for 20 minutes, and centrifuged at 4° C at 14000g for 2 minutes. The supernatant was saved as the nuclear extract and used in Western blotting assay.

Immunoprecipitation of TNFR1

After treatment of the cells with irradiated riboflavin for 24 and 48 hours, whole-cell lysates were prepared with lysis buffer (20 mM HEPES, pH 7.7; 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1% Nonidet-P40 [NP40], 20 mM p-nitrophenylphosphate, 1 mM *O*-Vanadate, 1 mM 4-(2-amino-ethyl)-benzolsulfonyl-fluorid-hydrochloride), 1 mM DTT, 10 µg/mL aprotinin, and 10 µg/mL leupeptin) and chilled on ice for 2 hours. After centrifugation, lysates were incubated overnight with anti-TNFR1 at 4° C and then rotated with Protein A-Sepharose at 4° C for 2 hours. The beads were washed 3 times with lysing buffer and twice with PBS. Next, the immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membrane as described in "Western blotting."

TNF-α assay - cytokine level was measured in the cell culture supernatants by using enzyme-linked

immunosorbent assay (ELISA) kits from Biosource.

Caspase 3 activity - caspase 3 activity was determined by the measurement at 405 nm of *p*-nitroanaline released from the cleavage of caspase 3 substrate (Ac-DEVD-pNA).

Kinomic array - the production of the array and the protocol of the kinome array have been described in detail earlier (Diks et al., 2004a,b; Lowenberg et al., 2005, 2006; van Baal et al., 2006; De Borst et al., 2007). In short, cells were washed in PBS and lysed in a non-denaturing complete lysis buffer. The peptide arrays (Pepscan, Lelystad, The Netherlands), containing up to 1176 different kinase substrates in triplo, were incubated with cell lysates for 90 min in a humidified incubator at 37°C. Subsequently, the arrays were washed in 2 M NaCl 1%Tween, PBS 1% Tween and water, whereafter slides were exposed to a phosphoimaging screen for 72 h and scanned on a phospho-imager (Fuji, Stanford, USA).

Statistical evaluation - The Western blots represent 3 independent experiments. Cell viability and TNF quantification data were expressed as the means ± standard errors of 3 independent experiments run in triplicate. Data for each assay were analyzed statistically by analysis of variance (ANOVA).

3. Results

Irradiated riboflavin induces differentiation in HL60 cells

In this report, we investigated the potential of irradiated riboflavin as a differentiation inductor, using human myelocytic leukemia cells (HL60 cell) as a model. HL60 cells were seeded at a density of 5 or 6 x 10^5 cells/mL and treated with 0.5 and 5µM irradiated riboflavin for 24 or 48h. Cell number and viability for each group were

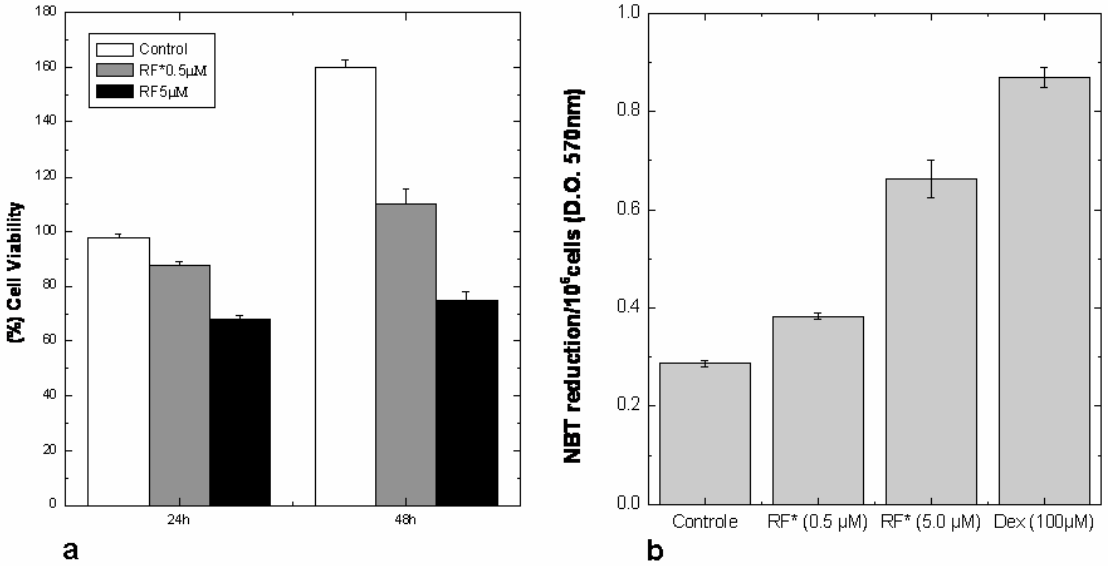


Figure 1 - Irradiated riboflavin causes differentiation of HL60 cells. (a) Cells viability was assessed by MTT reduction assay after treatment for 24 and 48h. **(b)** Cells were treated with 0.5 and 5 µM irradiated riboflavin or 100 µM dexamethasone, for 48 h. Cell differentiation was evaluated by NBT reduction.

determined. After treatment with the concentration of 5μM for 24h the cell viability decreased around 30%; and this effect was higher after 48h (Figure 1A). As can be seen in Figure 1b, irradiated riboflavin caused expressive HL60 cell differentiation which action was very significant when compared with the effect of dexamethasone, a typical cell differentiation inducer (Figure 1B).

Activation of TNFR1

Taking into account the importance to identify the molecular targets for new potential candidate to treat cancer, we evaluated a set of proteins involved in different cellular processes. For this purpose, basically two techniques were employed: western blotting and Pepchip. In general, we analyzed early and later effect of irradiated riboflavin on different signalling pathways.

Irradiated riboflavin caused an increase of TNFR1 expression only after 48h (Figure 2A).

According to this result, we directly assessed the activation state of this receptor using a co-immunoprecipitation assay. To this end, the TNF receptor 1 was immunoprecipitated and subsequently the association of TRAF2 and FADD to the receptor was studied using Western blotting. As TRAF2 and FADD association to the receptor is dependent on its activation, this assay allows direct assessment of TNF receptor 1 activation. As evident from Figure 2A, treatment of HL60 cells with riboflavin for 48h activated the TNF receptor. Importantly, the activation of TNFR1 was responsible for activation/cleavage of caspase 8 and PARP fragmentation after treating the cells for 48h. Interestingly, treated cells displayed low level of TNFα (Figure 2B).

In light of the connection between TNFR1 signaling and NFκB, the expression as well as this protein translocation into the nucleus were checked. Despite overexpressing NFκB, HL60 cells treated with irradiated riboflavin did not display activation of this transcriptional factor, as revealed by direct observation of the nuclear translocation of NFκB by western blot analysis of nuclear extracts (Figure 2D).

Modulation of protein phosphatases, protein kinases and cytoskeleton rearragement by irradiated riboflavin

Several cellular processes are regulated by protein kinases and protein phosphatases, including differentiation. Figure 3 shows the activation status of a set of protein kinases and phosphatases. Irradiated riboflavin treatment caused activation of PTPα as observed by an increase of the phosphorylated form of this protein, after 48h of treatment (Figure 3A). In accordance, its downstream molecules such as Raf, SHP2, Src and Stat3 were also activated (Figure 3A, 3B). Our findings indicate that this group of proteins did not display an important role in the early effect of irradiated riboflavin. In addition, the activity of PTEN and LMWPTP were not affected by riboflavin photoproducts. Nevertheless, an inhibition of PP2A was observed at 24h (Figure 3A).

Regarding to protein kinases, PKC

was inhibited at 24h and remained unchanged at 48h; Ras, c-Raf, MEK, ERK and p38 were not affected by irradiated riboflavin. However, p38 was activated at 24h. JNK seems essential for the cell differentiation, since we observed an expressive activation of this MAPK at 48h. In general, after 48h the cell proliferation cascade was inhibited but the survival signalling pathways remained activated at basal level (Figure 3C).

Microtubule and actin represent the major components of cytoskeleton, in hematopoietic cells. Taking into account the importance of cytoskeleton remodelling during cell differentiation we checked Src kinase and caspase 3 activities which display a pivotal role on the cytoskeleton/focal adhesion components regulation. According to our results the HL60 cells differentiation induced by irradiated riboflavin is reinforced by Src as well as caspase 3 activation.

Cell cycle arrest

According to differentiation process requirement, cells treated with irradiated riboflavin presented cell cycle progression arrest. Among cycle regulators, the initial cellular response against irradiated riboflavin was an upregulation of p21, however cyclin D was expressively downregulated after 48h (Figure 4). In agreement cdc2 was inhibited when 5μM of irradiated riboflavin was used (Figure 4).

Kinome profiling reveals that irradiated riboflavin attends 3 requirements for a desirable differentiation inductor: cell cycle arrest, survival pathway activation and proliferation inhibition

To investigate the effects of irradiated riboflavin on the kinome, HL60 cells were incubated for 48h with culture medium or irradiated riboflavin and subsequently analyzed using the peptide array. Afterwards, the radioactivity incorporated in the substrate phosphorylated by lysates of both experimental conditions was analyzed (Figure 5).

Besides the protein kinases which were affected by irradiated riboflavin, mentioned previously, kinome profiling provided additional information about 90 substrates which were expressively phosphorylated by kinases with distinct cellular localization: plasma membrane, cytoplasm, nucleus, cytoskeleton/microtubules and mitochondria (AMPK, cytokine inducible kinase, cdc2, EFG receptor, ABL, PKA, DNA-dependent protein kinase, Csk, ZAP70, AKT1, PKG-1, GSK3 beta, pyruvate dehydrogenase kinase, SHC, casein kinase 1, CDK5, TrkB tyrosine kinase, CDK2, PKC zeta, PAS kinase, ERK5, FAK, cyclinE-cdk2, Fyn, PI3K alpha, ribosomal protein S6 kinase alpha, calcium-calmodulin dependent protein kinase I, NIK, CaM kinase I, EphA8, CK-II, PRKG1, PRKA1, p34cdc2, CK2, casein kinase II, IKK beta and BMP receptor type IB).

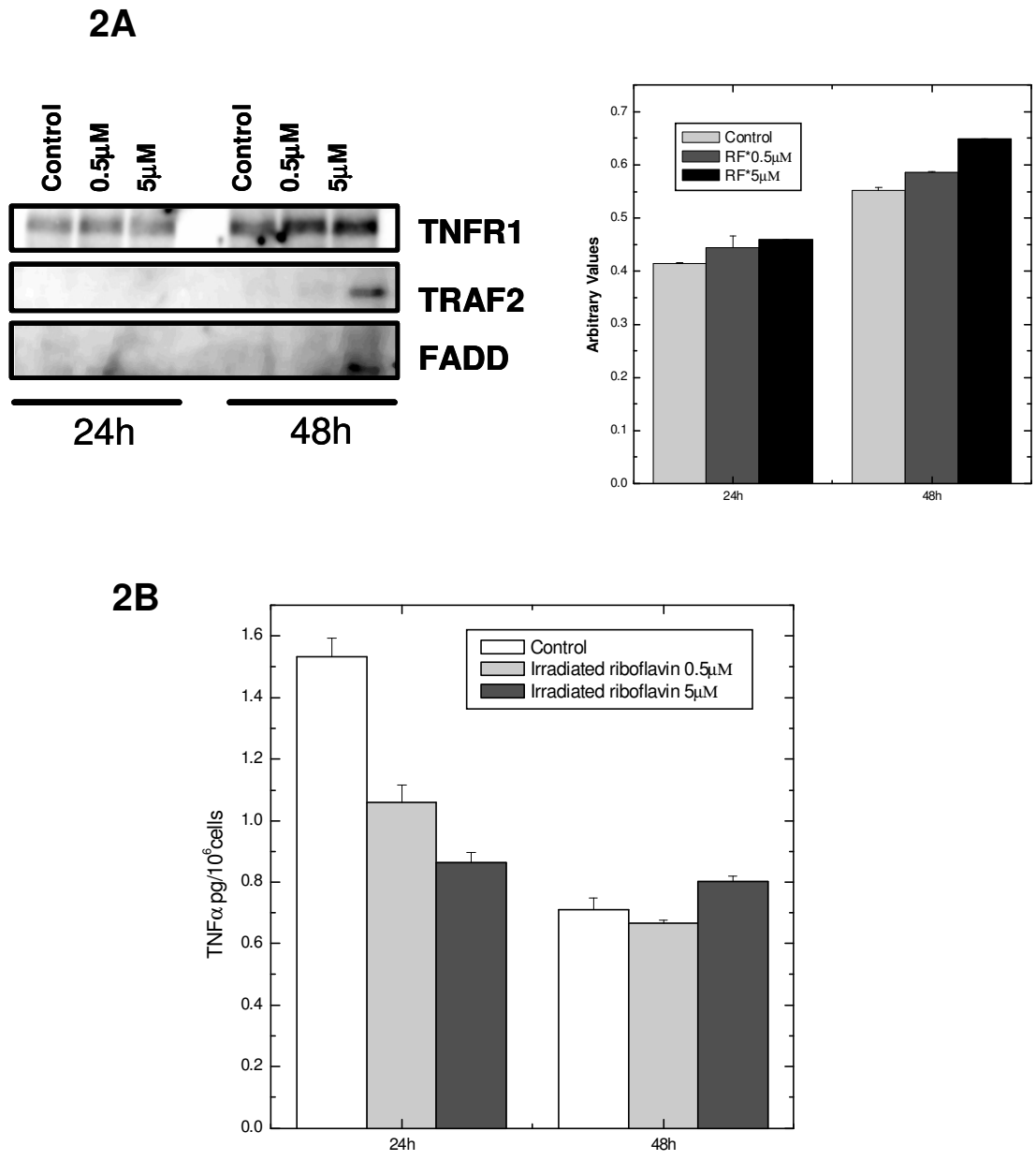


Figure 2. Activation of TNFR1 by irradiated riboflavin led to leukaemic cells differentiation. (A) TNFR1 expression, TRAF2 and FADD were co-immunoprecipitated with TNFR1 and analysed by western blotting. **(B)** TNFα quantification by ELISA.

2C

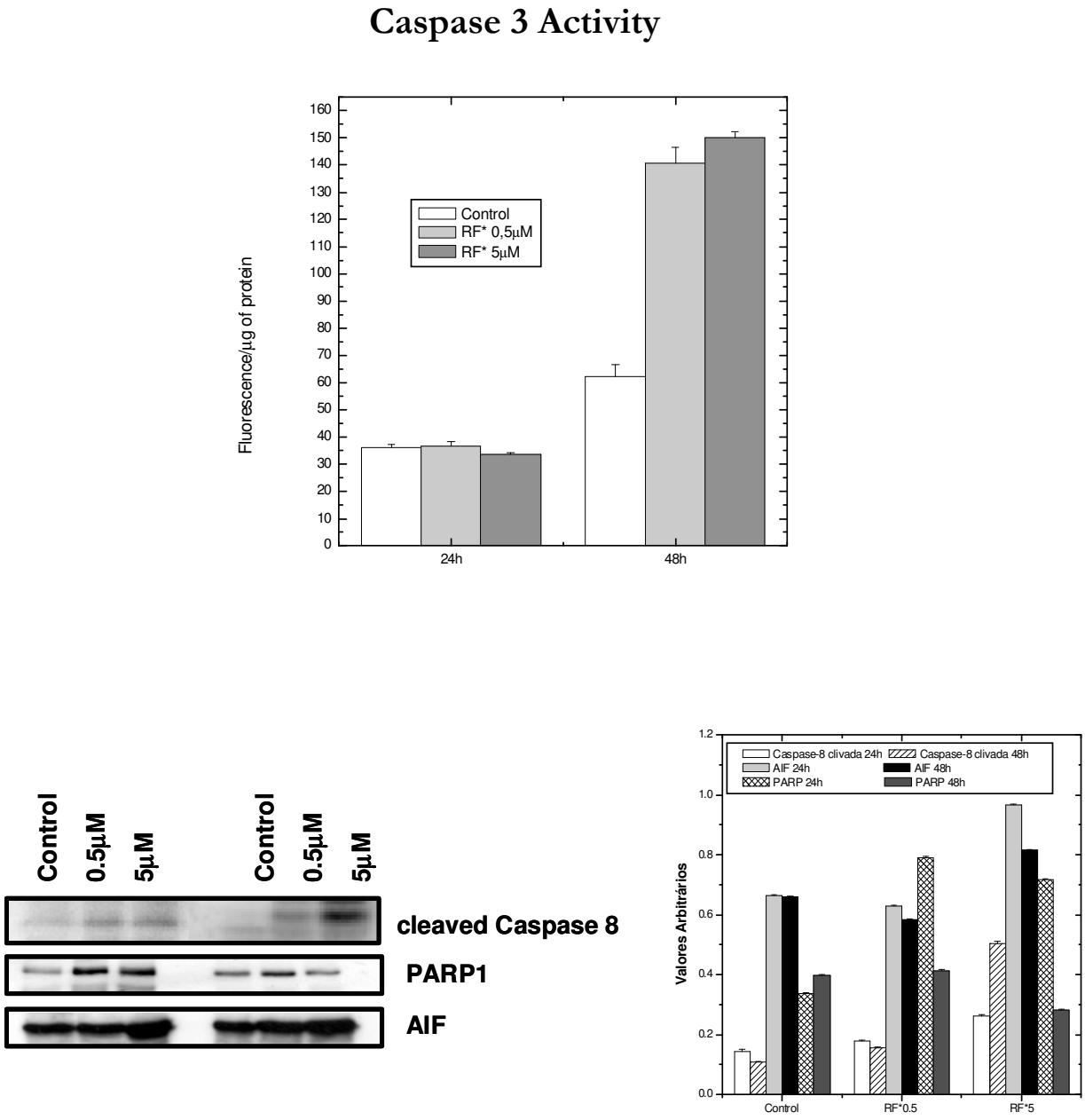
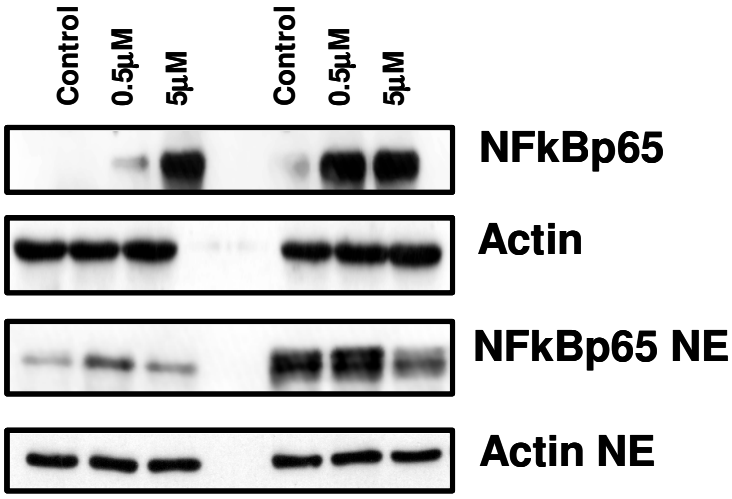


Figure 2. Activation of TNFR1 by irradiated riboflavin led to leukaemic cells differentiation. (C) caspase 8 zymogen, AIF and fragmented PARP were detected by western blotting; caspase 3 activity was colorimetric determined;

2D



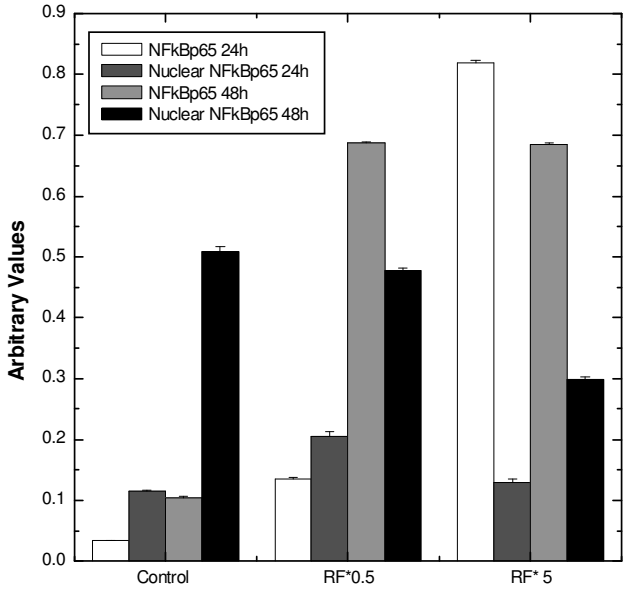
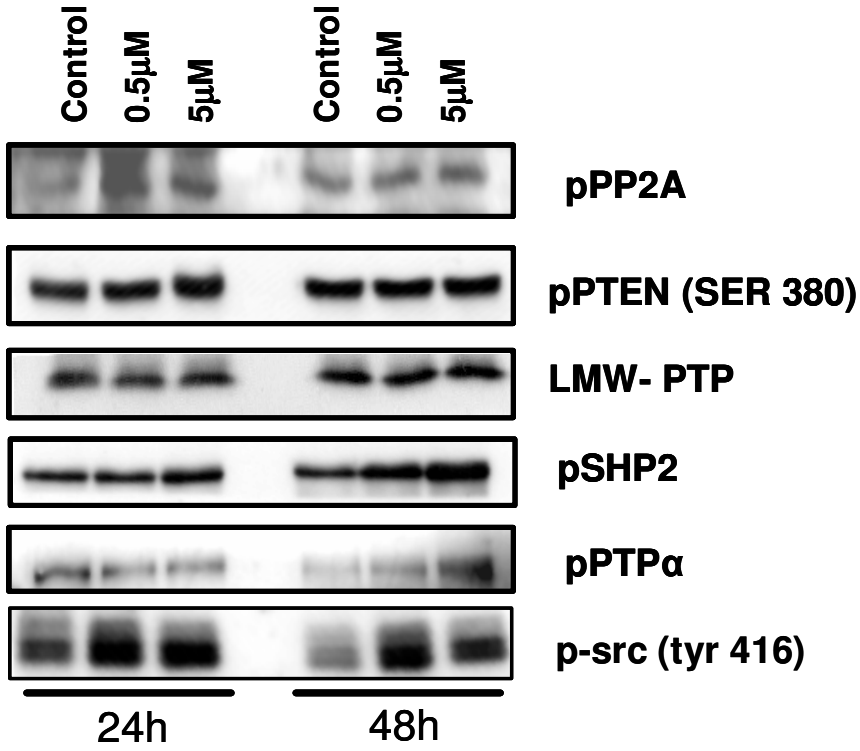


Figure 2. Activation of TNFR1 by irradiated riboflavin led to leukaemic cells differentiation. (D) NFKB expression as well its nuclear translocation were assayed by western blotting. NE = Nuclear extract.

3A



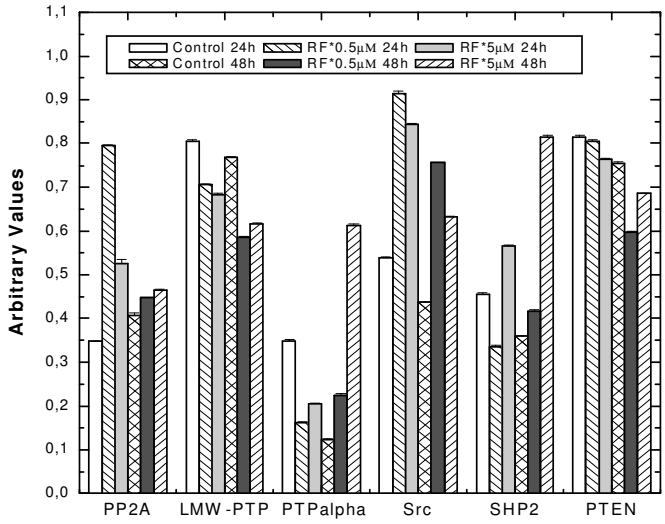
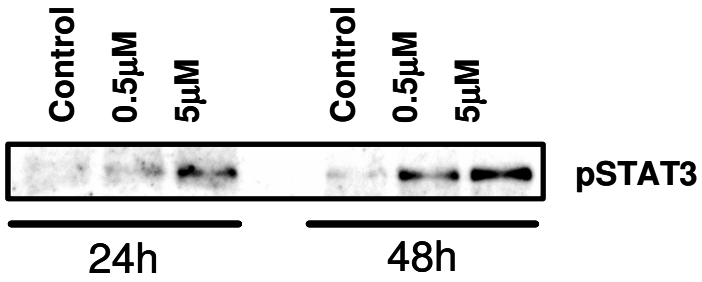


Figure 3. Modulation of protein phosphatases, protein kinases and STAT3 by irradiated riboflavin. Cells were treated with 0.5 and 5 µM irradiated riboflavin for 24 and 48h. **(A)** Expression and/or phosphorylation levels of phosphatasesand Src kinase; Expression and/or phosphorylation levels of kinases and phosphatases were evaluated by immunoblotting. Soluble lysates were matched for protein content and analyzed on Western blot.

3B



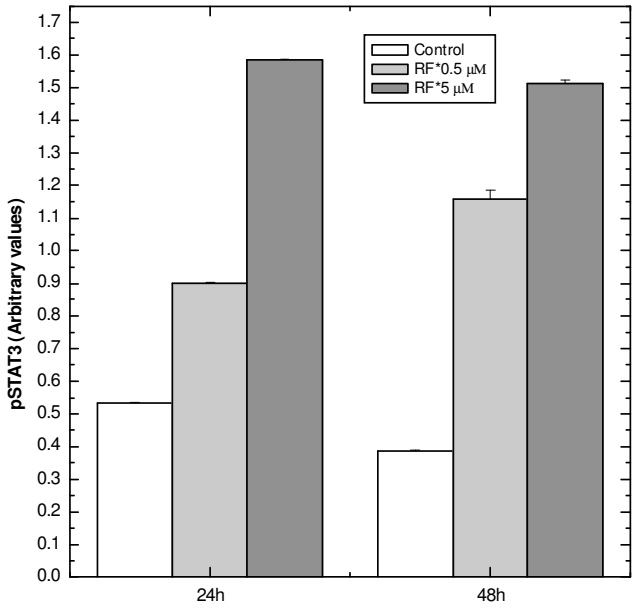


Figure 3. Modulation of protein phosphatases, protein kinases and STAT3 by irradiated riboflavin. Cells were treated with 0.5 and 5 µM irradiated riboflavin for 24 and 48h. **(B)** Phosphorylation status of STAT3; Phosphorylation levels of STAT3 was evaluated by immunoblotting. Soluble lysates were matched for protein content and analyzed on Western blot.

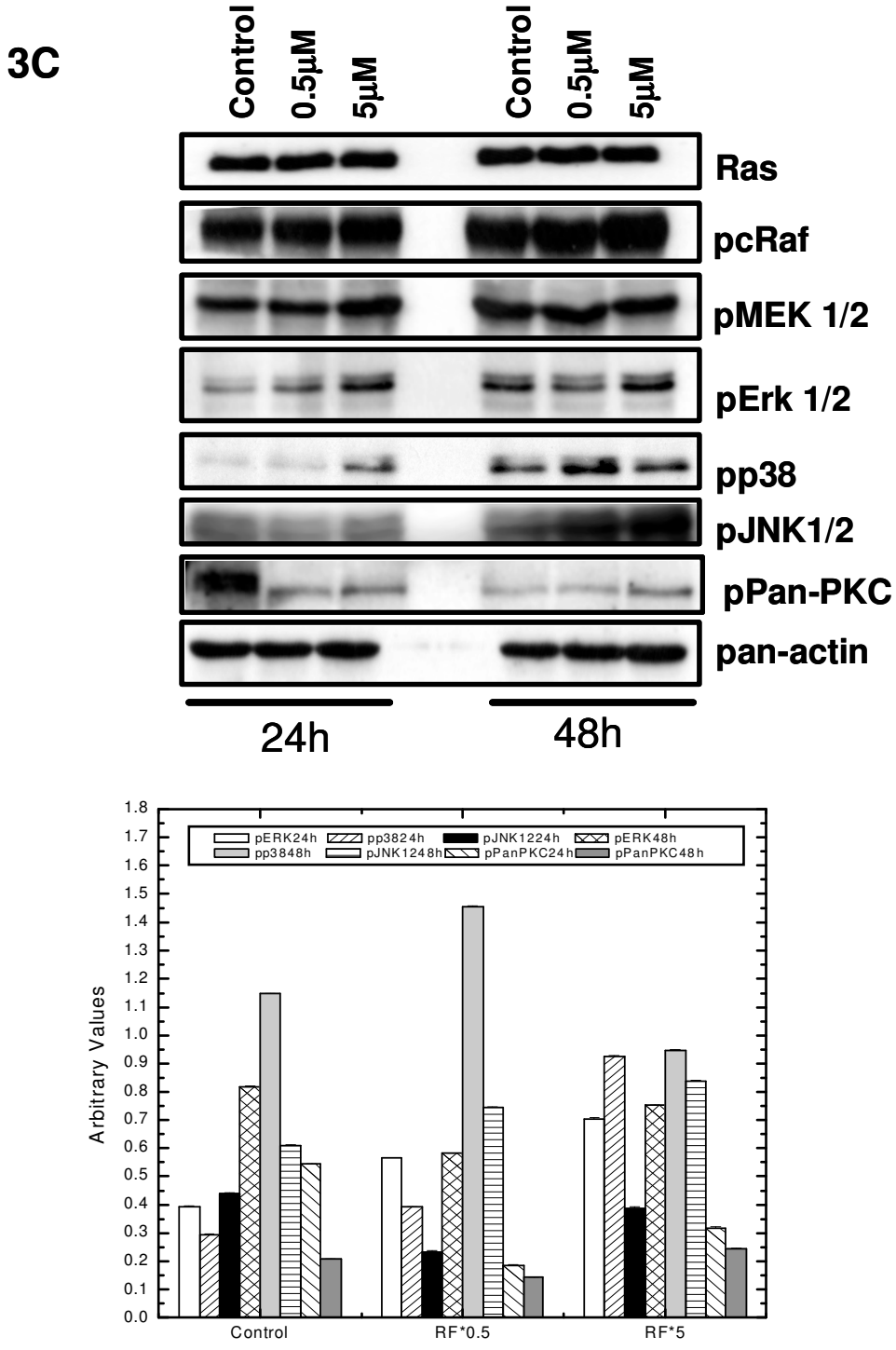
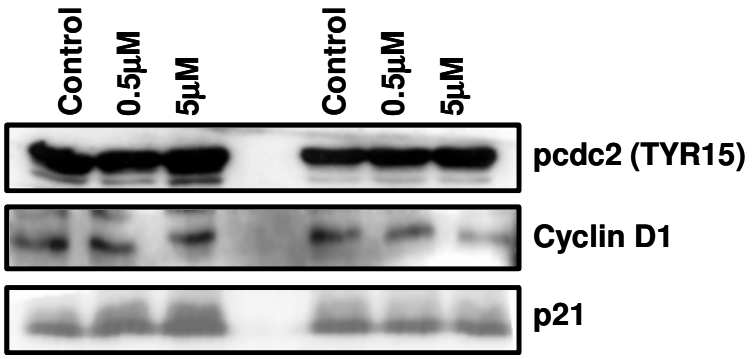


Figure 3. Modulation of protein phosphatases, protein kinases and STAT3 by irradiated riboflavin. Cells were treated with 0.5 and 5 µM irradiated riboflavin for 24 and 48h. **(C)** Expression and/or phosphorylation levels of Ras protein and kinases were evaluated by immunoblotting. Soluble lysates were matched for protein content and analyzed on Western blot.



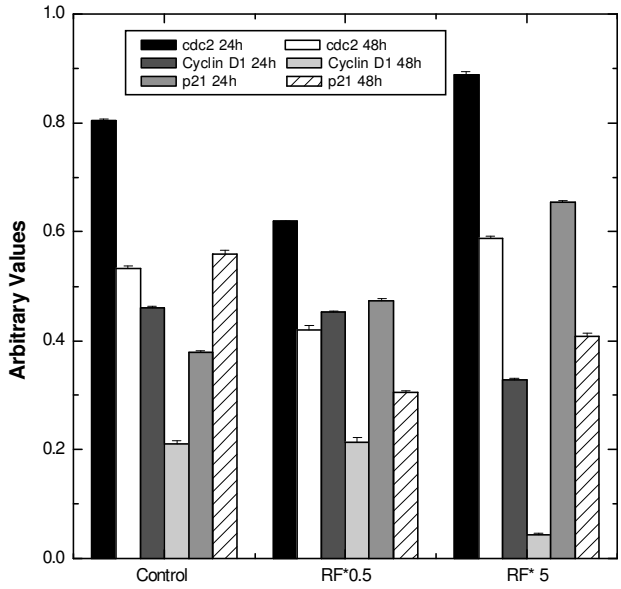


Figure 4. Inhibition of cell cycle progression by irradiated riboflavin. The expression of p21, cyclin D and pcdc2 (inhibitory site) was evaluated by immunoblotting. Soluble lysates were matched for protein content and analyzed on Western blot.

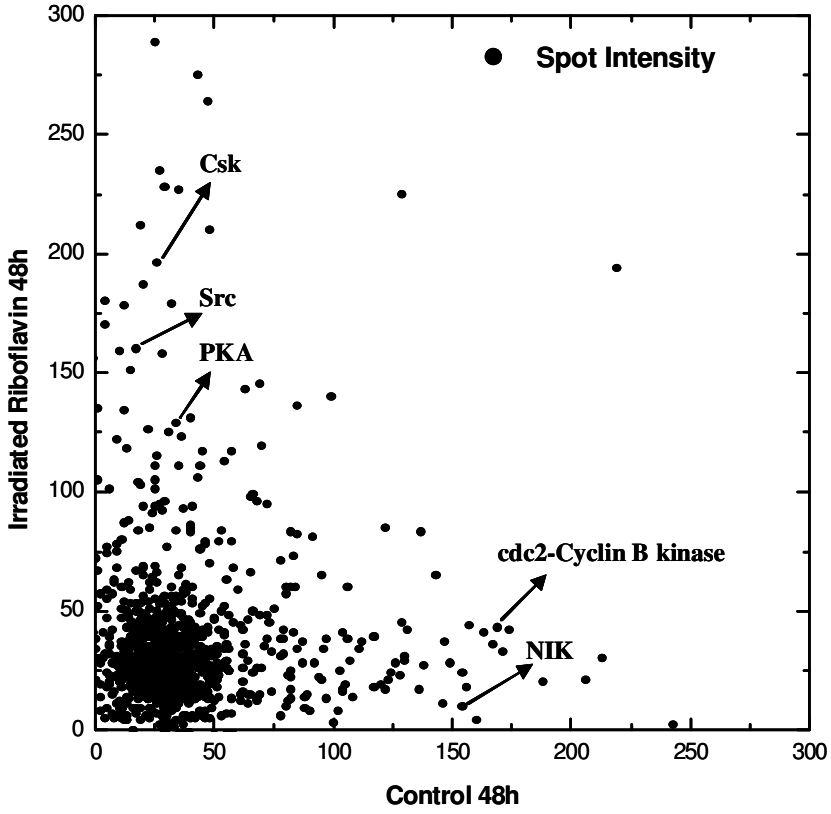


Figure 5. Peptide arrays reveal a multitude of cellular biochemical processes affected by irradiated riboflavin. The relationship between radioactivity incorporated in substrates phosphorylated (spot intensity) by vehicle-treated cells against the radioactivity incorporated in substrates phosphorylated by irradiated riboflavin-treated cells. The higher spot intensity value means the higher kinase activity. The dots indicated in the graphic correspond to the respective substrate (*see supplementary material in "Anexos").

*The cells lysates were put on PepChips and spot intensities were quantified. P-values derived from paired t-Tests performed on normalized intensities are given; n.s., not significant ($p > 0.05$). The kinases phosphorylating those substrates are indicated. ▲ means higher phosphorylation intensities after treatment with irradiated riboflavin 48h; ▼ means lower phosphorylation.

4. Discussion

Induction of apoptosis or differentiation is two promising cancer prevention and therapy strategies. It is generally accepted that activating fundamentally different anti-cancer activities, at the same time, would prevent the build-up of resistance. Unfortunately, some of drugs traditionally used for treating leukaemia do not present these desirable multiactivities, e.g., all-trans retinoic acid treatment is poorly compatible with chemotherapy (Zhou *et al*., 2007): chemotherapy acts in the S phase of the cell cycle whereas retinoic acid slows down the cell cycle, actually allowing cancer cells to escape chemotherapy. The results obtained in the present study, however, indicate a rare example that the same compound targets two important anti-leukaemic pathways at the same time, inducing cell death and differentiation simultaneously in a non-competing fashion. It is important to mention that these 2 effects are time- and dose-dependent.

In this context, irradiated riboflavin appears as an interesting approach to target cancer cells. Recently, we provided a wealth biochemical evidences about the multiple pathways by which irradiated riboflavin induces solid and hematopoietical cells death via apoptosis. Basically, the antitumoral activity of irradiated riboflavin is dependent on activation of caspase 8 caused by overexpression of Fas and FasL and also on mitochondrial amplification mechanisms, involving the stimulation of ceramide production by sphingomyelinase and ceramide synthase. The activation of this cascade led to an inhibition of mitogen activated protein kinases: JNK, MEK and ERK and survival mediators (PKB and IAP1), upregulation of the proapoptotic Bcl2 member Bax and downregulation of cell cycle progression regulators. These findings prompted us to investigate the capacity of lower concentration of irradiated riboflavin to induce leukaemia cells differentiation. Based on the previous results 0.5 and 5 μM riboflavin concentrations and treatment periods of 24 and 48h were chosen. HL60 cells treated with irradiated riboflavin for 48h demonstrated an expressive differentiation. The maturation process of these cells was monitored by NBT reduction, which is a typical compound commoly used to evaluate myeloid leukemia cells differentiation (Breitman, 1990; Kohroki et al, 1998).

Myeloid differentiation process is complex and involves several regulatory events depending on the differentiation inducer. Despite a huge number of study related to differentiation, detailed mechanism of this process are not completely defined yet. For instance, different mechanisms are described for diverse agents, including chemotherapic drugs (Chénais et al, 2000). In spite of absence of differentiation at 24h, our results claim that some important signaling events occur in this period. However, the major changing in signalling cascades were observed after 48h. Our findings indicate that a multitude of mediators are responsible for the irradiated riboflavin action, such as: activation of TNFR1 and PTPα cascades, downregulation of cyclin D, leading to cell cycle arrest; and cytoskeleton remodeling.

TNFα presents several biological functions, such as control of the proliferation, differentiation and apoptosis. The final cellular response towards TNFα is dependent on dose and growth phase. The reasons for these multifunctional effects are due to the presence of different types of TNF receptors, TNFR1 and TNFR2. The binding of the TNF to the TNFR1 causes trimerization of this receptor and favor the association with adaptors proteins, as TRADD, which bind TRAF2 and/or FADD (Hallenbeck, 2002; Deng et al 2003; Gaur and Aggarwal, 2003). TRAF2 activation is responsible for the NFκB activation and consequently anti-apoptotic protein expression, such as, IAP and Bcl2 (Karin and Lin, 2002; Wen et al 2003). On the other hand, when FADD is recruited by TNFR1 there is an activation of caspase 8, and in turn at least to final responses could take place, apoptosis or differentiation. According to our findings, irradiated riboflavin favored HL60 cells differentiation, since we detected an activation of caspase 8 and PARP fragmentation. Additionally, the level of TNFα was lower after treating the cells. These results are in agreement with the overexpression of NFκB, since the activation of TNFR1 can lead to an activation of this protein synthesis. On the other hand, our findings also demonstrated that NFκB did not translocate to the nucleus, which is also can explain the decrease of TNFα synthesis. NFκB is an ubiquitously expressed family of transcription factors controlling varied biological effects ranging from inflammatory, immune, and stress-induced responses to cell fate decisions such as proliferation, differentiation, tumorigenesis, and apoptosis. Ougolkov and collaborators (2007) have suggested that GSK3 is an important modulator of NFκB transcriptional activity by regulating the nuclear activity of both NFκB subunits p65/p50, in chronic lymphocytic leukemia B cells. Therefore, the inhibition of GSK3 showed by the kinome profiling, could be responsible for remaining NFκB inactive in the citosol, besides its overexpression. We also suggest that the remodeling of actin filament during HL60 cells differentiation could contributes for remaining NFκB inactive in the citosol. Despite the fact that the nuclear translocation constitutes the key step of NFκB activation, mechanisms underlying the nuclear transport of NFκB are uncertain; it is likely that they may involve association of the liberated RelA/p65 with cytosol structures such as actin cytoskeleton. The actin cytoskeleton is dynamic, and the rates of polymerization and depolymerization of actin are critical determinants of many cellular responses. Recently studies showed that NFκB can interact with actin and in turn regulate its translocation to the nucleus, in endothelial cells (Fazal et al., 2007). Additionally, the fact of cells treated with irradiated riboflavin present downregulation of TNFα as well as inhibition NFκB translocation, suggests that this vitamin and its photoproducts display anti-inflammatory property. Recently, Bertollo and co-workers (2006) demonstrated that riboflavin inhibits early and prolonged inflammatory responses towards LPS by unknown mechanism.

Cells respond to the microenvironmental stimulus by modifying the intracellular levels of phosphorylation and dephosphorylation (Tamura et al., 2004). In fact, the protein phosphorylation and dephosphorylation balance is the base of the control of several biological events regulated by extracellular effectors, such as: hormones, mitogens, carcinogens, cytokines, neurotransmitters, toxic compounds or differentiation inducers (Ostman and Bohmer, 2001; Ferreira et al., 2006). Irradiated riboflavin led to PTPα activation, this phosphatase is a main positive regulator of FAK and in turn plays a central role in the cytoskeleton organization (Chen et al., 2006; Feng, 2007). Microtubules and actin represent the major components of cytoskeleton, in hematopoietic cells. These proteins are submitted to a constant remodelling under different situations (Veselska *et al*., 2003). The results showed previously together with activation of Src, SHP2 and caspase 3, reinforce the action of irradiated riboflavin as a differentiation inductor. The protein tyrosine phosphatase SHP2 is an important regulator of the MAPK ERK or integrin pathways. Therefore, SHP2 modulates cell adhesion, migration, and cytoskeleton architecture (Feng, 2007). It has been suggested that SHP2 is connected with integrin function, because it can regulate the FAK phosphorylation level. Moreover, the α-actin rearrangements in focal complexes could be also dependent on SHP2 (von Wichert et al., 2003). Cell morphology rearrangement induced by irradiated riboflavin was also reinforced by the expressive activation of caspase 3. Caspase 3 is recently shown to exert several non-apoptotic functions as well. Several reports have demonstrated that caspases are involved in the terminal differentiation of a variety of cell types. Additionally, caspase inhibitors interfere with the maturation at an early stage of differentiation. This protease plays a pivotal role for the cystoskeleton remodelling, since can cleave actin (Zemati et al., 2001; Lamkanfi et al, 2007).

Inhibition of serine threonine phosphatase 2 (PP2A) in the first 24h of treatment with irradiated riboflavin 5μM can play an important function for the cell survival, once this enzyme is associated with dephosphorylation of the pro- and anti-apoptotic proteins. PP2A is the main serine/threonine phosphatase involved in the regulation of several cellular process, including different signal pathways, cell cycle progression, DNA replication, gene transcription and translation (Van Hoof and Goris, 2003; Hahn, 2004). The inhibition of PP2A is consistent with the capacity of irradiated riboflavin to induce leukaemia cell differentiation. Freire et al (2003) showed that the treatment of HL60 cells with inhibitors of PTPs and Ser/Thr phosphatases (pervanadate and okadaic acid) induced initially cell differentiation and later apoptosis. Usually, differentiation inducers are known for induce apoptosis. During the maturation process of granulocytes and erythrocytes, apoptosis happens as a physiological event.

Notably, after 24 and 48 h treatment the activity of protein kinases involved in proliferation/survival (cRaf, MEK1/2 and ERK1/2) remained unchanged in the presence of irradiated riboflavin. However, Src

protein kinase and JNK MAPK were activated after 48 h treatment. Usually, ERK 1/2 activation is correlated to cellular response to growth factors and survival signalling. However, the same is not observed for JNK and p38 MAPK. These MAPKs seem to be related to apoptosis cell death induction in presence of cellular stress stimulus (genetoxic events, thermal shock and irradiation) (Huh et al, 2004; Wada and Penninger, 2004). Nevertheless, the real functions displayed by MAPKs are extremely dependent on the cellular context as well as the cellular type. For instance, JNK has been cited in the literature as an important differentiation mediator. Wang at al (2003) has shown that JNK is essential to lead HL60 cells differentiation into monocytes triggered by diidroxi-vitamina D3.

According to our data the dominant cellular response towards irradiated riboflavin 5µM is differentiation process instead of proliferation. This hypothesis is reinforced for the inhibition of the cell cycle progression in presence of irradiated riboflavin since p21 protein was upregulated after 24h of treatment. This protein inhibits the complex formation of cyclin-CDK, a positive modulator of cell cycle progression. On the other hand, we observed a decreasing of the cyclin D1 expression after 48h treatment. Cyclin D is required for cell cycle progression from G1 to S phase. Cell cycle arrest is one of the requirements for differentiation, in addition, an increasing of phosphorylated STAT3 was observed after treating HL60 with irradiated riboflavin. It was reported that the maximal activation of STAT3 is required for leukaemia cell differentiation induced by Phorbol Myristate Acetate (PMA) and Interleukin 10 (IL10) which caused an increase of expression of cJun, cFos (Makuta et al., 2003). This information is in agreement with our data, since we detected an increase of phosphorylated STAT3 as well as an activation of JNK. Several molecules have been found to interact with activated STAT3. Among them, cellular cJun forms a complex with STAT3 and activates promoter that contains both STAT3- and cJun-binding sites (Tonozuka et al., 2004).

Establishing the molecular mode of action of compounds with clinically useful properties is difficult if there is no information as to the biochemical details of the biological effect observed. The present study has shown that techniques which generate comprehensive descriptions of cellular signaling pathways may substantially contribute to this field. Over the last 5 years, array and mass spectrometry technologies have enabled the determination of the transcriptome and proteome, and such information will likely be of significant value to the elucidation of the molecular mechanisms that govern the effects of pharmacological compounds (Kell, 2006). However, defining those proteins that participate in signaling pathways affected by such compounds may provide more direct insight into the mechanism underlying the clinical effects of such compounds, as also observed in the present study employing an adaptation of array technology that measures enzymatic activity in a parallel fashion. We feel that the results obtained show that kinome profiling using metabolic

arrays is a highly promising tool for the rapid evaluation of the effect of pharmacological intervention on signaling pathways.

Lastly, Pepchip technique appears as a useful approach for identifying molecular targets as well as to predict which pathway is positive or negatively modulated under diverse conditions.

In summary, our findings spotlighted irradiated riboflavin as an interesting and promising antileukemic agent, mainly due to present some characteristics which might causes less side-effects when compared with the traditional chemotherapics used as a differentiation inducers.

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Biochemical differences between erythroblast leukemia cell and its counterpart with resistance phenotype: kinases and phosphatases profiles

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Running Title: Kinomic profiling of erytroblastic cell lines

Abstract

Many of the difficulties in designing an effective therapeutic strategy for conquering cancer cell resistance arise from the limited data that are available from the mediators which take part in this complex phenomenon. Therefore, a deeper understanding of the mechanisms underlying the multiple layers of cellular function regulation and how they are altered during resistance acquisition will be critical in identifying the next generation of therapeutic targets. In this paper, using kinase microarray approach, we identified some kinases which displayed differential activities as well as protein phosphatases, in drug-resistant leukaemia cell line (Lucena) and the parental cell line K562. In addition, we observed that phosphatase from Lucena cells present higher affinity and specificity towards flavin mononucleotide. In general, all kinases/phosphatases which presented higher activities on Lucena cells are involved in signaling pathways responsible for cellular proliferation, survival and consequently, might be related with resistance acquisition. And importantly, we can speculate that flavin metabolism might play a role in the resistance acquisition and be useful target for preventing and overcome resistant cancer cells. Therefore, our findings pointed out some cell signalling mediators which might be worth to investigate as candidates for conquering cancer resistant cells.

Keywords: kinome profiling, protein kinases, protein phosphatases, leukaemia, flavin metabolism

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Introduction

The clinical management of myeloid leukaemia remains problematic. Although initially most of these leukaemias react favourably to chemotherapy, the chemotherapeutic treatment outcome of most adult acute myeloid leukaemia remains unacceptable. The major problem here is the build-up of resistance against therapy. The present and identifying proposal aims at exploring a novel avenue for counteracting and reversing such chemotherapy resistance. The molecular details by which cancer cells mediate resistance against chemo- and other therapy are now fairly well understood and involves overexpression of transporters (e.g. P-Glycoprotein), induction of autophagy, activation of the biotransformation enzymatic complex and an increase of intracellular vesicle trafficking. However, the molecular mechanisms, which direct the leukaemic cell to acquire these properties remain obscure at best (Higgins, 2007). Therefore, the biochemical characterization of multidrug resistance cells may provide a novel approach for counteracting and reversing such chemotherapy resistance.

Interestingly, however, recently novel technical approaches have been developed that allow the generation of comprehensive descriptions of cellular metabolism without the need for a priori assumptions as to the signaling pathways affected (Diks *et al*., 2004a,b). We pioneered an approach involving peptide arrays exhibiting consensus phosphorylation sequences for cellular kinases that allows simultaneous assessment of the activity of the total complement of kinases present in the human genome (the so-called kinome) - (Diks *et al*., 2004a,b; Goddard and Reymond, 2004a,b; Irish *et al*., 2004). Hence we decided to test whether this technology would allow us to identify metabolic differences between two chronic myelogenous leukaemia cell lines: drug-sensitive and - resistant cell lines (K562 and Lucena, respectively). Besides, protein tyrosine phosphatases were also analyzed, the results of which are presented in this study. We provide a wealth of information as kinases with different cellular localization which presented higher activities in resistant leukaemia cell, but also we identified protein phosphatases which were differentially expressed in both cell lines.

Materials and methods

Cell line and Reagents

K562 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and Lucena was obtained as described previously (Rumjanek *et al*., 2001). Polyclonal antibodies against LMWPTP, SHP2 and PTEN were purchased from Abcam or Cell Signalling Technology (Beverly, MA). Phosphatase substrates and inhibitors were from Sigma.

Cell Culture

K562 and Lucena cells were routinely grown in suspension in RPMI 1640 medium supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10 % heat-inactivated fetal bovine serum, at 37^{o}C in a 5 % CO_2 humidified atmosphere.

Western bloting analysis

Cells (3 x 10^7) were lysed in 200 µL of lysis buffer [50 mM Tris–HCl (pH 7.4), 1% Tween 20, 0.25 % sodium

deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitors (1 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl-fluorid-hydrochloride)] for 2 h in ice. Protein extracts were cleared by centrifugation and protein concentrations were determined using the Lowry method (Lowry and Lopez, 1945). An equal volume of 2X sodium dodecyl sulphide (SDS) gel loading buffer [100 mM Tris–HCl (pH 6.8), 200 mM DTT, 4 % SDS, 0.1 % bromophenol blue and 20 % glycerol] was added to samples which were subsequently boiled for 10 min. Cell extracts, corresponding to 3 x 10^5 cells, were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to PVDF membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (2%) in Tris-buffered saline (TBS)-Tween 20 (0.05 %) and incubated overnight at 4 °C with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05 %), membranes were incubated with antirabbit or antimouse horseradish peroxidase-conjugated secondary antibodies, at 1:2000 dilutions (in all Western blotting assays), in blocking buffer for 1 h. The detection was made using enhanced chemiluminescence (ECL).

Pepchip analysis

Kinome assay were performed in K562 and Lucena cells at the same passage (2.5 x 10^5 cells). The production of the array and the protocol of the kinome array have been described in detail earlier (Diks *et al*., 2004a,b; Lowenberg *et al*., 2005, 2006; van Baal *et al*., 2006; De Borst *et al*., 2007). In short, cells were washed in PBS and lysed in a non-denaturing complete lysis buffer. The peptide arrays (Pepscan, Lelystad, The Netherlands), containing up to 1176 different kinase substrates in duplo, were incubated with cell lysates for 2 h in a humidified stove at 37 °C. Subsequently, the arrays were washed in 2 M NaCl, 1% Triton-X-100, PBS, 0.1% Tween and water, thereafter slides were exposed to a phospho-imaging screen for 24-72 h and scanned on a phospho-imager (Fuji, Stanford, USA).

Phosphatase assay

The phosphatase extracts were obtained by lysis of the cells with acetate buffer 0.1 mM (pH 5.5). Then, the enzyme activity was measured in a reaction medium (final volume, 1.0 ml) containing 100mM acetate buffer (pH 5.5), 5.0 mM *p*-nitrophenylphosphate (pNPP) and cell extract enzyme. After 30 minutes incubation at 37 °C, the reaction was stopped by adding 1.0 ml of molybdate and 0.2 mL of ascorbic acid. The amount of phosphomolybdic complex was measured at 660nm.

Statistical analysis

All experiments were performed in triplicate and the results shown in the graphs represent the means and standard errors. Data from each assay were analyzed statistically by ANOVA. Multiple comparisons among group mean differences, in kinomic analysis, were checked with Turkey post hoc test. Differences were considered significant when the p value was less than 0.05. Western blottings represent 3 independent experiments.

Results and Discussion

Among the major problems in dealing with leukaemic disease is the build-up of resistance against therapy (Martelli *et al*., 2003; O´Hare *et al*., 2006; Diehl *et al*., 2007). The term multidrug resistance (MDR) is classically used to define a resistance phenotype where cells become resistant simultaneously to different drugs with no obvious structural resemblance and with different cellular targets. MDR is always multifactorial, with at least two resistance mechanisms present in the same tumor cell. This may include resistance associated with decreased drug accumulation, altered intracellular drug distribution, increased detoxification, diminished drug-target interaction, increased DNA repair, altered cell-cycle regulation, and uncoupling of the pathways linking cellular damage with programmed cell death. In this paper we compared K562 and its counterpart with resistance phenotype in regard to protein phosphatase and kinase profiles.

Kinome profiling

As shown in the Figure 1A, the kinase array profile displayed difference when both cellular lysates were compared. Subsequently, we analyzed the radioactivity incorporated in substrates phosphorylated by Lucena lysates against the radioactivity incorporated in substrates phosphorylated by lysates of K562 cells. Phosphorylation of 239 peptides was significantly different when Lucena and K562 cells were compared ($P < 0.05$; see supplementary information for a full description of results obtained). In general, all kinases which presented higher activities are involved in signaling pathways responsible for cellular proliferation, survival and consequently, resistance acquisition. According to their biological functions and with some evidences provided from the literature, these kinases identified by kinome assay (receptor tyrosine kinases, cyclin dependent kinases, MAPK, PKA, PKC, AKT, polo like kinase, janus kinases, Src and FAK) might be interesting targets to address cancer cells.

Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase that localizes to the focal adhesions and coordinates signals from integrins, cytokines, growth factor receptors, and oncogenes playing an important role in cell motility and survival through its ability to signals for both phosphoinositide-3 kinase (PI3K) and mitogen-activated protein kinase (ERKs). Solid tumoral cells often present an overexpression of FAK or keep the protein in an active state through its phosphorylation in Tyr-397, which have been associated with increased motility, invasiveness, proliferation and protection against cytotoxic stimuli such as γ radiation, drugs, and oxidative stress (Hiscox et al, 2006, 2007). Although the pivotal role of FAK as anti-apoptotic, cell migration and drug-resistance inductor agent is well established for solid tumor cells, little is known about the expression and function of FAK in leukemic cells. However, studies of Gotoh's group (Gotoh et al, 1995) have been shown that FAK is constitutively phosphorylated and activated, probably by BCR-ABL, in Philadelphia chromosome-positive leukemia cells lines, including K562 cells, while Recher and co-workers (2004),

demonstrated that FAK is aberrantly expressed and activated in acute myeloid leukemia (AML) cells and that FAK^+ AML cells displayed significantly higher migration capacities and resistance to daunorubicin, compared with FAK^- cells. Interestingly, the authors also showed that downregulation of FAK in $CD34^+$ significantly decreased migration and daunoribicin resistance in these cells. Additionally, the transfection of human mielocytic cell line HL60 with FAK induces these cells, otherwise sensitive to apoptosis, to become highly resistant to apoptosis induced hydrogen peroxide and etoposide, an event that seems associated with the FAK-mediated activation of PI3K/Akt pathway and the concomitant activation of NFκB and induction of the inhibitors of apoptosis proteins (IAPs) [Sonoda et al, 2000]. In agreement, our results showed an increased expression of PI3K and c-IAP in Lucena which reinforces the potential role of FAK overexpression/overactivity in the establishment of MDR event upon K562. Indeed, FAK has been involved in the acquisition of drug resistance induced by tumor cell adhesion to components of extracellular matrix or cell-cell interactions, an event known as cell adhesion-mediated drug resistance (CAM-DR). The increased adherent potential in tumor cells has been demonstrated to prevent cell death through a number of mechanisms and CAM-DR has been pointed as a phenomenon that may play an important role as an early anti-apoptotic effector allowing the emergence of acquired MDR after initial therapy, as already demonstrated in myeloma cells and others (Shain et al, 2001). In this context, FAK increased activity may be an early event associated with the mechanisms involved in the transformation of K562 cells in the Lucena phenotype through acquired adhesion properties that facilitate further transforming mutations and the acquisition of MDR. Given the fact that initial success in MDR therapy with chemosensitizers often culminates in relapse due to the multifactorial nature of this phenotype, our results pointed out FAK as attractive target for leukemic resistant cell therapy since this protein seems involved in the mechanisms that allow cell survival after initial treatments. Therefore, it constitutes a potential target for a more rational therapy focused not only in the reversion of the MDR but, most importantly, in the inhibition of the initial mechanisms involved in the MDR acquisition.

However, these authors observed that cellular adhesion mediated-drug resistance is not mediated by FAK. In solid tumor the activation of Src and FAK is responsible for enhancing cellular migration (Hiscox et al, 2006, 2007).

1A

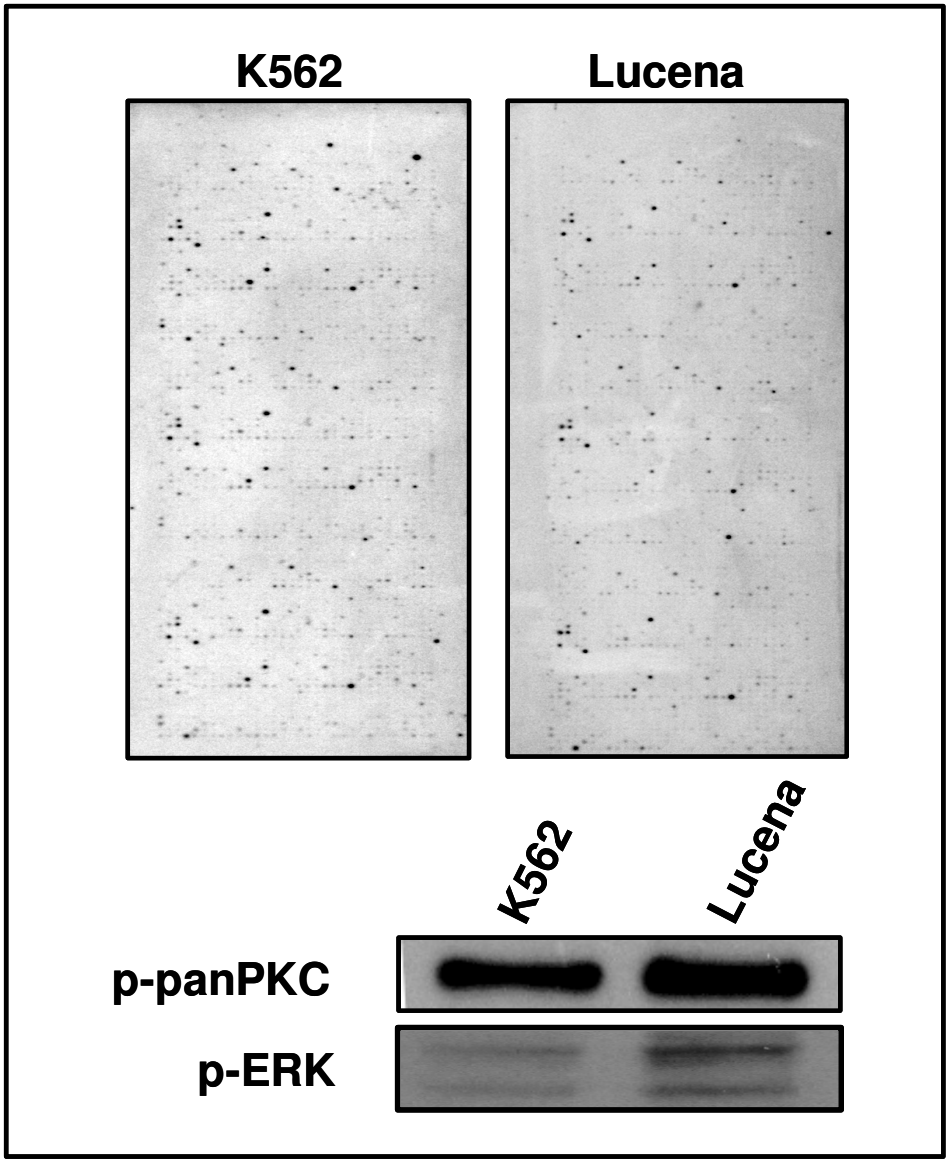

Figure 1A. Peptide arrays reveal kinase activity differences between erytroblastic leukaemic cell lines. (A) Example of a peptide array substrate phosphorylation by lysates of K562 and Lucena cells; and validation of the activation of PKC and ERK by western blotting.

1B

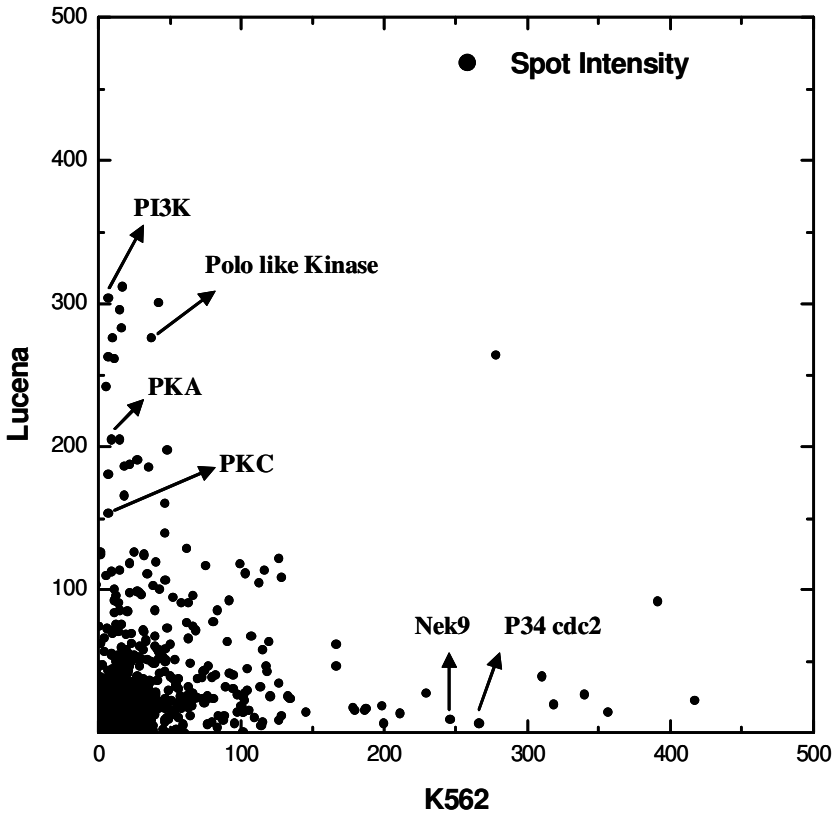

Figure 1B. Peptide arrays reveal kinase activity differences between erytroblastic leukaemic cell lines.(B) The relationship between radioactivity incorporated (spot intensity) in substrates phosphorylated by Lucena cell lysate against the radioactivity incorporated in substrates phosphorylated by K562 cell lysate are represented in the graphic. The higher radioactivity value means the higher kinase activity. The dots indicated in the graphic correspond to the respective kinase substrates (*see supplementary material in "Anexos")..

*The cells lysates were put on PepChips and spot intensities were quantified. P-values derived from paired t-Tests performed on normalized intensities are given; n.s., not significant ($p > 0.05$). The kinases phosphorylating those substrates are indicated. ▲ means higher phosphorylation intensities in the resistant cell (Lucena); ▼ means lower phosphorylation.

JAK - The development and function of hematopoietic cells depends on complex signaling pathways that are mediated by numerous cytokines and their receptors. The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway is prominent both in normal hematopoiesis and in hematological malignancies. STAT proteins mediate cell growth, differentiation, apoptosis, transformation, and other cell functions. In addition constitutive activation of the JAK-STAT pathway has been reported in various types of leukemias such as acute myelogenous leukemia, and multiple myeloma. Our results indicate an increased level of the active phosphorylated form of JAK2 in Lucena cells, which is accompanied by the increased phosphorylation and kinase activity of both erythropoietin receptor and STAT5a. Interestingly, a series of studies has reported the simultaneous expression of erythropoietin and its receptors in several different types of human haematological and non-haematological cancer cells, an event that is often associated with a poor prognostic and disease free survival. It has been suggested that in these tumor cells, the coexpression of erythropoietin receptor and its ligand erythropoietin functions as an autocrine or paracrine mechanism that activate erythropoietin signaling pathways, that acts mainly through activation of JAK2/STAT5 and the anti-apoptotic transcription factor NFκB. This enables cancer cells to modulate its growth leading to an increase in the proliferation rate associated with a more aggressive phenotype (Sawyer and Penta, 1996, Arcasoy et al, 2005; Lay et al, 2005; Saintigny et al, 2007).

Chronic myeloid leukemia cells, such as K562, are characterized by the Philadelphia chromosome translocation that causes expression of BCR-ABL, a deregulated tyrosine kinase. Imatinib mesylate, also known as STI571, Gleevec, is a therapeutically used inhibitor for the BCR-ABL enzyme that causes apoptosis in the BCR-ABL$^+$ cells such as in the case of K562 cells. Interestingly, Kirschner and Baltensperger (2003) related the acquisition of resistance to imatinib promoted by the action of erythropoietin in K562 cells highlighting the potential of erythropoietin and its receptor to induce drug resistance in leukemic cells. The authors showed that, in fact, the exposition of K562 to imatinib in presence of erythropoietin is able to invert the sensibility of these cells to the BCR-ABL inhibitor making them resistant to the drug-induced apoptosis. This fact seems to be associated with the ability of erythropoietin to prevent inhibition of MAPK and PI3K/Akt pathways induce by imatinib. Our results reinforce the hypothesis of Epo-mediated drug induced and additionally, suggest that Epo can not only induce imatinib resistance in K562 cells but, also, the cytokine can be associated with the vincristine resistance mechanisms acquired by these cells. These results bring important implications in the current therapeutic protocols for cancer treatment since EPO is also used to treat anemia and fatigue in cancer patients receiving radiation therapy and chemotherapy. If EPO treatment can contributes to MDR development is still a controversial issue since although it is known that the presence of the cytokine can induce MDR, it is also known that

some MDR phenotype can occur even in the phosphorylation/activation of EPO receptor and its increased binding to JAK2, in the absence of its ligand (Sawyer and Penta, 1996; Kirschner and Baltensperger, 2003).

Polo like kinase – Polo-like kinases (PLKs) are a family of serine-threonine kinases essential during mitosis and in the maintenance of genomic stability. Members of this family have multiple functions during mitosis and have a significant role in ensure check-point controls, thus representing a group of kinases whose cellular level and activity are absolutely critical for the precise regulation of cell division. As a consequence, PLKs are also targets used for cancer cells to evade cell cycle regulatory mechanisms and, in fact PLK1, the best characterized member of PLKs, is overexpressed in a variety of human tumors, which have been associated with poor patient prognosis (Strebhardt and Ullrich, 2006).

The importance of PLK1 in the control of mitosis is well demonstrated by its own transcriptional regulation. PLK1 transcripts accumulate in S phase, peak in M phase and decrease in G1 phase which is accompanied by changes in the protein level and activity that are low in G1, increased in S and G2/M phases and is rapidly reduced in late phases of mitosis. Thus, PLK1 is a key player in G2/M and M phases of cell cycle as the case of G2/M specific molecules cyclin B1, cyclin A, Cdc2 and Cdc25C (Martin and Strebhardt, 2006). Indeed, entry into mitosis by mammalian cells is triggered by the activation of the cdc2/cyclin B holoenzyme through its specific dephosphorylation in key residues by the cdc25C phosphatase. PLK1 contributes to this process through phosphorylation and activation of cdc25C which allows the enzyme to dephosphorylate inactive cdc2/cyclin B, a required event for cell cycle progression (Roshak et al, 2000). Also PLK1-mediated activation of cdc25c leads to the dephosphorylation of p53 renders its protein inactive.

As already mentioned, the deregulated expression of PLK1 has been detected in many types of human tumors and in these cases the enzyme is a key player in the tumorigenesis process. Additionally, some new works have brought new insights about the role of PLK1 in the mechanisms of drug resistance. In agreement non-small-cell lung cancer (NSCLC) patients whose tumor shows high PLK1 expression had poorly curative prognostic which have been associated with PLK1-induced drug resistance (Wolf et al, 1997). Recently, Spankuch et al (2007) demonstrated that treatment of breast cancer cells with siRNAs targeting PLK1 improved the sensitivity toward paclitaxel and Herceptin in a synergistic manner. In all experiments, very low concentrations across a wide range of clinically relevant concentrations were sufficient to induce an antiproliferative effect. The combination of PLK1-specific siRNAs with modern breast cancer drugs seems to represent rational combinations to be tested in preclinical trials.

Importantly, Lin et al (2006) showed that inhibition of PLK1 in K562/A02 cells, a classical MDR human tumor cell line with the overexpression of the drug efflux protein, P-glycoprotein, leads to cell cycle arrest at G2/M phase and also was able to restore the cells

sensitivity to adriamycin-induced apoptosis. In agreement the results obtained in our work demonstrated that Lucena presents higher PLK activity than its non-resistant counterpart K562 and reforces the hypothesis that PLKs are important effectors in MDR.

RTK – The class of receptor tyrosine kinase (RTK) plays important roles in the generation of physiological cell responses triggered by numerous hormones, growth factors and other bio-response modulating factors that control cell proliferation, differentiation, kinesis, metabolism and survival. Approximately 20 different RTK families have been identified that share a similar structure: a ligand-binding extracellular domain, a single transmembrane domain and an intracellular tyrosine kinase domain. The generation of signals by RTKs requires the binding of a ligand to the extracellular receptor domain which leads to the activation of the intrinsic tyrosine kinase activity of the receptor enabling its interaction with adjacent intracellular receptor domains. Subsequently, a crosswise intermolecular phosphotransferase reaction catalyses the phosphorylation of tyrosine residues that changes the receptor conformation stabilizing it in an active state that enables the recruitment of cytoplasmic signaling molecules, through their phosphotyrosine binding domains, to the cell membrane, the starting point of a multitude of signaling pathways such as that of the MAPKs activation (Weiss et al, 1997).
Given the critical roles of RTKs, deregulation of the receptor's functions and their downstream effectors (ras, JAK/STAT), have emerged as significant components to contribute to the degeneration of a variety of physiological process and the development of malignant cell transformation (Weiss et al, 1997). In fact, deregulation of receptor tyrosine kinase (RTK) activity has been implicated in the pathogenesis and progression of a variety of human leukemia and the role of individual receptors in such processes has been receiving increasing attention. Mutations of the FLT3, c-Kit, c-FMS, K-Ras, N-Ras, BRAF and CEBPA genes in the RTK/Ras-BRAF signal transduction pathway are frequent in acute myeloid leukemia (AML). Additionally activating mutations of RTKs and their downstream effectors are common in pediatric AML and their presence may identify a population of poor outcome presenting lower complete remission rate and overall survival than mutation-negative patients. (Meshinchi et al, 2003; Christiansen et al, 2005)
The comparison of the kinome profile between K562 and Lucena showed that Lucena presents an overall increase in the activation of RTKs, such as colony -stimulating factor receptor 1 (CSFR1 or G-CSFR), Kit, EGFR and VEGFR, confirming the participation of these receptors in the development of a more aggressive phenotype presented by Lucena cells. In special, our results showed a remarkable activation of EGF and VEGF receptors in Lucena indicating the importance of these receptors for the MDR development and/or for the stabilization of the phenotype. In agreement many MDR cell lines overexpress the EGFR and overactivation and/or deregulation of this receptor have been related to the

progression of solid tumors, including invasion, angiogenesis, metastasis, and is associated to a more apoptosis-resistant phenotype. Indeed, data from the literature have shown the association of EGF-induced signaling to the multidrug resistance caused by the reduction of intracellular drug accumulation through P-glycoproteins encoded by members of the mdr gene family. How the activation of EGFR can stimulate the resistant phenotype in tumoral cells is still a not completely known issue but it seems that the receptor can regulate the phosphorylation status and function of P-Glycoprotein (P-gp) through activation of phospholipase C (PLC) which can directly phosphorylate and activate the P-gp contributing to the development of MDR phenotype (Hirsch-Ernst et al, 1995; Yang et al, 1997).

Like EGFR, VEGFR overexpression/overactivation also has been associated to drug resistance in solid and hematological tumors (Podar and Anderson, 2005, Karp et al, 2004. Although initially the importance of VEGF was associated with its paracrine actions leading to neovascularization of the tumor involved tissue sites and the development of more aggressive disease status, recently autocrine pathways regulated by the VEGF binding to its receptors in the tumor cell itself have shown to increase the survival of malignant cells through induction of drug resistance phenotypes. Indeed, in B-Chronic Limphocytic Leukemia (B-CLL), an incurable disease predominantly characterized by apoptosis resistance in the CLL/B cells, and in the Adult T-cell leukemia/lymphoma (ATLL), an aggressive T cell lymphoproliferative disorder where the patients present high plasma levels of VEGF is associated to intrinsic resistance of tumor cells to chemotherapy. The interruption/ blockage of the VEGF-based pathways has shown to lead to increased sensitivity of these tumor cells to drug-induced cell death (Mukherjee et al, 2007; Kchour et al, 2007). The mechanisms involved in the VEGF/VEGFR-induced drug resistance in leukemic cells are still little known but Lee and co-workers showed that in CLL B cells the process requires the VEGFR-induced activation of STAT molecules. The authors showed that CLL B cells spontaneously secrete VEGF and have constitutively active VEGFR receptors R1 and R2 enabling the autocrine stimulation of VEGF-induced signaling pathways to upregulate the expression of the anti-apoptotic Bcl2-family member Mcl-1 and the inhibitor of apoptosis XIAP, events associated with decreased cell death. Additionally, the authors suggest that the VEGF/VEGFR signaling involves the activation and perinuclear translocation of STAT3 in an event likely independent of the JAK-Src, PI3K or ERK signaling pathways and promoted by VEGFR-mediated endocytosis. These findings have important implications for VEGFR receptor signaling, apoptotic resistance, and enhanced survival in provided by this RTK receptor in leukemic cells.

Although the involvement of VEGFR in drug resistant constitutes a well defined event for leukemic cells, as demonstrated by the examples above, until now little was explored about the implications of VEGFR overexpression/overactivation in chronic myeloid leukemia. Indeed only

recently, Cui and collaborators (2007) demonstrated that VEGF-induced pathways are also important effectors for the adriamycin-resistance development in K562 cells and that the reduction of VEGFR expression in K562/ADR efficiently reverted the MDR I these cells. Our results are in agreement with Cui suggesting that also in these groups of leukemia VEGFR has a pivotal role.

Protein kinase C (PKC) comprises a family of serine/threonine kinases which can be divided in into three groups based on their activating factors: (i) the classical (or conventional) PKC isoforms (cPKCs) α, β1, βII, γ; (ii) the novel PKC isoforms (nPKCs) δ, ε, θ, η, μ/PKD and (iii) the atypical PKC isoforms (aPKCs) λ and ζ. The cPKC isoforms require negatively charged phospholipids like phosphatidylserine, diacylglycerol and calcium for optimal activity while the nPKC isoforms also require negatively charge phospholipids and diaglycerol but no calcium for the optimal activity. Finally, the aPKCs only requires negatively charged phospholipids but neither calcium nor diacylglycerol for optimal activity (Martiny-Baron and Fabbro, 2007). PKC is activated downstream of PLCγ, an enzyme which is activated by G-protein coupled receptors and RTKs and, therefore, is involved in the transduction of signals for cell proliferation, differentiation, apoptosis, migration and angiogenesis. Unsurprisingly, disruption of PKC regulation is implicated in tumorigenesis, maintenance of malignant phenotype and drug resistance (Mackay and Twelves, 2007). Indeed, chemical tumor promoting agents, such as phorbol 12-myristate 13-acetate (PMA), have been identified as specific activators of PKC while the potentiation of malignant phenotypes appears to be mediated by activation of selective PKC isoenzymes or through altered isoenzyme profile compared to the originating tissue (Koivunen et al, 2006). The participation of PKC pathways in resistance to chemotherapeutic treatments has been recognized for a long time and have been related to the ability of the enzyme in modulate multi-drug transporters and also acts as a regulator of apoptosis.

As mentioned formerly, the activation of PLC downstream to RTKs-induced signaling pathways has been considered a strategy of tumor cells to acquire resistance to death induction since PLC is a potential candidate for direct phosphorylation of P-pg. However, P-gp has numerous potential phosphorylation sites for a number of kinases and, PKC, which is activated as a result of PLC activity, also has been proposed as one of the candidates involved in the regulation of P-gp and a potential target for therapy against MDR cells (Idriss et al, 2000; Martiny-Baron and Fabbro, 2007).

The involvement of PKC and regulation of apoptosis is demonstrated by the actions of bryostatin 1 in the modulation of ara-C-induced apoptosis in human leukemic cells. Chronic exposure of these cells to bryostatin 1 leads to PKC downregulation with consequent alteration in the balance between anti-apoptotic and pro-apoptotic effector molecules leading to an increase of ara-C-sensibility by the leukemic cells (Grant, 1997). Jiffar et al. (2004) demonstrated that overexpression of PKC alpha promotes Bcl2 phosphorylation and chemoresistance in human acute

leukemia cells. Recent findings suggest the potential role for PKC beta in the pathogenesis of B-cell malignancies since the expression levels of this enzyme is often in B lymphoid malignancies and is associated to worse prognostic consequent to anti-apoptotic and B-cell proliferation pathways induced by the high PKC beta expression (Li et al, 2007).

In K562 cells PKC also acts as an important anti-apoptotic factor and drug resistance inductor. Zhang et al, 2007, showed that the treatment of K562 with Trichosanthin lead to the growth arrest in these cells promoted by downregulation BCR-ABL and its downstream signals with consequent inhibition of key kinases, among them, PKC. Our results support the finding of Zhang et al (2007) since the increased activation/expression of PKC is a characteristic of Lucena cells that probably is associated with its more resistant phenotype. However, it is necessary a more accurate study of PKC expression and activation in our MDR model and in the other cancer cells. More than this, it is very important that the future studies have attention from the different members of PKC family and their respective actions in the cells studied. Indeed the relationship between PKC and MDR phenotype is a more complex issue than primarily thought. Data from the literature have revealed that PKC activation is not always associated with resistance but can also increase sensitivity to chemotherapy. In fact, while expression of PKCα and PKCγ increases the resistance of human uterine sarcoma cells to paclitaxel, elevated expression of PKCλ leads to reversal of the resistance. Likewise, PKCα overexpression has been associated with increased MDR expression although in the MDA-MB-231 breast cancer cells its expression confers sensitivity to retinoic acid treatment. These findings demonstrate the relevance of type of active isoforms rather than total PKC activity in the response to anticancer drugs and also demonstrate the ability of these isoenzymes to induce associated different outcomes during the treatment depending of the specific tumor type cells (Lorenzo and Dennis, 2003).

Protein kinase A (PKA) – overexpression of the R subunit of PKA (in particular, RI) is associated with high proliferation and malignant transformation in normal breast cell and with poor prognostic and resistance to antiestrogens in established breast cancer (Miller, 2002). Michalides et al (2004) and Zwart et al (2007) showed that phosphorylation of serine-305 in the hinge region of estrogen receptor alpha (ERalpha) by PKA induced resistance to tamoxifen in breast cancer. In other words, when ERalpha is phosphorylated, tamoxifen bound but then failed to induce this receptor inactive conformation. In clinical samples, these authors also found that downregulation of a negative regulator of PKA, PKA-RIalpha, was associated with tamoxifen resistance prior to treatment. The involvement of PKA and tumor drug resistance in tumoral cells is also demonstrated by works showing that MDR1 expression can be modulated by type 1 PKA-dependent transcription factors Glazer and Rohlff, 1994). These findings indicate that PKA inhibition may prove useful in reducing drug resistance in human cancer cells.

The involvement of PKA in the resistance of leukemia cells to death-inducers molecules is still a not studied issue and few works have been done in this area (Cho-Chung et al, 1999)

Phosphatases profile

The ubiquitous nature of protein phosphorylation/dephosphorylation underscores its key role in signal transduction pathways associated to the control of critical process, such as metabolism control, proliferation, survival, differentiation and death, for the cell homeostasis. Integrated networks of intracellular signaling pathways act via cascades of sequential phosphorylation or dephosphorylation reactions which are governed by the action of protein kinases (PKs) and protein phosphatases (PPs), respectively. Thus PKs and PPs have a pivotal role in regulating normal cell physiology and deregulation/disfunction of its enzymes is involved in the development of numerous inherited and acquired human diseases (Ferreira et al, 2006). Although yet until recently the study of the intracellular signaling associated with the generation and progression of cancer was focused mainly in the deregulated action of PKs, which typically initiate signaling cascades, PPs have progressively gained more attention in this scenario thanks to improvement of the knowledge about the signaling mechanisms behind the generation, maintenance and progression of the tumoral cells that demonstrated that PPs, specially protein tyrosine phosphatases (PTPs) not only have the ability to reverse the effects of PTKs but also can act as positive regulators of signaling pathways associated with the growth, proliferation and survival. In our work we compared the expression and/or activation of four PPs, whose changes in normal function have been found in cancer cells, among the K562 and Lucena cells with the aim to analyze the possible association of its phosphatases with the MDR phenotype development. Our results showed that Lucena presents increased expressions of the Low molecular weight protein tyrosine phosphatase (LMWPTP) and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) while the protein tyrosine phosphatase alpha (PTPα) was less expressed in this resistant cell (Figure 2). Additionally, the results demonstrated that Lucena has higher phosphorylated/activated form of SH2 domain-containing phosphatase SHP2 and, by the contrary, less phosphorylated/inactivated form of phosphoprotein phosphatase 2A (PP2A).

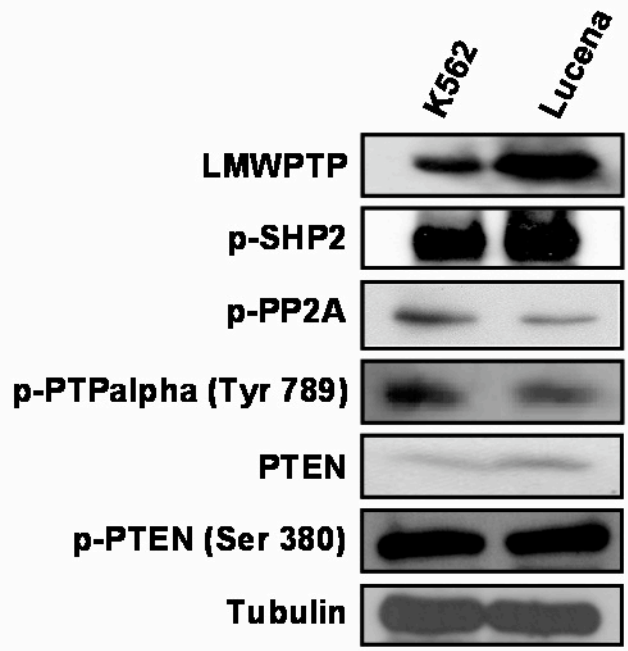

Figure 2. Protein phosphatases expression/activity on sensitive- and -resistant leukaemia cells. Soluble lysates were matched for protein content and analyzed on Western blot

SHP2: SHP2 is a protein tyrosine phosphatase (PTP) encoded by the PTPN11 gene in humans. The enzyme is a positive component of most, if not all, growth factor, cytokine, and extracellular matrix receptor signaling pathways, and is required for the full activation of the Ras/ERK cascade. In addition SHP2 can regulate Src family and PI3K activity, and may affect the NFκB and NFAT pathways. Interestingly the SHP2 has a close relation with some types of leukemia where the gene is often mutated in several pediatric occurrence of the disease, mainly in the juvenile myelomonocytic leukemia (JMML) where SHP2 gain-of-functions mutations account for ~ 35% of cases. SHP2 mutations are also found in childhood myelodisplastic syndrome, B-ALL and acute myelogenous leukemia (AML). Overexpression of wild type SHP2 has recently been described in adult CML and gain-of-functions mutations are present in 1 a 5% of adult AML cases but, in general, SHP2 mutations are uncommon in adult leukemia and constitute a rare event in solid tumors (Scherr et al, 2006, Mohi and Nell, 2007).

The inhibition of SHP2 expression was showed to be an effective mechanism to counteract BCR-ABL-dependent proliferation in CML cells. In addition, co-repression of both BCR-ABL and SHP2 seems to operate in a synergist manner inducing stronger inhibition in cell lines and CML cells. Given the known BCR-ABL inhibitors-induced resistance, as in the case of Imatinib, the targeting of SHP2 activity has been suggested to be a valid tool for the improvement of the therapy of CML cells resistant to imatinib treatment (Scherr et al, 2006). In agreement our results showed higher levels of phosphorylated/activated SHP2 in Lucena reforcing the participation of these PTP in the CML generation and also in the mechanisms associated to the transformation of the primary leukemic cells in drug resistant species.

PTPα - The receptor protein tyrosine phosphatase alpha (PTPα) is a 130 KDa transmembrane PTP which is implicated in the activation of Src family kinases, and regulation of integrin signaling, cell adhesion, and growth factor responsiveness (Ardini et al., 2000; Chen e al .2006). Indeed, upon phosphorylation (Ser184, Ser204) and activation, PTPα shows to dephosphorylate the inhibitory site

Tyr527 in Src kinase allowing the kinase to autophosphorylate in Tyr416 and reach full activation necessary to interaction with FAK and stimulation of focal adhesion complex formation. Subsequently, the complex Src/FAK activated promotes the phosphorylation of PTPα in Tyr789 that is required for cell spreading, cytoskeleton rearrangement and cell migration promoted by the integrin signaling (Brandt, 2003; Chen et al, 2006). Since mutations that increase activity of Src kinase family members are implicated in human cancers deregulated activity of PTPα is also supposed to be associated with tumor formation and progression (Zheng et al, 1992). However it has a lack of data from the literature to prove this direct relation showing the necessity to more extensive studies about the implications of PTPα activity in cancer development. Ardini et al. (2000) analyzed PTPα levels in primary human breast cancer and found that approximately one-third of the samples presented strong elevated levels of PTPα. Interestingly, the enhanced PTPα expression was correlated with delayed growth and reduced tumor aggressiveness demonstrating that high PTPα levels correlate with low tumor grade and positive estrogen receptor status in breast carcinoma cells. In agreement our results showed that K562 has higher level of PTPα expression in relation to Lucena and reinforces the findings of Ardini about the possible effects of PTPα as a negative regulator of tumorigenicity. Indeed, the opposite roles of PTPα in cell growth regulation is not an exclusive characteristic of PTPα, and the functions of other PTPs, such as SHP2 and CD45, in growth control may be lineage- and signal-specific. Furthermore, up to now it is not known all the physiological substrates of this phosphatase, besides Src tyrosine kinase family members, which underscores other possible roles of PTPα in physiologic and pathophysiological states. In relation to our results it is important to consider that we analyzed the expression of PTPα phosphorylated at Tyr789, a site known to be a target for Src/FAK phosphorylation. Since catalytic activity of PTPα has been attributed to PKC δ phosphorylation of the enzyme and shown to be independent of Tyr789 phosphorylation, our results cannot show the real difference in PTPα activity levels between K562 and Lucena but only show that in the Lucena cells the PTP phosphorylation by Src/FAK complex is lower than in K562 cells. Regulation of Src activity is a complex mechanisms and PTPα is not the only phosphatase involved. The results obtained through PepChip indicate that RTK/Src/FAK signaling is highly activated in Lucena thus probably other PTPs are the main responsible for the regulation of Src activity in this cell. How PTPα is "deviated" from the Src/FAK complex action, which are the novel targets of PTPα in these situations and what is the final consequence of this event for PTPα functions and cell behaviour are interesting questions that need to be further studied.

LMWPTP - Low molecular weight protein tyrosine phosphatases (LMW-PTPs; also known as acid phosphatase locus 1, ACP1) are a family of 18-kDa enzymes involved in cell growth regulation, cytoskeleton rearrangement and modulation of the immune response. In fact, members of this family are able to associate and

dephosphorylate many growth factors such as platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), macrophage-colony-stimulating factor receptors (MCSFR), insulin receptor, and ephrin receptors, thus downregulating many of the tyrosine kinase receptors functions that lead to cell division. Additionally, LMW-PTPs modulate the signal transduction by T cell receptor (Bottini et al, 2002a*)* and contributes to the regulation of cell division, adhesion and spreading through cytoskeleton rearrangement which have been associated to the ability of LMW-PTPs to regulate the small GTPase Rho and p125FAK (Raugei et al, 2002; Rigacci et al, 2001). Although some researches groups have contributed to the increase of knowledge about LMW-PTPs functions and its relation to pathological states, at the moment little is known about the exact biological functions of LMW-PTP family besides important relates in the literature about the implications of deregulated activities and expression of these group of enzymes in common diseases, including allergy, asthma, obesity, myocardial hypertrophy, and Alzheimer's disease (Bottini et al, 2007, Bottini et al, 2002b, Bottini et al, 2000).

LMWPTP has been recognized as a possible positive mediator in tumour onset and progression. In different tumor types the overexpression of this phosphatase is generally prognostic for a more aggressive cancer (Malentacchi et al., 2005; Chiarugi et al., 2004). In agreement Lucena cells have higher expression levels of LMWPTP than K562 reinforcing the association between LMWPTP expression and tumor aggressiveness. However it is important to mention that LMWPTP plays different physiological function depending on this phosphorylation state and level of enzymatic activity. Indeed phosphorylation LMWPTP, by Src, Lck and Fyn kinases, can lead to different effects on enzyme behavior. Tyr131 phosphorylation by Src Kinase induces an approximate 25 fold increase in LMW-PTPs specific activity enabling the enzyme to dephosphorylate receptors and signaling negatively regulating cell survival, proliferation, spreading and migration. Phosphorylation of Tyr132, contrary to the effect of Tyr131 phosphorylation, does not affect the enzyme activity but can be a starting point for the recognition of LMW-PTPs by SH2 domain-containing proteins like Grb2 and the assembling of signaling complex whose action are intimately associated with promotion of cell survival and proliferation (Tailor et al, 1997; Bucciantini et al, 1999).

Thus, although our results have been shown high LMWPTP expression in Lucena cells it is necessary to perform more studies about the real levels of catalytic activity and phosphorylation in the cell models analyzed.

PP2A – PP2A is a serine/threonine phosphatase that, together with the protein phosphatase 1(PP1), account for more than 90% of all serine/threonine phosphatase activity in mammalian cells. PP2A exists as a stable core dimer comprised of a regulatory subunit A and a catalytic subunit C that when associated with a thirty subunit, named subunit B, can originate approximately 75 different PP2A heterotrimers and heterodimers. There are four families of subunit B that differently influence the sub-cellular localization, activity and

substrate specificity of the PP2A holoenzymes, a fact that can be associated with ability of PP2A to regulate diverse signaling pathways, sometimes leading to opposite effects depending on the cell type and cellular context (Hahn, 2004; Boudreau et al, 2007).

Some reports suggest that PP2A possesses anti-tumor activities by regulating negatively cell cycle progression and inactivating the anti-apoptotic protein bcl2, others highlight a role for PP2A in tumor progression, whose disruption by selective inhibitors may suppress the tumor growth (Schweyer et al., 2007). In agreement PP2A appears to be essential for T leukemia and myeloid cell lines survival and its inhibition through the PP2A-selective inhibitor okadaic acid is able to trigger apoptotic death in these cells. (Boudreau et al, 2007). Our results showed that Lucena has higher levels of activated PP2A than K562 indicating that in these cell lines PP2A activity is also associated with survival induction. Additionally, our results suggest that PP2A may be involved in the resistant phenotype of Lucena and, consequently, one more target for combating MDR phenotypes in myeloid leukaemias.

We also determined the kinetic parameters of the total phosphatase activity in the lysates of K562 and Lucena (Table 1). The substrates used in this study were chosen based on the literature. p-nitrophenyl phosphate is a synthetic substrate currently used in phosphatase studies; in regard to FMN, recently some authors have suggested the importance of its metabolism in cancer cells (Herwaarden et al 2007). Interestingly, we observed that the resistance leukaemic cells (Lucena) displayed higher phosphatase activity for both substrates. Intriguingly, the affinity as well as the specificity of the enzyme by FMN was more expressive for Lucena cells (Table 1). The implication of the flavin metabolism with the MDR has been already reported by Herwaarden and collaborators (2007). These authors demonstrated that the multidrug transporter breast cancer resistance protein (BCRP/ABCG2) is strongly induced in the mammary gland during pregnancy and lactation, which is responsible for pumping riboflavin into milk. In light of this information and our findings, we can speculate that flavin metabolism might play a role in the resistance acquisition and be useful target for preventing and overcome resistant cancer cells.

Many of the difficulties in designing an effective therapeutic strategy for conquering cancer cell resistance arise from the limited data that are available from the mediators which take part in this complex phenomenon. Therefore, a deeper understanding of the mechanisms underlying the multiple layers of cellular function regulation and how they are altered during resistance acquisition will be critical in identifying the next generation of therapeutic targets. In this cenario, we feel that the results obtained show that kinome profiling using metabolic arrays is a highly promising tool for the rapid evaluation of cellular metabolism. Besides our findings highlighted some mediators which might me addressed for conquer resistance cancer cells.

Table 1: Kinetic parameters of phosphatases isolated from Lucena and K562 cells
Phosphatase activity was determined by using FMN and PNPP as substrates as described in method section.

Cell Line	Vmax (µmol/min/mg)	Km (mM)	Vmax/Km
K562	PNPP = 0.450 FMN = 0.574	PNPP = 0.27 FMN = 0.92	PNPP = 1.67 FMN = 0.62
Lucena	PNPP = 0.970 FMN = 0.753	PNPP = 1.1 FMN = 0.34	PNPP = 0.88 FMN = 2.22

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PATENTES NACIONAIS

Os trabalhos com riboflavina na potencialização da ação de antineoplásicos e com inibidores de proteínas fosfastases na reversão da resistência a quimioterápicos propiciaram duas patentes nacionais.

1. (PI0505220-3) - Ferreira, C.V.; Souza, A.C.S.; Aoyama, H.; Queiroz, K.C.S.; Peppelenbosch, M.P. Potencialização da Ação de Antineoplásicos pela associação com Riboflavina. 2005.

2. (PI0604691-6) - Ferreira, C.V.; Aoyama, H.; Queiroz, K.C.S.; Peppelenbosch, M.P.; Silva, M.A.S.; Jucá, M.B. Novo uso médico, composições farmacêuticas e método de reversão da resistência a quimioterápicos utilizando inibidores de proteínas fosfatases. 2006.

3. Discussão

3. Discussão

No presente trabalho foram abordados três aspectos da atividade antitumoral da riboflavina: indução da apoptose das células de câncer prostático, indução da diferenciação de células leucêmicas e aumento da biodisponibilidade intracelular do quimioterápico mitoxantrona.

Riboflavina irradiada como indutora da apoptose de células de câncer prostático

Câncer de próstata é a principal causa de morte entre os homens, esse fato se deve muitas vezes, ao diagnóstico tardio e também à alta capacidade invasiva desse tumor. Portanto, a prevenção, novas formas de diagnóstico e protocolos terapêuticos são objetos de vários grupos de pesquisas. Nesse trabalho utilizamos como modelo experimental para se analisar o efeito da riboflavina irradiada, a linhagem de células PC3, a qual apresenta algumas características que a tornam um excelente modelo de câncer de próstata: alta capacidade invasiva, hormônio independente e é não responsiva à intervenção farmacológica.

A ação da riboflavina irradiada como antitumoral foi anteriormente descrita pelo nosso grupo, para células da leucêmica mielocítica (Souza et al., 2006). No entanto, esse tipo de abordagem para tumores sólidos não tinha sido avaliado. De forma interessante, verificamos que a riboflavina irradiada apresenta mecanismos moleculares similares para as células PC3, responsáveis pela indução da apoptose. Evidenciamos também para as células PC3 a implicação da sinalização FasL-Fas. Os resultados obtidos apontam os fotoprodutos da riboflavina como candidatos para terapia do câncer de próstata, considerando-se que os efeitos tóxicos dos fotoprodutos sobre as células tumorais foram observados em concentrações menores que 40 µM. É importante ressaltar que nessas condições, os fotoprodutos não apresentaram toxicidade em células não

transformadas (hepatócitos humanos e células de músculo liso prostático de rato). A indução da apoptose como sendo o principal tipo de morte celular induzida pela riboflavina irradiada foi confirmada pela ativação da caspase 3 e baixos níveis da proteína inibitória da apoptose, cIAP. Esta importante ação biológica dos fotoprodutos pode ser explicada pelas nossas observações de que vários eventos não necessariamente ligados a vias de morte celular são alvos dos mesmos. Provavelmente, esses eventos moleculares em conjunto, definem o destino celular caracterizado pela apoptose, tendo-se em vista que a inibição do Fas ou da produção da ceramida, foram capazes de "desligar" o processo apoptótico. Ressalta-se que o pré-tratamento com o anticorpo anti-Fas neutralizante resultou em completa inibição da morte celular, indicando que a via extrínseca é o principal mediador no processo apoptótico induzido pela riboflavina irradiada. Este resultado é consistente com o aumento da expressão do FADD observado em células PC3 tratadas com os fotoprodutos da riboflavina.

A fosforilação reversível de proteínas, regulada por proteínas quinases e proteínas fosfatases, é a principal forma de regulação covalente de proteínas, e portanto, da regulação de diversas funções celulares, tais como proliferação, diferenciação e transformação celular. Os fotoprodutos da riboflavina induziram uma diminuição da atividade da proteína fosfatase PP2A, que pode ser crucial para a indução da apoptose. Este resultado está de acordo com outros trabalhos que mostraram uma indução de apoptose por inibidores da PP2A (Souza *et al*, 2006).

Especialmente interessante é a observação de que os fotoprodutos podem diminuir o potencial metastásico de tumores sólidos. Esta ação é sustentada pela diminuição da atividade das MMPs 2 e 9, aumento da expressão do TIMP1 (modulador negativo das MMPs) e diminuição da expressão do VEGF. Outro fato que reforça essa hipótese foi a detecção de

inibição da MAPK p38. Por causa da característica das MMPs em degradar componentes da matriz extracelular, certamente estas enzimas estejam implicadas com a metástase tumoral. Portanto, vários relatos na literatura atestam o envolvimento destas proteases com a angiogênese, invasão tumoral e estabelecimento do tumor em sítios secundários (Justo and Ferreira, 2005; Choueiri et al, 2006; Deryugina and Quigley, 2006). Neste contexto, inibidores fisiológicos das MMPs e alguns produtos naturais têm sido considerados importantes moduladores negativos da invasão de células tumorais. A MAPK p38 apresenta diversas funções celulares, recentemente Ye e Yuan (2007) relataram que a inibição da mesma promovia um impedimento da angiogênese. Baseado nessas observações, hipotetizamos que a inibição da p38 pela riboflavina irradiada poderia portanto, prevenir o potencial metastásico das células PC3.

Riboflavina irradiada como indutora da diferenciação das células leucêmicas

A eficiência terapêutica da leucemia é drasticamente prejudicada pela característica das células leucêmicas em adquirirem resistência a fármacos estruturalmente não relacionados (Martelli et al., 2003; O´Hare et al., 2006; Diehl et al., 2007). Em geral é desejável que substâncias com ação antitumoral apresentem diferentes alvos moleculares e dessa forma, possam prevenir a aquisição da resistência. No entanto, os fármacos tradicionalmente utilizados no tratamento da leucemia não apresentam essa "multiatividade", em termos de alvos moleculares. Sob esse aspecto, o ácido retinóico que é o fármaco de escolha para o tratamento da leucemia, apresenta baixa eficiência quimioterápica devido ao fato do mesmo diminuir a velocidade da progressão do ciclo celular e conseqüentemente, permitir que subpopulações de células possam desenvolver formas de "escape" da indução de morte (Zhou et al.,

2007). Portanto, novos compostos que apresentem “multiatividade antitumoral” são altamente promissores na área oncológica.

Nesse cenário, a riboflavina irradiada surge como um candidato em potencial para a terapia de tumor de células hematopoiéticas, devido a capacidade da mesma induzir apoptose (Souza et al., 2006) e também diferenciação das células leucêmicas.

Os mecanismos de diferenciação de células mielóides são complexos e envolvem vários eventos regulatórios dependendo do indutor. No entanto, as vias de indução da diferenciação não estão bem elucidadas, não havendo uma via comum para o mecanismo de ação de vários compostos, incluindo alguns agentes antineoplásicos (Chénais et al, 2000). Na maioria dos estudos do mecanismo indutor de diferenciação em células leucêmicas é utilizada a linhagem da leucemia mielocítica humana (HL60), porém mesmo nessas células os eventos moleculares que levam à diferenciação ainda não estão bem esclarecidos (Shimizu *et al*, 1998).

Baseando-se em dados prévios, o estudo da diferenciação das células HL60 foi planejado para ser analisado após 24 e 48h utilizando concentrações sub-letais da riboflavina irradiada (0,5 e 5,0 µM). Nessas condições, identificamos alterações em diferentes vias de transdução de sinal.

Em geral as células respondem aos estímulos do meio ambiente através de vias transdução de sinais intracelulares as quais utilizam a fosforilação/ desfosforilação a fim de promover modificação de proteínas rápida e reversível (Tamura et al., 2004). De fato, o balanço entre fosforilação e desfosforilação de proteínas é a base para o controle de diversos eventos biológicos disparados por efetores extracelulares como hormônios, mitógenos, carcinógenos, citocinas, neurotransmissores, substâncias ou metabólitos tóxicos e agentes indutores da diferenciação (Ostman and Bohmer, 2001; Ferreira *et al*, 2006).

Proteínas quinases desempenham importantes funções em diversos processos celulares, incluindo diferenciação. Analisando-se o padrão de ativação das proteínas quinases fica evidente que após 48h de tratamento com a riboflavina irradiada, proteínas envolvidas na proliferação/sobrevivência (cRaf, MEK1/2, ERK1/2 e PKC) estão discretamente ativadas em relação ao controle. No entanto, a proteína quinase Src e a MAPK JNK encontraram-se significativamente ativadas. Em geral, a ativação da via de sinalização de ERK1/2 tem sido correlacionada com a resposta celular frente a fatores de crescimento e sobrevivência, controlando eventos como proliferação, diferenciação e sobrevivência celular. Contrariamente, a ativação das vias de sinalização celulares mediadas por JNK e p38 MAPK estão ligadas ao estímulo de morte celular por apoptose desencadeado por estresse celular tais como agentes genotóxicos, choque térmico e irradiação (Huh et al, 2004; Wada and Penninger, 2004). Contudo, as reais funções desempenhadas pelas MAPKs mostram-se extremamente dependentes do contexto bem como dos tipos celulares. Estudos com agentes citotóxicos promotores de apoptose mostram grande variação no padrão de ativação de MAPKs bem como variação de funções que são dependentes das concentrações da droga usada, tempo de exposição e do "status" de ativação de outras vias de sinalização. Ainda em relação à JNK vários relatos na literatura apontam esta MAPK como importante mediadora da diferenciação. Wang e colaboradores (2003) demonstraram que a atividade da JNK é essencial no processo de diferenciação de células HL60 em monócitos induzida por diidroxi-vitamina D3.

Microtúbulos e actina representam os principais componentes do citoesqueleto, e em células hematopoiéticas, estão sujeitos à constante remodelação frente a diferentes situações (Veselska et al., 2003). De acordo com os nossos resultados a diferenciação das células HL60 pela riboflavina irradiada é reforçada pela expressiva ativação da Src, a qual desempenha papel importante na regulação de proteínas da adesão focal e do citoesqueleto. Alguns

autores têm reportado que a alteração dos microtúbulos leva à ativação da Src quinase (Cambien et al., 1999), o que corrobora com os nossos resultados.

Os resultados em conjunto indicam que o processo celular predominante quando as células foram tratadas com riboflavina irradiada 5µM, é a diferenciação e não a proliferação. Essa hipótese é reforçada pelo fato que observamos inibição da progressão do ciclo celular na presença da riboflavina irradiada 5 µM. A proteína p21 foi up-regulada após 24h de tratamento. A proteína p21 apresenta papel essencial na regulação do ciclo celular, uma vez que a mesma inibe a formação do complexo ciclina-CDK, fator essencial para a progressão do ciclo celular. De forma interessante a expressão da ciclina D1 diminuiu após o tratamento por 48h, com a riboflavina irradiada. Confirmando assim, a inibição do ciclo celular, já que essa proteína é requerida para a progressão do ciclo celular para a fase G1. A interrupção do ciclo celular é um evento crucial para a ocorrência da diferenciação. O nível da cdc2 fosforilada no sítio inibitório também apresentou-se levemente aumentado na presença da riboflavina irradiada, fator que reforça a parada do ciclo celular.

Células HL60 tratadas com riboflavina irradiada apresentaram inibição da PP2A nas primeiras 24h. A inibição da proteína fosfatase tipo 2 (PP2A) nas primeiras 24h de tratamento das células com a riboflavina irradiada pode ter uma função importante para a sobrevivência celular, uma vez que a ativação dessa enzima muitas vezes está relacionada com a desfosforilação de proteínas pró- e antiapoptóticas culminando na ativação da apoptose. A PP2A é a principal proteína serina/treonina fosfatase envolvida na regulação de vários processos celulares incluindo diferentes vias de transdução de sinais, progressão do ciclo celular, replicação do DNA, transcrição gênica e síntese de proteínas (Van Hoof and Goris, 2003). Tal diversidade de funções é explicada em parte pela sua complexidade estrutural e de regulação (Hahn, 2004). A função indutora da diferenciação da PP2A é consistente com seu papel na progressão do ciclo

celular, crescimento e sobrevivência celulares, inibindo direta ou indiretamente a quinase cdc2, MAPKs e PKB/Akt. Além de regular negativamente o crescimento celular, a atividade da PP2A também contribui para a natureza transiente das ativações de proteínas quinases ativadas por mitógenos (MAPKs) por fatores de crescimento, o que evita uma ativação constitutiva suficiente para causar transformação tumorigênica (Schontal, 2001).

A PTEN permaneceu ativa nessas condições, no entanto observamos expressiva ativação da SHP2 (24 e 48h) e PTPα (48h). A expressão da LMWPTP não foi afetada em nenhum dos períodos analisados. Estudos de diferenciação de HL60 induzida por ácido retinóico (Treigyté et al, 2000) mostram a ocorrência de aumento constante de proteínas fosforiladas em tirosina ao longo do processo de diferenciação sendo que nos estágios finais, o nível de tirosinas fosforiladas cai drasticamente nos neutrófilos maduros que, subseqüentemente, iniciam processo apoptótico culminando com a morte celular após diferenciação. Freire et al (2003) demonstraram que o tratamento de células HL60 com inibidores de PTPs e Ser/Thr fosfatases, pervanadato e ácido okadaico, respectivamente, também induziu a diferenciação e posterior morte celular por apoptose. Geralmente os indutores de diferenciação em células leucêmicas são conhecidos por induzir apoptose. Durante o processo de maturação normal de células mielóides, a apoptose ocorre como evento fisiológico sendo bem conhecido que granulócitos maduros e eritrócitos desencadeiam apoptose espontânea ao final do tempo de vida. Os dados mencionados anteriormente mostram que a atuação de proteínas tirosina quinases está relacionada ao processo de diferenciação de células HL60 e que no processo apoptótico subseqüente ocorre diminuição de proteínas fosforiladas em tirosina provavelmente por atuação de proteínas tirosina fosfatases específicas.

A proteína tirosina fosfatase homóloga à tensina (PTEN) é uma importante supressora de tumores (Kim and Mak, 2006). Em muitos tipos de tumores humanos como o de próstata, cérebro, mama, endométrio e rins é possível encontrar essa proteína mutada ou mesmo deletada do genoma (Huang, 2001). A PTEN desempenha uma importante função regulatória de vias de transdução de sinal responsáveis pela proliferação celular, por atuar na regulação negativa da fosfatidilinositol 3 quinase (PI3K). PI3Ks catalisam a síntese do fosfatidilinositol 3,4,5 fosfato, modulador alostérico positivo da proteína quinase AKT (Baker, 2007).

A proteína tirosina fosfatase SHP2 está presente no citoplasma, e por apresentar dois domínios SH2 apresenta importante papel como mediadora "downstream" de receptores tirosina quinases e de citocinas. Desta forma, essa enzima pode regular vias de sinalização da cascata da MAPK ERK ou de integrinas, desempenhando portanto, regulação da adesão, migração e arquitetura do citoesqueleto (Feng, 2007). Especula-se que a SHP2 regula a função da integrina por regular a fosforilação de FAK. Além disso, o rearranjo de α-actina em complexos focais poderia ser diretamente dependente de SHP2, com a inibição da atividade de FAK (von Wichert et al., 2003). A ativação da SHP2 após o tratamento das células HL60 com a riboflavina irradiada sugere portanto, a participação desta proteína fosfatase no processo de diferenciação induzido pela riboflavina.

A PTPα é uma proteína fosfatase tipo receptor a qual é o principal modulador positivo da proteína quinase Src e conseqüentemente ativa a proteína quinase de adesão focal (FAK). Desta forma, a PTPα desempenha papel central na sinalização que culmina na organização do citoesqueleto (Chen et al., 2006; Feng, 2007). Portanto, a ativação dessa fosfatase pela riboflavina irradiada, é outro fator que explica a ação dessa vitamina como indutora da diferenciação de células leucêmicas.

O TNFα apresenta diversas funções biológicas, dentre as quais, controle da proliferação, diferenciação e apoptose. A natureza dos efeitos causados pelo TNFα é dose e fase de crescimento-dependente. Estas diferenças são em parte devido a presença de dois tipos de receptores para o TNFα, tipo 1 e 2 (TNFR1 e TNFR2). A ligação do TNFα ao TNFR1 causa trimerização deste receptor e em seguida ocorre o recrutamento da proteína adaptadora TRADD, na qual se liga TRAF2 e/ou FADD (Hallenbeck, 2002; Deng et al 2003; Gaur and Aggarwal, 2003). Ativação de TRAF2 é responsável pela ativação do NFκB e consequente expressão de proteínas anti-apoptóticas, tais como IAP e Bcl2 (Karin and Lin, 2002; Wen et al 2003). A ativação da caspase 8, como mencionado anteriormente, é o ponto de partida para ativação da cascata de diferenciação ou apoptose.

Células HL60 tratadas com riboflavina irradiada por 48h tiveram um aumento significativo da expressão do TNFR1. Além disso, verificamos através da co-imunoprecipitação, ativação desse receptor, uma vez que após imunoprecipitação do TNFR1 detectamos as proteínas FADD e TRAF2. Como mencionado anteriormente, o TNFR1 pode desencadear dois tipos de respostas celulares principais: diferenciação e/ou apoptose. Na presença da riboflavina irradiada a resposta dominante foi a diferenciação, uma vez que não observamos ativação significativa da caspase 8. Em relação ao nível da PARP total (intacta), observamos um aumento após o tratamento da células por 24h, no entanto, após 48h as células tratadas com riboflavina 5μM apresentaram uma diminuição da PARP intacta. Esse resultado indica a ocorrência da degradação da PARP, resultado concordante com a ativação da caspase 3. O envolvimento das caspases em outros processos que não a apoptose, tem sido demonstrado em diferentes tipos celulares (Lamkanfi et al, 2007). Nesse contexto, o envolvimento da caspase 3 na diferenciação de células eritróides e osteoblásticas tem sido reportada por diferentes grupos de pesquisas (Mogi and Togari, 2003; Carlile et al., 2004). Adicionalmente, tem sido demonstrado que

inibidores de caspases interferem na maturação de progenitores da linhagem eritróide nos estágios iniciais de diferenciação. Nesse processo caspases 2, 3 e 9 são "transientemente" ativadas e estão envolvidas na clivagem de proteínas nucleares tais como laminina, PARP e acinus (Zemati et al., 2001; Lamkanfi et al, 2007).

Após a comprovação da ativação do receptor TNFR1, a próxima estratégia experimental foi determinar o nível de TNFα. De forma interessante, observamos uma diminuição desta citocina. Esses resultados foram reforçados com a observação de aumento da expressão do fator NFκB, pois a síntese desta proteína é regulada pela ativação do TNFR1. Entretanto, nossos resultados mostram que esta proteína não é translocada para o núcleo, o que explica a diminuição no nível de TNFα, já que um dos genes alvos desse fator de transcrição é o gene do TNFα. Ougolkov e colaboradores (2007) sugeriram que a GSK3 é um importante modulador da atividade transcricional do NFκB, por regular a atividade das subunidades p65/p50 do NFκB, em leucemia linfocítica crônica. Nesse trabalho, a permanência do NFκB no citosol também foi evidenciada à partir da detecção da inibição da GSK3 através da técnica do Pepchip. Desta forma, hipotetisamos que a reorganização do citoesqueleto principalmente decorrente do rearranjo dos filamentos de actina durante a diferenciação das células HL60, poderia também contribuir para a permanência do NFκB no citoplasma. O filamento de actina é uma estrutura dinâmica, ao ponto em que a polimerização e despolimerização são eventos críticos para muitas respostas celulares. Recentemente, estudos mostraram que o NFκB pode interagir com actina o que pode regular sua translocação para o núcleo, quando células endoteliais foram utilizadas como modelo (Fazal et al., 2007).

Os resultados mostrados anteriormente juntos com a observação da ativação da Src, SHP2 e caspase 3, confirmam a ação da riboflavina irradiada como indutor de diferenciação. A proteína tirosina fosfatase SHP2 é um

importante regulador das vias de MAPK ERK e integrina. SHP2 também modula a adesão e migração celular assim como a arquitetura do citoesqueleto (Feng, 2007), sendo esta função sugerida devido a seu papel regulador sobre a FAK. Outro importante resultado relacionado com a reorganização da morfologia celular foi a expressiva ativação da caspase 3, sendo esta recentemente citada por suas funções não apoptóticas. Vários estudos têm demonstrado que as caspases estão envolvidas com diferenciação terminal de vários tipos celulares. Estas proteases dão sua contribuição a este processo por desempenhar importante papel na reorganização do citoesqueleto (Zemati et al., 2001; Lamkanfi et al, 2007).

A ativação da STAT3 foi um outro interessante evento observado, já que a máxima ativação desta é requerida para a diferenciação induzida pelo PMA e IL10, os quais promovem um aumento da expressão do cJun, cFos (Makuta et al., 2003).

De acordo com os nossos resultados sugere-se o envolvimento dos receptores, TNFR1 e da proteína tirosina fosfatase tipo receptor (PTPα), no processo de indução de diferenciação, bem como das proteínas ciclina D, JNK, Src quinase e da proteína tirosina fosfatase SHP2. Portanto, proteínas com diferentes localizações celular foram afetadas, culminando com a diminuição da proliferação, manutenção da sobrevivência celular, interrupção da progressão do ciclo celular e reorganização do citoesqueleto, efeitos metabólicos essenciais para a ocorrência da diferenciação.

Aspectos bioquímicos das células da leucemia eritrocítica

Dentre as diversas dificuldades encontradas para tratar o câncer, a aquisição de resistência a quimioterápicos permanece como um tema a ser

explorado (Martelli et al., 2003; O´Hare et al., 2006; Diehl et al., 2007). A resistência a múltiplos fármacos é definida como um fenômeno de alta complexidade, onde as células tornam-se resistentes a diferentes fármacos estruturalmente não relacionados.

Um outro aspecto abordado nesse trabalho foi a identificação de diferenças bioquímicas entre dois tipos de linhagens da leucemia eritroblástica, K562 e K562 resistente (Lucena). As proteínas quínases e proteínas fosfatases foram alvos dessa investigação.

O perfil quinásico obtido com o estudo de microarranjos de substratos para proteínas quinases (Pepchip) exibiu diferenças no nível de fosforilação de 307 peptídios dos 1.024 contidos no chip. Verificamos que as quinases com maior atividade na Lucena estão envolvidas com vias de sinalização responsáveis por proliferação celular, sobrevivência e consequentemente, aquisição de resistência. Dentre as quinases mais ativas vale ressaltar a tirosina quinase tipo receptor, quinases dependentes de ciclinas, MAPK, PKA, PKC, AKT, “Polo like” quinase, Src e FAK.

Fosforilação/desfosforilação de proteínas exercem um papel central nas vias de transdução de sinal e estão associadas ao controle de processos comuns, tais como: metabolismo, proliferação, sobrevivência e morte, com o objetivo de manter a homeostase celular (Ferreira et al, 2006). Embora os estudos de sinalização associados com desenvolvimento e progressão do câncer têm ao longo dos anos, dado ênfase às proteínas quinases, que tipicamente iniciam as cascatas de sinalização, recentemente as proteínas fosfatases têm adquirido grande importância neste cenário. Nesse trabalho abordamos de forma inédita a importância das proteínas fosfatases para o fenótipo da resistência de células leucêmicas. Os resultados evidenciaram que a Lucena apresenta níveis elevados da LMWPTP, ativação da SHP2 e da PP2A. Ressaltamos ainda, a alta especificidade e afinidade das fosfatases isoladas das

células Lucena, pela flavina mononucleotídeo (FMN). Algumas indicações na literatura têm apontado a relevância do metabolismo de flavinas em células tumorais (van Herwaarden et al., 2007).

Produção técnica

O desenvolvimento do projeto de doutoramento forneceu subsídio em termos de conhecimento que permitiram o depósito de duas patentes. Apesar dos dados que fundamentaram essas duas produções técnicas não poderem ser descritos detalhadamente nesse trabalho, é válido mencionar que essas duas patentes corroboram todos os resultados apresentados nesta tese, bem como atestam o caráter inédito da mesma, principalmente sob o aspecto do potencial da riboflavina, bem como da modulação de proteínas fosfatases como estratégias para o tratamento do câncer.

4. Conclusão

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Os fotoprodutos da riboflavina apresentam-se como alternativas interessantes para o tratamento do câncer. Os fotoprodutos da riboflavina foram capazes de induzir morte de células do câncer de próstata, modularam a expressão/atividade de mediadores importantes para a ocorrência da metástase, tais como: MMPs 2 e 9, TIMP1 (modulador negativo das MMPs) e VEGF.

Além disso, frente as células leucêmicas, os fotoprodutos foram capazes de induzir diferenciação ou morte celular. Processos que têm sua ocorrência dependente da dose utilizada. De forma interessante, podemos ressaltar a indução da diferenciação pelos fotoprodutos. É importante considerar que para alguns tipos de leucemia a terapia da diferenciação apresenta-se como alternativa interessante e eficiente no sentido de diminuir a agressividade da terapia antileucêmica.

Ainda no presente trabalho foi possível avaliar diferenças na atividade/expressão de proteínas responsáveis pelo balanço entre fosforilação/desfosforilação entre a K562 e Lucena (K562 resistente). Os dados obtidos neste trabalho apontaram um grupo interessante de proteínas que pode estar desempenhando funções cruciais no processo de resistência a quimioterápicos. Outro aspecto relevante foi a observação de uma possível implicação do metabolismo de flavinas com o fenótipo de resistência tumoral. Já que no extrato protéico total das células Lucena foi identificada uma fosfatase com alta especificidade para a flavina mononucleotídeo (FMN), quando comparado com a célula parental (K562).

Diante do exposto ressaltamos a contribuição deste trabalho no sentido de definir vias de sinalização potencialmente interessantes na modulação de processos relacionados com a progressão e resistência tumoral.

Podemos ressaltar ainda que atualmente muito tem sido alcançado com objetivo de se obter terapias mais eficientes e ao mesmo tempo melhor

toleradas. Os avanços científicos têm contribuído de forma inestimável para o melhoramento e desenvolvimento da terapia de diversas doenças. Sob esse aspecto, a visão dos profissionais de saúde, pesquisadores, bem como dos órgãos que regulamentam o "lançamento" de novos fármacos também tem sofrido mudanças positivas. Destacamos aqui, o novo objetivo do FDA, em relação a quimioterápicos, que é garantir a qualidade de vida do paciente. Isto somente será possível com a identificação de alvos moleculares bem como definição do mecanismo de ação do candidato a fármaco no microambiente celular. Esse trabalho além de atender a essa "nova demanda", permitiu sob o ponto de vista da transdução de sinal, a definição e identificação de alvos moleculares de três processos celulares que apresentam grande relevância na área de pesquisa do câncer: diferenciação, morte e reversão da resistência. Sob esse aspecto os resultados apresentados nesse trabalho, corroboram com a importância da transferência de informações da pesquisa básica para aplicação no desenvolvimento de fármacos "inteligentes", que possam garantir novas abordagens terapêuticas e qualidade de vida do paciente.

5. Referências Bibliográficas

Referências Bibliográficas

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6. Anexos

Tabela 1 – Diferenças estatisticamente significante da fosforilação das seqüências consenso de peptídios entre os grupos controle 48 e riboflavina irradiada 48 horas.

Spot	Kinase	Peptide	Ratio	T-value	▲▼
683	Protein kinase C epsilon	LLREASARDRQ	0.0	0%	▼
685	AMPK	IRSSMSGLHLV	6.4	0%	▲
478	Cytokine inducible kinase Cell cycle checkpoint kinase CHK2 MAPK14 MARK3	QLLASTPNGLD	14.2	0%	▲
940	CDC2	IVPGKSPTRKK	3.3	0%	▲
389	EGF receptor	ATLDVYNPFET	3.3	0%	▲
803	Cdc2 kinase	GSKNKSPSKAA	0.1	0%	▼
421	NIK	DVDQGSLATSF	0.1	0%	▼
46	Casein kinase 2	AVHEDSGDEDG	0.5	0%	▼
105	AKT1	RLRTHSIESSG	0.1	0%	▼
634	PKA	KKKKPSRLKGD	0.3	0%	▼
660	ABL	RNEGVYTAIAV	12.0	0%	▲
905	pp60-src	DIDGQYAMTRA	8.6	0%	▲
668	PKA	AQRRHTLPASE	4.4	0%	▲
638	PKA	KARKKSSAQLL	2.5	0%	▲
696	MAPK4	TKWYRSPRLLL	0.2	0%	▼
714	Lyn	FSGGLYGGLPP	0.1	0%	▼
615	CDC2 PP2A	YQQRNSPGVPT	2.0	0%	▲
380	Casein kinase II	LEVSDSESDEA	0.4	0%	▼
63	PKC	SRKGYSRKGFD	0.3	0%	▼
130	Focal adhesion kinase 2	IESDIYAEIPD	0.2	0%	▼
404	ABL	EEGEGYEEPDS	16.5	0%	▲

150	DNA-dependent protein kinase catalytic subunit	TLQTRTQEGSL	5.6	0%	▲
577	c-Src	DDEDAYGNYDN	0.1	0%	▼
630	Cyclin-dependent kinase 4	HERYSSPTAGS	5.6	0%	▲
673	AKT1	QPRATSLDSAL	0.2	0%	▼
463	Protein kinase C alpha	WGAGNSLRTAL	0.2	0%	▼
693	AKT1	RSRDPSLMVDF	0.0	0%	▼
428	CDC2	ETPAISPSKRA	7.9	0%	▲
402	JNK1	EEIYLTPVQRP	0.0	0%	▼
717	Csk	QQQEVYGMMPR	1.7	0%	▲
709	ZAP70	EQETFYEQPPL	4.4	0%	▲
209	Cyclin A Cyclin B CDC2 Cyclin dependent kinase 2 Cyclin E	SASPYTPEHAA	8.6	0%	▲
623	AKT1	RRRAISETEEN	14.7	0%	▲
356	PKG-1	KKRKRSRWNQD	2.6	0%	▲
534	CaMK2	FESIESYDSAD	0.2	0%	▼
370	PKA	IKRQLSMTLRG	0.2	0%	▼
628	Glycogen synthase kinase 3 beta Tumor endothelial marker 8	ENNVLSPLPSQ	0.3	0%	▼
	DNA-dependent protein kinase catalytic subunit 208900				
558	Casein kinase II	GGADDSAEEGD	0.4	0%	▼
695	Pkb	RPRNYSVGSRP	-312.8	0%	▼
322	c-Src ABL	GWMVHYTSKDT	0.1	0%	▼
372	Glycogen synthase kinase 3 beta	GHSNSSPRHSE	4.7	0%	▲
1018	PKA	LPRASSLNENV	15.0	1%	▲
395	TRKA	IENPQYFSDAA	0.3	1%	▼

607	Pyruvate dehydrogenase kinase, isoenzyme 1	QSKRSTMVGTP	7.2	1%	▲
190	PKA PKG	AERRNSILTET	-0.1	1%	▼
335	SHC	LVNRHYAKISD	5.5	1%	▲
591	ZAP70	TGSVDYLALDF	39.0	1%	▲
574	PKA	QLRRPSDRELS	49.7	1%	▲
572	AMPKK	GEFLRTSAGSP	0.1	1%	▼
366	Casein kinase 1, delta	MGQAGSTISNS	8.8	1%	▲
426	Protein kinase C alpha PKM Protein kinase C, gamma Protein kinase C, beta-1	AKIQASFRGHM	0.1	1%	▼
527	Pka pkc	RNPGFYVEANP	2.0	1%	▲
666	p90rsk	KGGKYSVKDKE	0.5	1%	▼
473	RK	GDDEASATVSK	-274.6	1%	▼
706	erbb2	SPQPEYVNQPD	-0.2	1%	▼
955	PKC	AKKTLSEVERD	0.2	1%	▼
589	Lyn	ASKRSYQFWDT	0.2	1%	▼
942	Cyclin dependent kinase 5	KSEPISPPRDR	3.6	1%	▲
355	ATK1	RKRRWSAPESR	0.1	1%	▼
719	TrkB tyrosine kinase	IENPQYFGITN	5.7	1%	▲
207	EphB1	PGMKIYIDPFT	0.2	1%	▼
102	Casein kinase 1, alpha 1	PQSDPSVEPPL	0.3	1%	▼
830	Ribosomal protein S6 kinase alpha 3/PKA/PKC	EPKRRSARLSA	-0.8	1%	▼
140	EGF receptor	SANAIYSLAAR	0.4	1%	▼
595	CDk2	DNTPHTPTPFK	3.7	1%	▲

738	RPL10	QKIHISKKWGF	0.1	1%	▼
637	Protein kinase A	SQRRESFLYRS	3.8	1%	▲
362	PKC zeta	YFRYLSEVASG	4.4	1%	▲
831	PKC	RPDPSSFSRPR	0.0	1%	▼
600	c-Src	TTSQLYDAVPI	9.4	1%	▲
476	ERK2	GTNPGTPPAST	0.2	1%	▼
427	cdc2-kinase	SSTPLSPTRIT	2.5	1%	▲
40	Cyclin dependent kinase 5	ARPPASPSPQR	0.3	1%	▼
649	Src kinase	ITEEDYQALRT	4.0	1%	▲
536	PAS kinase	GKLFYTFAGTI	4.0	1%	▲
614	LIMK1 TESK1 TESK2 LIMK2	MASGVAVSDG	3.4	1%	▲
378	PKA	QERRGSNVALM	2.9	1%	▲
498	PKC CaM-Kinase II	LIPQQSINEAI	1.6	1%	▲
410	Phosphorylase kinase, muscle, alpha-1 subunit	EFRRLSISAES	-988.6	1%	▼
218	ERK5	DPRLLSPQQPA	1.7	1%	▲
332	MAP3K10	MNEVTYSTLNF	0.5	1%	▼
201	Csk	AQDEFYRSGWA	7.5	1%	▲
333	Lyn	TAEPDYGALYE	0.3	1%	▼
975	Tyrosyl kinase	ENFDDYMKEVG	-81.6	1%	▼
205	EGF receptor	SFLQRYSSDPT	2.6	1%	▲
707	FAK	EEEHVYSFPNK	2.1	1%	▲
309	CDK2-cyclin E	EAKRKSPKKKE	0.5	1%	▼
736	c-Src	FFVIEYVNGGD	122.3	1%	▲

947	Protein kinase C	RDRHLSFSGSG	5.9	1%	▲
769	Insulin receptor	EDIKSYYTVRQ	0.2	1%	▼
465	cyclin E-CDK2	GSVEQTPKKPG	5.6	1%	▲
897	Protein tyrosine kinase TXK	QVKALYDFLPR	0.1	1%	▼
619	Protein kinase C alpha PROTEIN KINASE C beta 1 PROTEIN KINASE C beta 2 Protein kinase C, eta	GLMQQQKSFR	-64.5	1%	▼
898	SYK	ADENYYKAQTH	0.0	1%	▼
765	PKA	RDTRDSEAQRL	0.5	1%	▼
731	Fyn	SLESLYSAASM	2.7	1%	▲
974	Ret	AFPVSYSSSGA	0.2	1%	▼
438	Casein kinase I	PVRAYSAEVVT	4.0	1%	▲
740	CK2, alpha 1	LLEDDSDEEED	0.3	1%	▼
705	EGFR kinase	LKSPAYRDLAA	0.2	2%	▼
946	PKC	EKRKNSILNPI	0.7	2%	▼
609	Cdpk	RFIIGSVSEDN	0.2	2%	▼
206	Hepatocyte growth factor receptor	YDKEYYSVHNK	0.4	2%	▼
938	Protein kinase C alpha	KTVNESASLRE	0.3	2%	▼
241	PKA	SVRRASSADDI	6.1	2%	▲
654	Phosphatidylinositol 3-kinase, catalytic subunit, alpha	EDLSAYASISF	1.9	2%	▲
119	JNK1	LPGALSPLYGV	103.0	2%	▲
146	AKT	RSRRLTFRKNI	0.1	2%	▼
58	Ribosomal protein S6 kinase alpha 3/PKA/PKC	EPQRRSARLSA	2.8	2%	▲
532	Calcium-calmodulin dependent protein kinase I kinase	GSVLSTAAGTP	2.7	2%	▲

93	PKC	FLSSPTYRALL	0.1	2%	▼
360	3-Phosphoinositide dependent protein kinase 1	SDTTTTFAGTP	0.4	2%	▼
925	CK2, alpha 1	SVSVETQGDDW	0.1	2%	▼
1	NIK MAP3K7 MEKK1 IKK alpha	ELDQGSLATSF	0.3	2%	▼
114	CaM Kinase I,PKA	LRRRLSDSNFM	9.2	2%	▲
791	CDC2	EGAAATPERMA	2.8	2%	▲
896	ERK1	PTAPLSPMSPP	15.9	2%	▲
645	EGF receptor	PGLDEYNPFSD	0.3	2%	▼
843	EphA8	AEPHTYEEPGR	3.5	2%	▲
697	Protein Kinase C	PAARASKKILL	0.1	2%	▼
661	ERK2	RTAPYTPNLPH	0.4	2%	▼
406	Beta-adrenergic receptor kinase 2	ALDFRTPRNAK	0.4	2%	▼
1011	CK-II	QVSSLSESEES	4.5	2%	▲
702	PKA	ELEGISPDELK	79.8	2%	▲
157	ERK	SILPFTPPVVK	2.5	2%	▲
225	Activin A receptor, type I TGF beta receptor, type I	SPHNPISSVS	0.2	2%	▼
267	EphB2	TIEDSYTKIAS	0.5	2%	▼
882	PRKA1	SKRRNSEFEIF	2.3	2%	▲
99	PRKG1	PFRRPSTYGIP	2.3	2%	▲
611	Interleukin 2	LDSRLSPPAGL	2.0	2%	▲
505	PKA	PLVQRGSANGL	1.9	2%	▲
277	PKC	LNRIQTQIRVV	1.9	2%	▲
621	ERK1 ERK2 MEK1 MAP2K2	RRRFSSLHFMV	0.1	2%	▼

126	T cell acute lymphocytic leukemia 1	VKRRPSPYEME	1.3	2%	▲
439	Protein kinase	SERRGSHPYID	4.4	3%	▲
790	Casein Kinase	HYLDETEQWEK	0.3	3%	▼
196	Fyn	PATTIYVAATE	0.4	3%	▼
566	Casein kinase II, beta	DVHMVSDSDGD	0.4	3%	▼
475	nb	AKKEESEESDD	0.0	3%	▼
128	GSk3	STPAPSRTASF	0.7	3%	▼
985	AKT1	DHYRYSDTTDS	0.0	3%	▼
178	PKA, AKT1	ELRRMSDEFVD	0.7	3%	▼
321	FER, Fyn	VNLINYQDDAE	0.4	3%	▼
145	MAP kinase	PVVTATPSATA	-0.1	3%	▼
698	PKA PKC	LERNLSFEIKK	13.5	3%	▲
286	p34cdc2/cyclin B	IPTGTTPQRKS	3.8	3%	▲
39	PKN	GLYSRSGSLSG	0.4	3%	▼
181	CK2	EEKQKSDAEED	2.4	3%	▲
462	c-abl kinase	QESEDYSQPST	8.2	3%	▲
969	Lck Csk	EANSHYGHNDD	0.2	3%	▼
81	CDC2-kinase	VDPMLTPEERH	1.7	3%	▲
429	CK2	PQSPGSPLEEE	2.9	3%	▲
829	JNK1	LLKLASPELER	-2.9	3%	▼
684	CDK4, CDC3	PSPLPSPTASP	-0.3	3%	▼
316	Casein kinase II	DTLSDSDDEDD	0.4	3%	▼
258	FMS-related tyrosine kinase 3	DNEYFYVDFRE	0.2	3%	▼

217	Dual-specificitytyrosine phosphorylation-regulated kinase 2	SHLRNSPEDKR	0.4	3%	▼
44	PDK1	QARANSFVGTA	0.4	3%	▼
69	CK2, alpha 1 p38 MAP kinase PKR Cyclin dependent kinase 2	FKTEGPDSD	0.2	3%	▼
802	PAK2	RRRLSSLRAST	0.4	3%	▼
987	Casein kinase II	KEEKGSPLNAA	2.6	3%	▲
54	CDC2	YSQGASPQPQH	3.2	4%	▲
728	Casein kinase 2	EEEADSAFGDD	2.7	4%	▲
385	Casein kinase II	HVDNEYSQPPR	0.4	4%	▼
323	Csk Fgr PTPase	STEPQYQPGEN	1.8	4%	▲
155	Cyclin dependent kinase 5	HFTMRSPFKAD	0.2	4%	▼
852	c-Src	VLDDQYVSSVG	0.2	4%	▼
917	ERK2	IEQWFTEDPGP	0.3	4%	▼
117	AKT1	RRRAASMDSSS	0.1	4%	▼
749	CK2-beta	HWQQQSYLDSG	0.4	4%	▼
635	PKC-beta	LKPGSSHRKTK	0.7	4%	▼
978	HGF	DVPLLTPSSKE	0.1	4%	▼
510	PKC	GTLRTSISVER	3.3	4%	▲
162	ATM	QEEGSSQGEDS	0.2	4%	▼
90	PAK3	NQKYMSFTSGD	-0.9	4%	▼
500	SDK1	GARRSSWRVIS	0.4	4%	▼
127	Protein kinase C alpha	KFRTPSFLKKN	0.4	4%	▼
826	MAPKAP kinase 2	IDRTESLNRSI	0.1	4%	▼
298	Cell cycle checkpoint kinase	TQRQNSAQLGM	0.4	4%	▼

382	PKA	RTRRISQTSQV	70.8	4%	▲
602	CK1	DDGEFSDDSGA	0.2	4%	▼
761	PKA	VRRRQSVELHS	0.1	4%	▼
531	CaMKII	SSPPGTPSPAD	0.2	4%	▼
922	IKK beta	APAYLSSPLAL	18.1	4%	▲
868	ATR	QGISFSQPTAP	0.1	4%	▼
62	PKA	YQRRASDDGKL	5.5	5%	▲
112	PKR	GFTRKSVRKAQ	0.3	5%	▼
980	EGF receptor Bcr/Abl	MFPRNYVTPVN	3.5	5%	▲
596	SYK c-Src	PDHQYYNDFPG	2.7	5%	▲
364	Phosphoinositide-3-kinase, catalytic subunit, gamma	TEDQYSLVEDD	0.2	5%	▼
397	Protein kinase C, zeta	AATMKTFAGTP	23.2	5%	▲
953	Protein Kinase C	DIQQLSSEEND	-13.5	5%	▼
642	SYK	VSFNPYEPELA	0.3	5%	▼
641	ZAP70 Protein tyrosine kinase TXK	FEEDDYESPND	-0.1	5%	▼
704	CK2, alpha 1	SQSPHSPDSSQ	0.1	5%	▼
470	PKR	TRSKGTLRYMS	0.6	5%	▼
936	Protein kinase A	IFRRPSLPAIS	1.6	5%	▲
665	Pyruvate dehydrogenase kinase, isoenzyme 1	EKKAYSFAGTV	0.1	5%	▼
369	Protein kinase A	RKRKNSRVTFS	1.9	5%	▲
935	Bone morphogenetic protein receptor, type IB	GALSNSESIPT	3.2	5%	▲
680	Cyclin dependent kinase 2	SPYSLSPVSNK	2.4	5%	▲

Tabela 2 – Diferenças estatisticamente significante da fosforilação das seqüências consenso de peptídios nas células K562 e Lucena.

Spot.	Kinase	Peptide	Ratio	T-value	▲▼
494	Phosphorylase kinase muscle gamma-1	ILRKVSGHPNI	2.9	0%	▲
482	CK2	KRRGASDLSSE	0.1	0%	▼
483	Protein kinase C	QGSDVSLTAAK	5.4	0%	▲
654	Phosphatidylinositol 3-kinase, catalytic subunit, alpha	EDLSAYASISF	18.4	0%	▲
517	DYRK1B	LGQRIYQYIQS	0.1	0%	▼
418	Cam KII	GKNRPSSGSLI	0.2	0%	▼
365	AKT1	RGRGSSVGGGS	0.1	0%	▼
507	cdc2-cyclin B kinase	LYRSPSMPENL	0.0	0%	▼
759	MARK	KSKIGSTDNIK	0.2	0%	▼
660	ABL	RNEGVYTAIAV	0.1	0%	▼
218	ERK5	DPRLLSPQQPA	0.2	0%	▼
1017	Protein kinase C	RFYPESSYKST	0.0	0%	▼
682	Protein kinase C alpha	QNLMQSVKETV	0.1	0%	▼
246	AKT2	RERLASTNDKG	27.7	0%	▲
457	Lck	LNEEWYVSYIT	0.1	0%	▼
609	Cdpk	RFIIGSVSEDN	0.1	0%	▼
877	AKT1	RPRRRSSAVSL	-37.5	0%	▼
756	CKII	MRRQRSAPDLK	0.3	0%	▼
453	Mnk1 Protein Kinase C	ATKSGSTTKNR	0.1	0%	▼

698	PKA PKC	LERNLSFEIKK	0.1	0%	▼
981	AKT1	RKRRPTSGLHP	-2.4	0%	▼
366	Casein kinase 1, delta	MGQAGSTISNS	38.7	0%	▲
102	Casein kinase 1, alpha 1	PQSDPSVEPPL	10.6	0%	▲
480	c-Src	PRSTHTAYIK	0.3	0%	▼
420	Cyclin dependent kinase 5	APRQSSPSKSS	15.8	0%	▲
435	PKC	PSAYGSVKAYT	86.4	0%	▲
458	Lyn	RRAKHYVELLV	0.4	0%	▼
503	MARK	QAKVGSLDNVG	0.3	0%	▼
952	p74raf-1	DSMANSFVGTR	0.2	0%	▼
897	Protein tyrosine kinase TXK	QVKALYDFLPR	0.1	0%	▼
190	PKA PKG	AERRNSILTET	0.0	0%	▼
406	Beta-adrenergic receptor kinase 2	ALDFRTPRNAK	0.3	0%	▼
771	PDGF receptor, beta	RKGHEYTNIKY	0.1	0%	▼
254	PKC	GFSRKSHTFLP	0.0	0%	▼
245	p34cdc2	PEFPLSPPKKK	0.0	0%	▼
470	PKR	TRSKGTLRYMS	8.0	0%	▲
424	Cyclin dependent kinase 11	AAPASSSDPAA	0.2	0%	▼
767	cAMP-dependent protein kinase A	TKRNSSPPPSP	9.9	0%	▲
249	CaMK-I, PKA, S6K	LARRPSYRKIL	0.4	0%	▼
937	MAPK14	LQEVLSSDENG	3.4	0%	▲
745	CDC2	SATIVSPPPSS	0.4	0%	▼
736	c-Src	FFVIEYVNGGD	0.3	0%	▼
233	CK2, alpha 1	LSRHSSPHQSE	5.6	0%	▲
425	CK2 Kinase	GSDSSSESEPE	-26.3	0%	▼

387	Bruton's tyrosine kinase	HVEDLYVEGLP	0.1	0%	▼
661	ERK2	RTAPYTPNLPH	8.0	0%	▲
402	JNK1	EEIYLTPVQRP	0.4	0%	▼
852	c-Src	VLDDQYVSSVG	2.5	0%	▲
948	Sterol regulatory element binding transcription factor1	SMPAFSPGPGI	0.3	0%	▼
485	ERK1	KSEPSSPDHGS	4.5	0%	▲
989	MAP kinase	GTLFSTTPGGT	0.2	0%	▼
407	dsDNA-activated protein kinase	PEETQTQDQPM	6.5	0%	▲
237	Casein kinase II, beta	RVRASSDGEGT	2.9	0%	▲
936	Protein kinase A	IFRRPSLPAIS	2.8	0%	▲
958	PKC	DDGRVSYPLAF	7.9	0%	▲
434	PKA	EAKRSSADKGV	0.3	0%	▼
957	Protein kinase C	HLRSESQRQRR	0.3	0%	▼
997	Polo like kinase	LAQAFSDVILA	5.7	0%	▲
674	PAK5	DIKSDSILLTS	0.0	0%	▼
220	PKC	TGLYKSQRPAV	21.5	0%	▲
714	Lyn	FSGGLYGGLPP	1.9	0%	▲
722	PDK1	MVRTQTESSTP	0.2	0%	▼
988	ERK2	HTGFLTEYVAT	20.6	0%	▲
849	Glycogen synthase kinase 3 beta	DEYNVTPSPPG	3.0	0%	▲
635	PKC-beta	LKPGSSHRKTK	4.0	0%	▲
202	Fgr	IKDDEYNPAQG	0.1	0%	▼
700	CaM kinase II	GPRLVSNHSLH	2.4	0%	▲
634	PKA	KKKKPSRLKGD	0.4	0%	▼

706	Erbb2	SPQPEYVNQPD	0.2	0%	▼
335	SHC	LVNRHYAKISD	6.7	1%	▲
212	EGF receptor Lck	DGKEIYNTIRR	5.9	1%	▲
74	Fyn	VLDDEYVSSFG	95.2	1%	▲
110	CaMK2	VPSYDSFDSED	5.2	1%	▲
243	Protein kinase C	MQPDNSSDSDY	3.2	1%	▲
204	Auto	NGRPDYIIVTQ	7.0	1%	▲
372	Glycogen synthase kinase 3 beta	GHSNSSPRHSE	0.6	1%	▼
502	Calcium/Calmodulin dependent protein kinase II gamma	LQRYSSDPTGA	0.5	1%	▼
145	MAP kinase	PVVTATPSATA	10.3	1%	▲
49	ERK2	YVSILSPKEVS	0.2	1%	▼
1005	CK2, alpha 1	GSDSDSEVDKK	4.4	1%	▲
680	Cyclin dependent kinase 2	SPYSLSPVSNK	3.9	1%	▲
213	Serum/glucocorticoid regulated kinase AKT1	RPRSATWPLQR	0.2	1%	▼
917	ERK2	IEQWFTEDPGP	29.5	1%	▲
187	GSK3-beta	DSGIHSGATTT	0.2	1%	▼
697	Protein Kinase C	PAARASKKILL	-16.2	1%	▼
473	RK	GDDEASATVSK	0.4	1%	▼
256	CKII	GSEGDSESGEE	0.3	1%	▼
486	CaMKIV	LGRTQSAPLPQ	3.3	1%	▲
898	SYK	ADENYYKAQTH	5.9	1%	▲
733	Insulin receptor EGF receptor c-Src PTK III	RKMKDTDSEEE	-93.2	1%	▼
749	CK2-beta	HWQQQSYLDSG	0.1	1%	▼
940	CDC2	IVPGKSPTRKK	0.1	1%	▼
732	ERK2	EFTSRTPKDSP	0.2	1%	▼

355	ATK1	RKRRWSAPESR	6.1	1%	▲
561	ERK2	LSYLQSPITTS	1.8	1%	▲
696	MAPK4	TKWYRSPRLLL	0.0	1%	▼
171	CDC2	PTTPLSPTRLS	0.2	1%	▼
17	PKA Protein kinase C alpha	KERRRTESINS	1.8	1%	▲
137	ABL	IGEGTYGTVFK	0.1	1%	▼
766	PKC	GLRRSSKFALK	0.0	1%	▼
998	CK 2	SFGEPSYPEVF	0.3	1%	▼
913	ERK	NYIPETPPPGY	4.7	1%	▲
468	EGF receptor	LPVPEYINQSV	0.3	1%	▼
649	Src kinase	ITEEDYQALRT	1.6	1%	▲
925	CK2, alpha 1	SVSVETQGDDW	0.6	1%	▼
419	CDC2	MSETVPPAPAA	0.8	1%	▼
323	Csk Fgr PTPase	STEPQYQPGEN	0.1	1%	▼
789	PKA	SSRRTTLAGTL	2.1	1%	▲
984	EGF receptor	TDRSPYEKVSA	3.0	1%	▲
828	Lck Fyn	DNSSDSDYDLH	2.2	1%	▲
806	CDK7	EFISLSPPHEA	3.4	1%	▲
179	protein kinase CK2	IRYIESLQELL	-43.0	1%	▼
469	AKT1	RKRPATDDSST	2.4	1%	▲
464	EphB3	IDPFTYEDPNE	-2.2	1%	▼
380	Casein kinase II	LEVSDSESDEA	1.9	1%	▲
1018	PKA	LPRASSLNENV	28.7	1%	▲
260	TrkB tyrosine kinase	PRQLNYIQVEL	3.9	1%	▲
653	Janus kinase 2	HAQDTYLVLDK	0.1	1%	▼

715	IL-12 MAP2K6 STAT4	RGDKGYVPSVF	0.0	1%	▼
786	CK2-beta	AMFPETLDEGM	3.0	1%	▲
42	PKA PKG	VLAQPSTSRKR	2.4	1%	▲
52	c-Src	GIWKASFTTFT	3.3	1%	▲
138	SYK	FEGFSYVNPQF	17.9	1%	▲
664	FES kinase	GVDGDYEDAEL	0.4	1%	▼
375	MAPK10	DYDDMSPRRGP	5.1	1%	▲
1011	CK-II	QVSSLSESEES	3.2	1%	▲
702	PKA	ELEGISPDELK	0.6	1%	▼
975	Tyrosyl kinase	ENFDDYMKEVG	0.4	1%	▼
150	DNA-dependent protein kinase catalytic subunit	TLQTRTQEGSL	2.3	1%	▲
971	MAP2K3	SEMTGYVVTRW	52.2	1%	▲
172	pRB kinase	GNIYISPLKSP	5.6	2%	▲
188	CaMK-II	ASRMESTGVMG	3.1	2%	▲
459	Discoidin domain receptor	LSNPAYRLLLA	8.0	2%	▲
174	Rho kinase	AVRLRSSVPGV	0.0	2%	▼
206	Hepatocyte growth factor receptor	YDKEYYSVHNK	0.2	2%	▼
548	ERK2	IAVRKSRDKAK	15.2	2%	▲
962	Hepatocyte growth factor receptor	FIGEHYVHVNA	0.2	2%	▼
1012	CK1, CK2	TDGNRSSHSRL	3.3	2%	▲
325	ZAP70	MPDNLYTFVLK	0.1	2%	▼
1001	CDC2	LRQLRSPRRAQ	2.0	2%	▲
727	CK2	SSTSVTPDVSD	0.2	2%	▼
510	PKC	GTLRTSISVER	3.3	2%	▲
324	Protein kinase, cAMP dependent, regulatory, type II,	FTRRASVAAEA	2.4	2%	▲

	beta				
	PKR GCN2 Eukaryotic translation initiation factor 2-				
699	alpha kinase 3 dsRNA-PK CKII HCR dsI PKC	LLSELSRRRIR	3.2	2%	▲
704	CK2, alpha 1	SQSPHSPDSSQ	0.7	2%	▼
645	EGF receptor	PGLDEYNPFSD	6.2	2%	▲
724	EGF receptor	LRLQDYEEKTK	7.7	2%	▲
437	CDC2 Kinase	KVGVSSRINEW	0.1	2%	▼
560	PAK1	PGRPLSSYGMD	0.3	2%	▼
1013	CK2	SRVTFSEDDEI	0.3	2%	▼
636	Fibroblast growth factor 2	PALPQYPHING	2.8	2%	▲
659	PK161	KIGEGTYGVVY	5.3	2%	▲
834	c-Src	PKDEVYSKYYT	0.4	2%	▼
950	CaMK II	LEPQKSLGDEG	4.4	2%	▲
914	JNK1	APAAPTPAAPA	0.4	2%	▼
	Protein kinase C, zeta Protein kinase C alpha PKC Beta				
825	II Protein kinase C delta	PPRRSSIRNAH	2.4	2%	▲
461	EGF receptor	DGENIYIRHSS	-0.1	2%	▼
671	Tpl2	DYIPGTETHMA	2.7	2%	▲
765	PKA	RDTRDSEAQRL	6.3	2%	▲
316	Casein kinase II	DTLSDSDDEDD	0.6	2%	▼
241	PKA	SVRRASSADDI	1.7	2%	▲
650	JNK1	KDGWVYYANHT	2.0	2%	▲
509	PKA	ETKGKSFEEIA	0.6	2%	▼
772	c-Mer	IYSGDYYRQGR	3.3	2%	▲
639	Protein kinase C	GLQMGSNRGAS	0.5	2%	▼

	Protein kinase, cAMP dependent, regulatory, type I,				
363	alpha	WRRKSSDRKGG	1.6	2%	▲
484	Never in mitosis gene A-related kinase 2	YPFALSKSSMY	0.3	2%	▼
995	Nb	KFDTNSHNDDA	4.2	2%	▲
373	AKT1	RARSTSLNERP	2.1	2%	▲
564	3-Phosphoinositide dependent protein kinase 1	GDRTSTFAGTP	5.9	2%	▲
83	G protein coupled receptor kinase 6	IEQFSTVKGVE	-1.2	2%	▼
438	Casein kinase I	PVRAYSAEVVT	5.2	2%	▲
389	EGF receptor	ATLDVYNPFET	2.2	2%	▲
226	PDK1	GATMKTFAGTP	0.1	3%	▼
721	CDK2-cyclin A	SEGLPTPTKMT	3.6	3%	▲
423	c-Src	PTQPTSASPSL	12.1	3%	▲
317	JNK1	RSGLASPSYVA	3.2	3%	▲
450	c-Src	IEDNEYTARQG	-2.1	3%	▼
953	Protein Kinase C	DIQQLSSEEND	3.9	3%	▲
24	Janus kinase 1 c-Src	GSAAPYLKTKF	1.9	3%	▲
	Protein kinase C alpha PKM Protein kinase C, gamma				
426	Protein kinase C, beta-1	AKIQASFRGHM	-0.1	3%	▼
506	ATR	RPRVTSGGVSE	-11.7	3%	▼
139	BTK, ITK, Tec	TSLAQYDSNSK	7.4	3%	▲
1023	MAPK14 EphB2	PVAPLSPARLQ	2.3	3%	▲
930	PAK1	SQKYMSFTDKS	29.7	3%	▲
181	CK2	EEKQKSDAEED	-39.8	3%	▼
148	Lyn kinase, Bruton's tyrosine kinase	LGSQSYEDMRG	0.0	3%	▼
10	FGF receptor 1	HHIDYYKKTTN	3.3	3%	▲

500	SDK1	GARRSSWRVIS	0.4	3%	▼
678	EphB2	KTPKDSPGIPP	4.7	3%	▲
924	CK2	ESLDQSMEEEE	2.6	3%	▲
201	Csk	AQDEFYRSGWA	3.6	3%	▲
185	Protein kinase C	GTRRGSPLLIG	2.9	3%	▲
433	Protein kinase C	SDSRKSMRQST	0.0	3%	▼
155	Cyclin dependent kinase 5	HFTMRSPFKAD	1.8	3%	▲
836	Csk	TPSAAYLWVGT	3.1	3%	▲
778	EGF receptor Hepatocyte growth factor receptor	HVSISYDIPPT	3.7	3%	▲
547	CK-1 CK-2	ADERTSLMSAE	0.3	3%	▼
364	Phosphoinositide-3-kinase, catalytic subunit, gamma	TEDQYSLVEDD	1.8	3%	▲
565	CDK2	GVERSSPSKAP	0.1	3%	▼
396	Colony stimulating factor 1 receptor	QGVDTYVEMRP	0.0	3%	▼
369	Protein kinase A	RKRKNSRVTFS	2.2	3%	▲
532	Calcium-calmodulin dependent protein kinase I kinase	GSVLSTAAGTP	1.3	3%	▲
798	Protein kinase A	PFRRHSWIAFD	2.2	3%	▲
605	PKC	RLRPRTRKVKS	3.1	3%	▲
126	T cell acute lymphocytic leukemia 1	VKRRPSPYEME	0.4	3%	▼
666	p90rsk	KGGKYSVKDKE	0.5	3%	▼
31	c-Src	AGSQKYAYFNG	0.2	3%	▼
689	Protein kinase C Protein kinase A	RSNPPSRKGSG	-4.6	4%	▼
811	CDC2	SLYASSPGGVY	2.7	4%	▲
804	Cyclin dependent kinase 2	PKEKDSPHMQD	5.6	4%	▲
142	SYK	PSEEGYQDYEP	2.3	4%	▲
422	EphB2	TKALQSPKRPR	6.1	4%	▲

597	JNK2, MAPK14	IVADQTPTPTR	2.1	4%	▲
250	PKC	TRVPPSRRGPD	3.6	4%	▲
879	Akt	RSRSGSIVELI	1.8	4%	▲
929	Casein kinase 1, epsilon	ASSLSSLSSAE	0.1	4%	▼
168	cGKI-beta	GQTPSSSSIPS	6.5	4%	▲
686	CDK5/p35	ENTFPSPKAIP	2.7	4%	▲
175	Nucleoside diphosphate kinase A	NIIHGSDSVES	8.5	4%	▲
1021	PKA	REKKFSTKSDV	2.2	4%	▲
922	IKK beta	APAYLSSPLAL	0.4	4%	▼
675	Aurora kinase A	EVPRRSGLSAG	7.7	4%	▲
508	CKII	NIVLLSAEEKK	2.7	4%	▲
50	PKA	LDRFLSLEPVK	2.5	4%	▲
524	BCR	EPAHAYAQPQT	0.4	4%	▼
949	AKT1	RERKSSAPSHS	0.6	4%	▼
412	Casein kinase II	DHIEVSDDEDE	0.4	4%	▼
210	Ca2+Calmodulin dependent protein kinase	IRRASTIEMPQ	0.5	4%	▼
632	Ribosomal S6 kinase 1	RRGSDSSEDIY	2.7	4%	▲
593	GSK3-beta	VDAAVTPEERH	19.0	4%	▲
258	FMS-related tyrosine kinase 3	DNEYFYVDFRE	3.3	4%	▲
946	PKC	EKRKNSILNPI	5.1	5%	▲
112	PKR	GFTRKSVRKAQ	3.6	5%	▲
996	Nek9	TTAAHSLVGTP	0.0	5%	▼
1002	MAT1	PLNSVSPSPLM	1.7	5%	▲
1007	PKG	RKRHNSISEAK	11.2	5%	▲
1024	CK2, alpha 1	ARVLGSEGEEE	0.6	5%	▼

131	Ron	ILDREYYSVQQ	3.4	5%	▲
414	DNA-dependent protein kinase catalytic subunit	GAGGYTQSPGG	46.1	5%	▲
197	JNK MAP kinase	GVITTTPTPPG	0.5	5%	▼
194	KIT	INGNNYVYIDP	0.7	5%	▼
130	Focal adhesion kinase 2	IESDIYAEIPD	2.3	5%	▲
399	FAK	KTNLSYYEYDK	0.1	5%	▼
321	FER, Fyn	VNLINYQDDAE	0.2	5%	▼
541	Rho-kinase	QQREKTRWLNS	3.1	5%	▲
693	AKT1	RSRDPSLMVDF	0.3	5%	▼
576	ck2	VEDNRSQVETD	0.4	5%	▼
969	Lck Csk	EANSHYGHNDD	0.2	5%	▼
610	CDC2	DPWGGSPAKPS	13.5	5%	▲
315	beta II PKC	LSPSPSSRVTV	0.7	5%	▼
62	PKA	YQRRASDDGKL	0.5	5%	▼
944	Cyclin D1 Cdk4	RPPTLSPIPHI	7.1	5%	▲
270	Protein tyrosine kinase 6	IKEDVYLSHDH	3.7	5%	▲