UNIVERSIDADE ESTADUAL DE CAMPINAS

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Avaliação de Citotoxicidade e Indução de Diferenciação e Apoptose em Células de Leucemia (HL60) pela Dimetilamida-crotonina

Dissertação apresentada ao Instituto de Biologia para obtenção do título de Mestre em Biologia Funcional e Molecular na área de Bioquímica.

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Dedico este trabalho,

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"A ciência é uma aventura de toda a raça humana para aprender a viver e talvez a amar o universo onde se encontra. Ser uma parte dele é compreender, é conhecer-se a si próprio, é começar a sentir que existe dentro do homem uma capacidade muito superior à que ele pensava ter e uma quantidade infinita de possibilidades humanas."
Isaac I. Rabi.

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ABREVIATURAS

- Apaf-1 – fator de ativação de protease apoptótica – 1
- Bax – proteína x associada ao Bcl-2
- Bcl-2 – célula B de linfoma 2 (B-cell lymphoma 2)
- Bcl-x – isoforma x de Bcl-2
- Bcl-x_L – forma longa de Bcl-x
- Bid – domínio de morte de interação com BH-3
- BH-3 – domínio-3 homólogo ao Bcl-2
- BrdU - bromodesoxiuridina
- CAT – catalase
- CD95 – receptor de morte celular (cell death), também chamado Fas ou Apo-1
- CD95L – ligante do receptor de morte celular CD95 (Faz ou Apo-1), também chamado FasL
- c-FLIP – proteína inibidora de FLICE
- ConA – concanavalina A
- dATP – 2'-desoxiadenosina 5'-trifosfato
- DHC – desidrocrotonina
- DCR – dimetilamida-crotonina
- DMSO – dimetilsulfóxido
- DPA - difenilamina
- DTNB - ácido 5,5'-ditio-bis-2-nitrobenzóico
- EDTA - ácido tetracético etilenodiamina dissódio
- EROs - espécies reativas de oxigênio
- FAB – grupo cooperativo French-American-British
- FADD – domínio de morte (death domain) associado ao Fas
- Fas – receptor Fas ou CD95 ou Apo 1
- FLICE – ICE tipo-FADD (também chamada caspase-8)
- GPX – glutationa peroxidase
- GSH - glutationa reduzida
- Hepes –(N-[2-hidroxietil] piperazina-N' –[2- ácido etanessulfônico])
- HL60 – linhagem de células da leucemia promielocítica humana

- IAP – proteínas inibidoras de apoptose
- ICE – enzima de conversão da interleucina – 1b (também chamada caspase-1)
- LMA – leucemia mielóide aguda
- LPA-leucemia promielocítica aguda
- MTT - [brometo de (3-(4,5-dimetiltiazol-2yl)-2,5-difenil tetrazólio)]
- NBD – nitro azul diformazan
- NBT – nitro azul tetrazólio
- PBMCs - linfócitos mononucleares humanos
- PHA - fitohemaglutinina
- PBS – solução tampão fosfato
- PBS-A – solução tampão fosfato sem cálcio
- PKC – proteína quinase C
- pNPP - p-nitrofenilfosfato
- Reagente A - Carbonato de Sódio (Na_2CO_3) 2% em NaOH 0,1M
- Reagente B - $CuSO_4.5H_2O$ 0,5% em tartarato de sódio 1%
- RPMI - Roswell Park Memorial Institute
- SFB - foro fetal bovino
- SOD - superóxido dismutase
- TBA – ácido tiobarbitúrico
- TBARS – substâncias reativas ao TBA
- TCA – ácido tricloro acético
- Tampão TE: EDTA + triton X-100
- TNF – fator de necrose tumoral
- TNF-R1 – receptor 1 fator de necrose tumoral
- TPA - acetato de tetradecanoilforbol
- TRX - tiorredoxina
- V79 – linhagem de células de fibroblastos de pulmão de hamster chinês

RESUMO

Atualmente, morte celular por apoptose é objeto de intensa pesquisa devido à susceptibilidade de células tumorais, incluindo linhagens de células de leucemia e de linfoma, a sofrerem este tipo de morte celular em resposta a determinados agentes anti-tumorais. O estresse oxidativo induzido, entre outros fatores, pela diminuição dos níveis de glutationa intracelular, está envolvido no mecanismo de ação de vários compostos, sinalizando as células ao processo apoptótico, envolvendo eventos mediados por cisteíno proteases (caspases). Neste estudo, nós examinamos a indução de diferenciação morfológica e morte celular por apoptose pela dimetilamida-crotonina (DCR) em células da leucemia promielocítica humana HL60. A DCR é um derivado sintético da desidrocrotonina (DHC), uma diterpeno lactona extraída das cascas de *Croton cajucara*, uma planta da região amazônica. A DCR apresentou um efeito inibitório similar ao composto original conforme avaliado por diferentes parâmetros de citotoxicidade e ambos os compostos induziram diferenciação fenotípica em células HL60. Em contraste, não foram observadas citotoxicidade ou alterações morfológicas associadas com apoptose em linfócitos de sangue periférico humano após tratamento com os compostos em estudo nas concentrações utilizadas (0 - 400μM). Com base nas alterações morfológicas, no padrão de fragmentação de DNA e na análise de simetria de membrana de células marcadas com anexina V/iodeto de propídeo por citometria de fluxo, DHC e DCR induziram apoptose em células HL60 com a mesma eficiência. Ambos os compostos promoveram a ativação de caspases–2, –6 e –9. A ativação de caspase-9 pela DHC e DCR em células HL60 sugere indução de apoptose através da via mitocondrial. Com o intuito de analisar o possível envolvimento do estresse oxidativo no mecanismo de toxicidade dos compostos, foram determinados os níveis de glutationa e ocorrência de peroxidação lipídica. O nível de GSH diminuiu cerca de 30% e a

produção de substâncias reativas ao ácido tiobarbitúrico (TBARS) aumentou 4 vezes após o tratamento com ambos os compostos em concentrações próximas ao IC_{50} (250 μM). A suplementação do meio de cultura com 15 mM de GSH protegeu as células da toxicidade induzida pelos compostos em estudo. Nossos resultados indicam que a DCR e a DHC induzem apoptose em células HL60 em parte pela conjugação com GSH e indução de estresse oxidativo, o qual seria responsável pela peroxidação lipídica e ativação da cascata de caspases através da via mitocondrial. Além disso, resultados de espectroscopia UV/Vis sugerem a formação de conjugados entre a GSH e o grupamento $O{=}CH{-}CH{=}CH_2$, presente na DHC e na DCR. Desta forma, os compostos estudados possuem características almejadas para quimioterápicos desencadeando indução de diferenciação morfológica e de apoptose em células da leucemia HL60.

ABSTRACT

Apoptosis is subject of intense research due to the susceptibility of tumor cells, including leukemia and lymphom cell lines that have been found to undergo this kind of cell death in response to antitumor agents. The oxidative stress induced, by others factors, by the decrease of glutathione levels is involved in the action mechanism of several compounds, signalling the cells to apoptotic process, involving events mediated by cystein proteases (caspases). In this study, we evaluated the morphological differentiation and cell death by apoptosis induction by dimethylamide-crotonin (DCR) in human HL60 promylocitic leukemia cells. The DCR is a synthetic derivative of dehydrocrotonin (DHC), a diterpene lactone isolated from the *Croton cajucara*, an Amazonian plant. The dimethylamide-crotonin presented a similar inhibitory effect that its parent compound evaluated by different endpoints of cytotoxicity and both compounds induced morphological differentiation on HL60 cells. In contrast, no cytotoxicity or morphological alterations associated with apoptosis were observed in human peripheral blood mononuclear cells (PBMC) after treatment with these studied compounds in the concentrations used (0-400 μM). Based on morphological changes and the pattern of DNA fragmentation and the symmetry analysis of cell membrane labelled with annexin V/propidium iodide by flow cytometry, DHC and DCR induced apoptosis on HL60 cells with similar efficiency. Both compounds were effective in triggering the activation of caspase-2, -6 and –9. The caspase-9 activation by DHC and DCR on leukemic cells suggests apoptosis induction by the mitochondrial pathway. In attempt to analyse the possible involvement of oxidative stress on the toxicity action mechanism of these compounds, we determined the GSH levels and lipid peroxidation occurrence. The glutathione levels decreased around 30% and the thiobarbituric acid substance reactive (TBARS) increased 4 times after treatment with both

compounds in concentrations around the IC50 value (250 μM). The culture medium supplementation with 15-mM GSH protected the cells from the toxicity induced by both compounds. The results indicate that DHC and DCR induce apoptosis on HL60 cells in part by conjugation with GSH and oxidative stress induction, which could be responsible for lipid peroxidation and caspases activation by the mitochondrial pathway. Moreover, the spectroscopic analyses of DHC or DCR in the presence of GSH 15 mM suggest the conjugate formation between GSH and O=CH-CH=CH_2 of DHC or DCR. In this way, the studied compounds have the desirable chemotherapic characteristics, triggering cell differentiation and apoptosis.

I - INTRODUÇÃO

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1. Desidrocrotonina e Dimetilamida-Crotonina

A espécie *Croton cajucara* Benth (família Euphorbiaceae), é uma planta nativa e endêmica da região Amazônica, ao norte do Brasil, onde é popularmente conhecida como "sacaca" e muito utilizada na medicina popular como infusões da casca e das folhas no tratamento de diversas doenças. Dentre essas destacam-se distúrbios hepáticos e renais, diabetes, diarréia, gastralgia, febre, icterícia, hepatite viral e malária (Van Den Bergh, 1982; Martins, 1989; Di Stasi *et al.*, 1994). Porém, evidências de ocorrência de hepatite tóxica também foram relatadas, principalmente no Estado do Pará, onde seu consumo é mais intenso (Maciel, 1997; Maciel *et al.*, 1998b; Maciel *et al.*, 2000).

Estudos anteriores revelaram a presença de esteróides, flavonóides e diterpenos tipo clerodane nas folhas e cascas de *Croton cajucara* (Maciel *et al.*, 1995, 1998a, 2000; Simões *et al.*, 1979; Itokawa *et al.*, 1989, 1990; Kubo *et al.*, 1991; Ichihara *et al.*, 1992), com atividades biológicas diversas, tais como antibiótica, anti-tumoral (Mc Chesney e Clarck, 1991; Siddiqui *et al.*, 1992; Hagiwara *et al.*, 1995), anti-inflamatória (Kitazawa *et al.*, 1979, 1980; Ichihara *et al.*, 1992; Carvalho *et al.*, 1996; Perazzo *et al.*, 1997; Maciel *et al.*, 2000).

Dentre os compostos encontrados em espécies de plantas do gênero *Croton*, a desidrocrotonina (DHC) (**figura 1**) é o nor-diterpeno tipo clerodane presente em maior quantidade no extrato das cascas de *Croton cajucara,* possuindo atividades hipoglicemiante (Farias *et al.*, 1997), anti-ulcerogênica (Souza-Brito *et al.*, 1998; Hiruma-Lima *et al.*, 1999, 2002), antiestrogênica (Costa *et al.*, 1999) e anti-tumoral (Grynberg *et al.*, 1999) comprovadas. Estudos recentes indicaram atividade anti-tumoral *in vivo* e *in vitro* nos dois modelos experimentais do Sarcoma 180 (S180) e do tumor ascítico de Ehrlich. Segundo Grynberg e colaboradores (1999), tanto a DHC quanto a crotonina (**figura 1**), presentes na

C. cajucara, induzem apoptose *in vitro* e produção de fator de necrose tumoral-α (TNF-α) em cultura de células do tumor de Ehrlich.

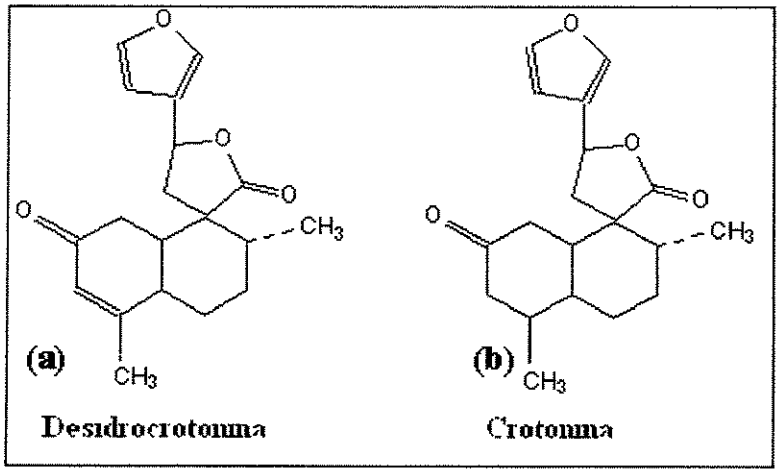

figura 1: Estrutura Química da Desidrocrotonina (a) e Crotonina (b).

Há cerca de três décadas várias observações sugeriram que sesquiterpeno-lactonas apresentam efeitos inibitórios contra vários tipos de câncer (Rodriguez *et al.*, 1976; Grynberg *et al.*, 1999; Cho *et al.*, 2000). Além disso, outros diterpenos isolados de *Cistus incanus e Laetia corymbulosa* também apresentaram efeito citotóxico em várias linhagens de células tumorais. Segundo Dimas e colaboradores (1999), tal efeito deve-se a interação entre os diterpenos e macromoléculas.

A estrutura da DHC contém três grupos funcionais altamente reativos, responsáveis tanto pela citotoxicidade da molécula quanto pela atividade biológica, uma α-metileno-γ-lactona, um sistema ciclopentanona α-β-insaturada e um grupamento O=C–C=CH_2, sendo este último um centro de alquilação essencial (Giordano *et al.*, 1992). Segundo Kupchan e colaboradores (1970), o sistema α-metileno-γ-lactona participa de uma reação do tipo Michael com nucleófilos biológicos e enzimas chaves em processos de proliferação e diferenciação celular. Neste sentido, Freire e colaboradores (2003a) demonstraram que a

DHC apresenta efeitos citotóxicos em células da leucemia mielóide humana HL60, com possível envolvimento de estresse oxidativo.

Em estudos anteriores foram avaliados os efeitos toxicológicos da DHC *in vitro e in vivo*. A observação de citotoxicidade dependente da atividade do citocromo P450 em cultura de hepatócitos sugeriu que a biotransformação hepática produz compostos mais tóxicos. Em adição, verificou-se em experimentos de toxicidade sub-crônica alterações hepáticas em ratos após o tratamento com a DHC. Dentre estas incluem-se o aumento significativo no peso do fígado, alterações histopatológicas como tumefação turva, degeneração microvascular, alterações nucleares e aumento da atividade da enzima gama-glutamil transpeptidase. Por outro lado, a avaliação dos efeitos tóxicos da DHC em fibroblastos V79 demonstrou uma citotoxicidade do tipo basal (inespecífica). Portanto, apesar da atividade anti-ulcerogênica comprovada, os resultados acima sugerem que o uso da DHC por períodos prolongados pode induzir a lesões hepáticas, representando um efeito tóxico seletivo (Rodriguez & Haun, 1999; Melo, 2000).

Com o intuito de melhorar as atividades biológicas da DHC e reduzir sua toxicidade foram sintetizados três derivados deste composto dos quais a dimetilamida-crotonina (DCR) (**figura 2**) apresentou atividade anti-ulcerogênica significativa em todos os modelos de úlcera gástrica (Melo, 2000; Melo *et al.*, 2003), e reduzida citotoxicidade em células V79 (Melo *et al.*, 2001) e em hepatócitos de ratos *in vitro* (Melo *et al.*, 2002).

2. Citotoxicidade

Toxicidade é um evento complexo que apresenta um amplo espectro de efeitos, desde simples morte celular até aberrações metabólicas complexas. A neuro, hepato e/ou nefrotoxicidade são exemplos de alterações funcionais e não necessariamente de morte

celular. Por isso, os ensaios de citotoxicidade *in vitro* devem abranger vários parâmetros que avaliem alvos diferentes (Roguet *et al.*, 1993; Melo *et al.*, 2000). Estudos *in vitro* fornecem importantes ferramentas para ampliar os conhecimentos sobre os efeitos citotóxicos causados por agentes químicos e para estimar estes efeitos em humanos (Eisenbrand *et al.*, 2002).

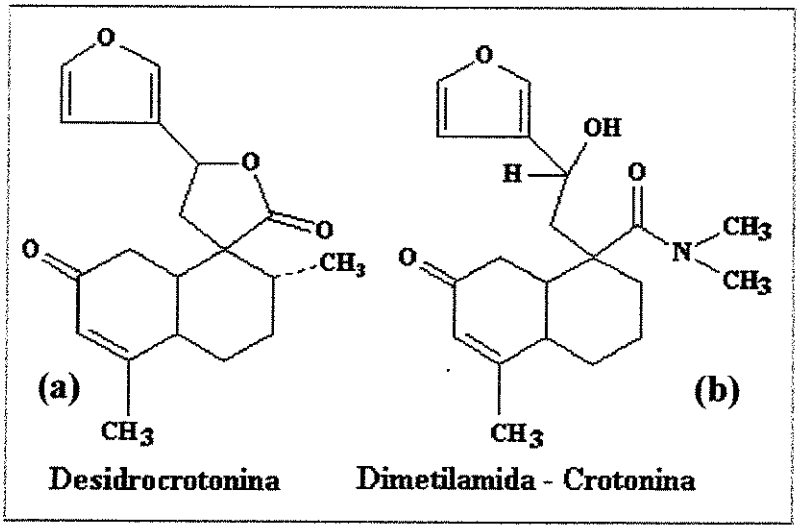

figura 2: Estrutura Química da Desidrocrotonina (a) e Dimetilamida - Crotonina (b).

Muitos dos compostos apresentam dificuldades de utilização em tratamentos terapêuticos pela instabilidade em sistemas biológicos *in vivo* ou pela necessidade de alta dosagem. Outros exibem sérios efeitos adversos em células normais (Honma *et al.*, 1998). Além disso, conforme descrito por Freshney (1994), a toxicidade é um fator limitante na liberação e consumo de fármacos e portanto, a análise de toxicidade *versus* atividade biológica de um composto é fundamental para determinar sua aplicação estabelecendo-se o índice terapêutico. Por motivos éticos e financeiros, em se tratando de utilização de animais para estudos toxicológicos, considera-se mais vantajoso o estudo *in vitro* de toxicidade (Melo *et al*, 2001). A possibilidade de trabalhar com linhagens estabelecidas solucionou o

problema da reprodutibilidade dos experimentos, uma vez que se pode utilizar uma mesma população celular por um longo período de tempo. Assim, além de resolver os problemas éticos e técnicos, os ensaios em culturas de células são ainda vantajosos pela rapidez de obtenção de resultados, pela reduzida quantidade de composto teste utilizado e pelo custo financeiro. Assim, uma grande variedade de testes empregando culturas celulares foi descrito nos últimos anos (Melo *et al.*, 2000).

Os testes de citotoxicidade *in vitro* permitem a determinação de citotoxicidade basal, assim como o estabelecimento de uma faixa de concentração biologicamente ativa para um determinado agente (Seibert *et al.*, 1996; Eisenbrand *et al.*, 2002). Nos fenômenos associados à citotoxicidade basal todos os tipos celulares apresentam uma sensibilidade similar e os valores de IC_{50} obtidos nos ensaios de citotoxicidade, avaliando diferentes alvos celulares, geralmente se encontram próximos (Clemedson *et al.*, 1996; 2002). Alguns testes, como por exemplo, a análise do conteúdo de proteína, a exclusão de azul de tripan, a redução do sal de tetrazólio MTT ([brometo de (3-(4,5-dimetiltiazol-2yl)-2,5-difenil tetrazólio)]) e a atividade fosfatásica adquiriram considerável atenção como indicativos de citotoxicidade. Esses ensaios fornecem informações sobre diferentes funções ou compartimentos celulares (Renzi *et al.*, 1993).

O MTT revela o correto funcionamento do sistema enzimático mitocondrial, principalmente a atividade de desidrogenases (Mosmann *et al.*, 1983; Denizot & Lang, 1986). No entanto, este ensaio, segundo alguns autores, avalia predominantemente o balanço redox $NAD(P)^+/NAD(P)H$ (Melo *et al.*, 2002). Portanto, compostos que interferem neste equilíbrio tais como agentes oxidantes ou redutores podem participar da ativação ou inibição da redução do MTT.

A exclusão do corante azul de tripan, juntamente com observações morfológicas, permite avaliar a integridade estrutural da membrana plasmática celular. Este ensaio mede a habilidade das células viáveis, com membrana plasmática intacta, em excluir o corante, permitindo a quantificação do número de células viáveis (Loveland *et al.*, 1992; Renzi *et al.*, 1993).

O conteúdo fornece uma estimativa do número total de células baseado na detrminação do conteúdo total dessas macromoléculas celulares (Hartree, 1972; Forsby *et al.*, 1991; Haun *et al.*, 1992). Finalmente, um outro parâmetro de citotoxicidade utilizado é a determinação da atividade de fosfatases, hidrolases que utilizam como substrato monoésteres e participam de diversos eventos de fosforilação envolvidos em mecanismos de transdução de sinais de controle do crescimento e diferenciação celular (De Paoli *et al.*, 1994; Uzunoglu *et al.*, 1999). Portanto a determinação da atividade fosfatásica constitui um importante aspecto de avaliação de viabilidade celular em ensaios de citotoxicidade, segundo demonstram recentes trabalhos (Aoyama *et al.*, 2000; Freire *et al.*, 2003a).

A combinação de agentes citotóxicos que induzem diferenciação celular e apoptose podem modificar a história da quimioterapia nos próximos anos, desenvolvendo tratamentos mais tóxicos para as células tumorais e mais eficientes, projetados em função das alterações biológicas dessas células.

3. Morte Celular por Apoptose

A morte celular por apoptose foi reconhecida morfologicamente como um fenômeno distinto de morte há mais de 20 anos por Kerr e colaboradores (1972). Este processo de morte celular possui um papel essencial na manutenção da homeostase tecidual e é importante em certas condições patológicas, incluindo câncer. O interesse de

pesquisadores por este tema aumentou substancialmente na última década com a descoberta dos genes envolvidos no controle da apoptose (Kerr, 1995; 2002), alterando conceitos em todos os aspectos da biologia celular, incluindo patologia, imunologia, biologia do desenvolvimento, carcinogênese, farmacologia e toxicologia. Após o reconhecimento do processo apoptótico como um mecanismo celular fundamental, a biologia da apoptose continuou a ser investigada avaliando-se as alterações morfológicas e bioquímicas características (Wyllie, 1985), a natureza das vias intracelulares (Hale *et al.*, 1996), a complexa biologia molecular de genes e elementos efetores (Baker & Reddy, 1996; Fraser & Evan, 1996), a sua relação no desenvolvimento embrionário (Brill *et al.*, 1999), o seu papel na homeostase celular (Raff, 1992), e o seu envolvimento na patogênese de doenças (Thompson, 1995), incluindo o câncer (Martin & Green, 1995; Green & Martin, 1995; Lee & Bernstein, 1995; Raffray & Cohen, 1997; Johnstone *et al.*, 2002; Makin, 2002; Reed, 2003). Com o surgimento de novos conhecimentos na biologia do câncer e consequentemente, na indução química da apoptose, os tratamentos tornam-se mais eficazes, pois terapias utilizando drogas anti-tumorais convencionais são pouco seletivas e efetivas (Hickman, 1996).

As mutações em genes que regulam o crescimento de células somáticas em vertebrados dirigem o desenvolvimento do câncer. Os processos governantes da gênese e progressão de câncer são evolucionários, nos quais a seleção natural atua sobre a diversidade adquirida e inerente de vários clones somáticos, prevalecendo as formas que apresentam vantagens propagativas. Processos de proliferação celular desregulada e morte celular por apoptose suprimida constituem suporte para a progressão neoplásica, comum a todos os tipos de câncer. Estes dois defeitos celulares devem ser explorados terapeuticamente (Bertram, 2001; Evan & Vousden, 2001).

O padrão de alterações morfológicas e bioquímicas celulares associadas com a programação normal de morte celular e certos processos patológicos *in vivo* inclui a formação de vacúolos citoplasmáticos, encolhimento e diminuição do contato entre células vizinhas, fragmentação da membrana nuclear e condensação cromatínica (Wyllie *et al.*, 1980; McConkey, 1998), despolarização de membrana mitocondrial, fragmentação internucleossomal do DNA e alterações na assimetria de fosfolipídeos de membrana plasmática (Curtin *et al.*, 2002). Quando a morte celular apresenta todas as características morfológicas e bioquímicas de apoptose, mas foi induzida por um determinado composto ou por um estímulo físico, não constitui um processo programado e sim uma resposta celular às mudanças ambientais (Maria, 1998). Por outro lado, a morte celular por necrose ocorre, geralmente, em resposta à injúria severa às células e é caracterizada morfologicamente por inchaço citoplasmático e mitocondrial, ruptura da membrana plasmática e liberação do conteúdo extracelular. Consequentemente, ocorre a geração de uma resposta inflamatória, que pode causar injúria e até morte de células vizinhas (**figura 3**) (Curtin, *et al.*, 2002).

O processo apoptótico envolve a participação ativa das células afetadas na cascata de autodestruição que culmina em degradação do DNA via ativação de endonucleases, desintegração nuclear e formação de "corpos apoptóticos" (Wyllie *et al.*, 1980; Wyllie, 1985; Arends *et al.*, 1990). Estes corpos apoptóticos são rapidamente retirados do tecido por macrófagos (Wyllie, 1985; Compton, 1992). A maioria das alterações morfológicas observadas por Kerr e colaboradores (1972), é causada por uma série de cisteíno proteases, chamadas caspases, que são ativadas especificamente em células em apoptose (Cohen, 1997; Minko *et al.*, 2001). Estas enzimas possuem um resíduo de cisteína no sítio ativo e clivam substratos que possuem resíduos de ácido aspártico em sequências específicas. A

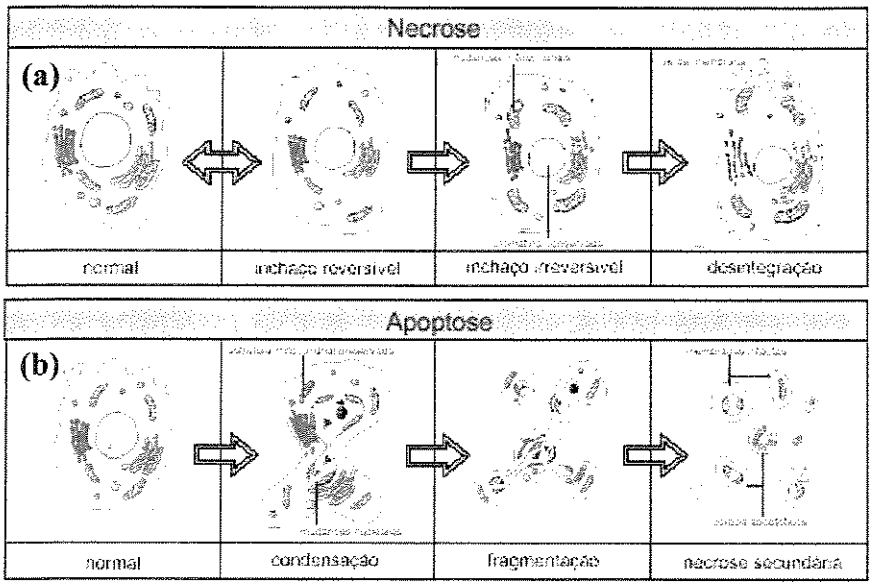

figura 3. Distinção das diferentes alterações morfológicas nos processos de morte celular por apoptose *versus* necrose. A necrose **(a)** é caracterizada pela perda de integridade de membrana plasmática, floculação da cromatina, inchaço seguido de lise celular com extravasamento do conteúdo intracelular e desintegração de organelas. O processo apoptótico **(b)** envolve alteração de permeabilidade de membranas, condensação cromatínica, encolhimento celular, formação de corpos apoptóticos sem desintegração de organelas.

especificidade pelos seus respectivos substratos é determinada por quatro resíduos amino-terminal no sítio de clivagem (Jacobson & Evan, 1994; Stennicke & Salvesen, 1998; Thornberry & Lazebnik, 1998; Hengartner, 2000). Evidências atuais sugerem que há várias rotas distintas para a ativação de caspases, dependendo do estímulo que desencadeia a maquinaria de morte (**figura 4**) (Slee *et al.*, 1999a,b; Hengartner, 2000).

Em geral, duas vias distintas podem estar ativas. A apoptose iniciada via receptores de morte (**figura 4(A)**) tais como Fas, também chamado de CD95 ou Apo-1 e TNF-R1 (receptor fator de necrose tumoral), que requerem pró-caspase-8 ou –10 no complexo. Porém, estímulos apoptóticos induzidos por agentes quimioterapêuticos em várias

linhagens de leucemia parecem ser independentes desta via (**figura 5**) (Eischen *et al.*, 1997; Debatin, 2000). Neste caso, a via mitocondrial (**figura 4(B) e 5**) é ativada predominantemente, com envolvimento de alterações de permeabilidade de membrana mitocondrial e liberação do citocromo c para o citosol, que se liga a dATP, Apaf-1 e pró-caspase-9, formando o complexo apoptossomo. A caspase-9 ativa (iniciadora) pode então

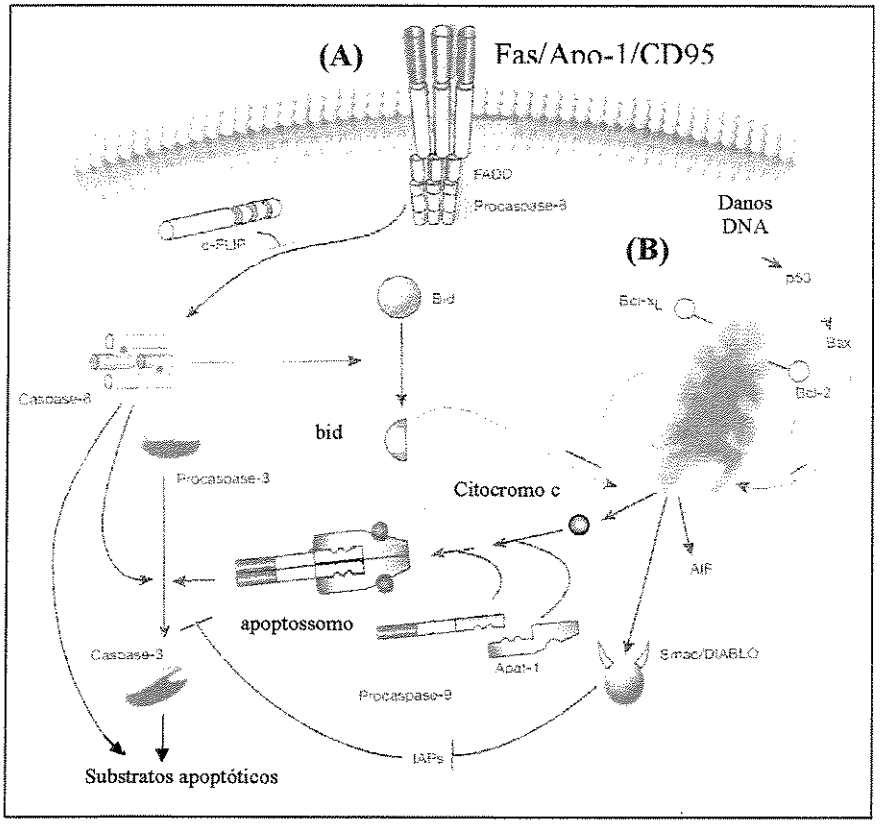

Figura 4. A via receptor **(A)** é desencadeada por membros da super-família de receptores de morte celular (tal como CD95/Fas/Apo-1). Ligantes específicos sinalizam agregação e formação de um complexo indutor de morte, que recruta pró-caspases através de proteínas de domínio de morte associada ao receptor. A via mitocondrial **(B)** é frequentemente ativada em resposta a danos no DNA, envolvendo a ativação de um membro pró-apoptótico da família Bcl-2 (Bax, Bid). Membros pró- e anti-apoptótico da família Bcl-2 regulam a liberação de citocromo c a partir da membrana mitocondrial interna. Este associa-se com Apaf-1, dATP e pró-caspase-9, formando apoptossomo. Caspases subsequentes são ativadas, culminando na clivagem de substratos específicos e morte celular por apoptose.

clivar as caspases efetoras subsequentes (-2, -3, -6, -7, -8, -9, e –10). Portanto, a ativação da caspase-9 mediada pelo citocromo c serve como um mecanismo de amplificação de sinais durante o processo apoptótico (Li *et al.*, 1997; Green and Reed, 1998; Slee *et al.*, 1999a,b; Desagher and Martinou, 2000; Kuida, 2000; Herr & Debatin, 2001).

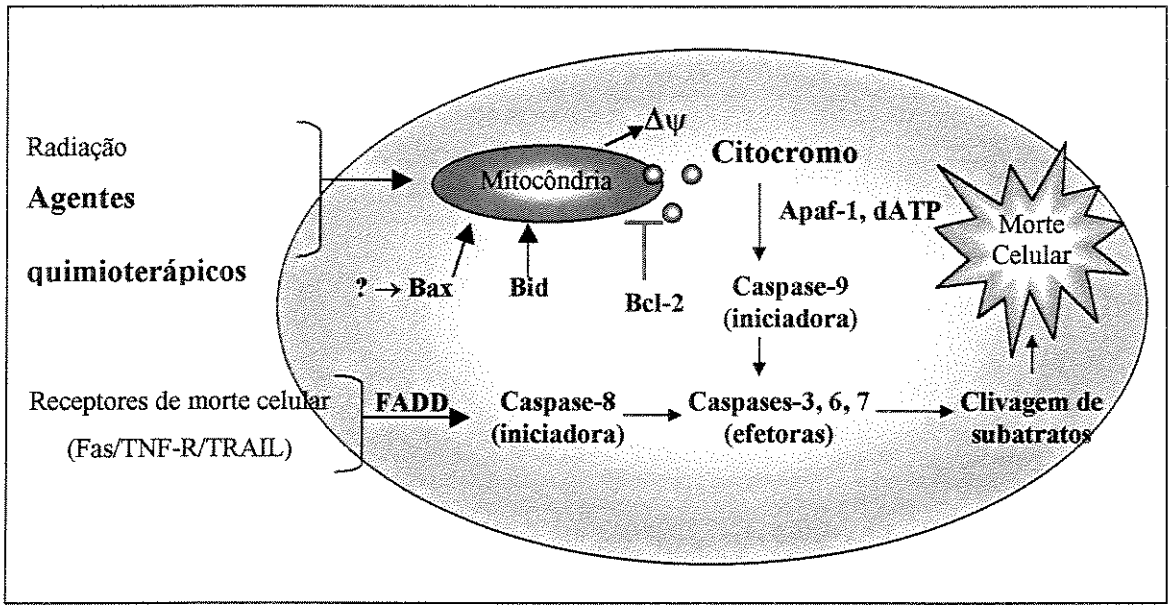

Figura 5. Estímulos apoptóticos induzidos por agentes quimioterápicos podem ser independentes da via receptor de morte celular. Neste caso, a via mitocondrial é ativada com envolvimento de alterações de permeabilidade de membrana mitocondrial e liberação do citocromo c para o citosol, que se liga a dATP, Apaf-1 e pró-caspase-9, formando o complexo apoptossomo. A caspase-9 ativa (iniciadora) pode então clivar as caspases efetoras subsequentes.

Uma grande variedade de estímulos pode induzir apoptose, dentre esses o estresse oxidativo provocado pela geração de intermediários oxidativos através da ação de alguns agentes anti-neoplásicos (Matés & Sanchez-Jimenez, 2000; Piwocka *et al.*, 2001). O mecanismo de indução pode ser via exposição a peróxido de hidrogênio (Ikeda *et al.*, 1999; Matsura *et al.*, 1999), ciclização-redox de quinonas ou agentes tiol-alquilantes (Slater *et al.*, 1995). Uma enorme quantidade de dados suportam o papel do estresse oxidativo no desencadeamento de apoptose (McConkey, 1998), sendo que as vias apoptóticas clássicas

envolvem um acúmulo moderado de espécies reativas de oxigênio. Assim como o cálcio, o estresse oxidativo pode inibir ou promover apoptose e até necrose, dependendo da intensidade do estímulo (Fernandez & Cotter, 1994).

O estado redox das células é uma conseqüência do balanço entre os níveis de espécies reativas de oxigênio (EROs) oxidantes e redutoras equivalentes. Elevação nos níveis de EROs acima da capacidade de tamponamento e atividade enzimática designada para modular os níveis dessas espécies resulta em estresse oxidativo potencialmente citotóxico. Sob esta condição pró-oxidante, radicais altamente reativos podem danificar o DNA, RNA, proteínas e componentes lipídicos, que podem levar a morte celular. Nesta situação de estresse oxidativo as células apresentam dois mecanismos de defesa importantes: -um tampão redutor tiol consistindo de pequenos peptídeos com moléculas sulfidrila redox-ativas: glutationa (GSH) e tiorredoxina (TRX)) e, -um sistema enzimático (superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidade (GPX)) (**figura 6**) (Yu, 1994; Gabbita *et al.*, 2000; Matés, 2000; Davis *et al.*, 2001; Curtin *et al.*, 2002).

A GSH (**figura 7**) é o mais abundante composto tiol de baixo peso molecular encontrado em plantas e animais (Meister & Anderson, 1984; Sies, 1999), em concentrações variando entre 0,1 – 10 mM (Schroeder *et al.*, 1996; Davis *et al.*, 2001). Este tripeptídeo apresenta diversas funções celulares em adição à suas propriedades antioxidantes participando na transdução de sinal, na expressão gênica e na apoptose (Larsson *et al.*, 1983; Vina, 1990; Cotgreave & Gerdes, 1998; Arrigo *et al.*, 1999; Sies, 1999; Voehringer, 1999; Davis *et al.*, 2001). Estes processos estão interrelacionados com o estado redox tiol, interações proteína-glutationa e proliferação celular (Cotgreave & Gerdes, 1998; Sies, 1999).

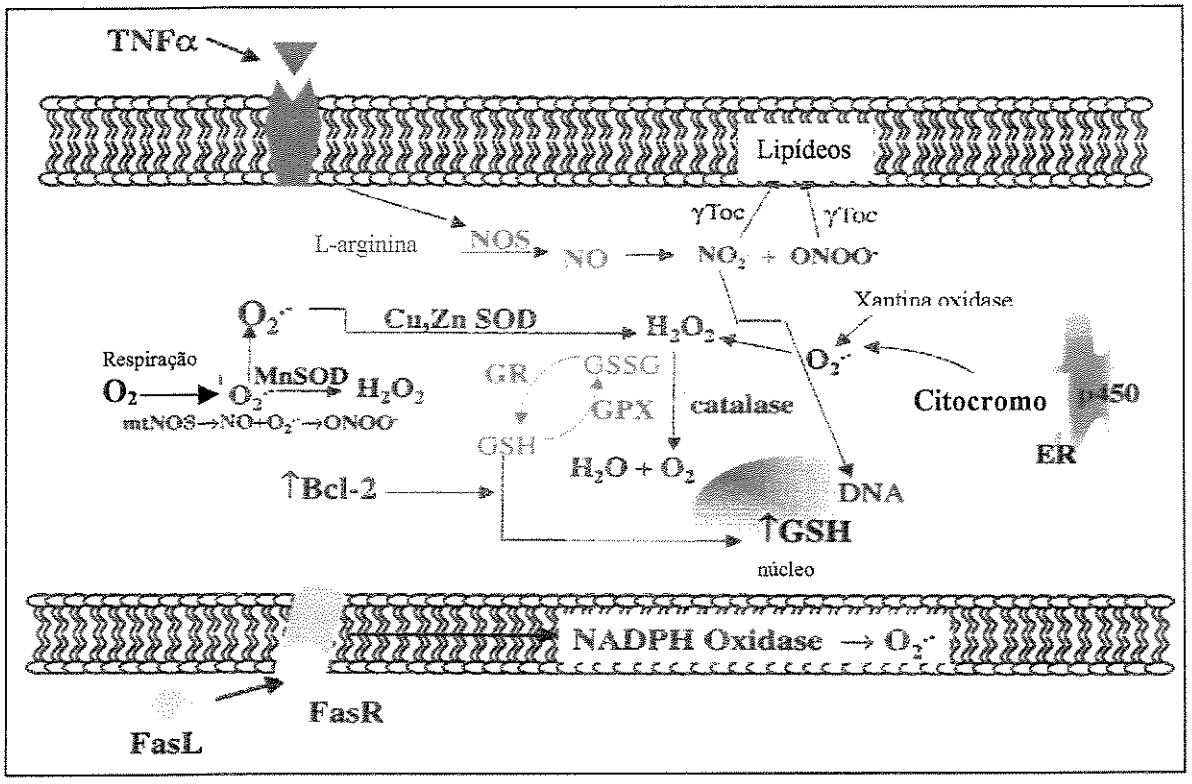

Figura 6. Fontes intracelulares de EROs e principais mecanismos de defesa antioxidante. As maiores fontes produtoras de EROs inclui a mitocôndria, retículo endoplasmático, membrana plasmática e citosol. A mitocôndria gera $O_2^{\bullet-}$ durante a respiração, que é convertido a H_2O_2 pela Mn-SOD. No citosol, $O_2^{\bullet-}$ é convertido a H_2O_2 pela Cu, Zn-SOD. As duas maiores defesas contra H_2O_2 são o ciclo redox GSH presente em ambos, citosol e mitocondria e catalase presente na fração peroxissomal. Outras fontes de $O_2^{\bullet-}$ incluem as enzimas xantina oxidase no citosol, NADPH oxidase na membrana plasmática e citocromo P450 no retículo endoplasmático. Bcl-2 pode funcionar como um antioxidante em alguns sistemas apoptóticos induzindo a relocalização de GSH no núcleo. NO pode ser produzido no citosol ou na mitocôndria por NOS. Adicionalmente, TNFα pode induzir ativação de NOS, resultando na geração de óxido nítrico (NO_2). NO_2 pode reagir com lipideos de membrana e podem causar mutações no DNA. Além disso, $ONOO^-$ pode induzir peroxidação lipídica.

Em condições normais, mais de 95 % da GSH nas células está reduzida; portanto o ambiente intracelular é, normalmente, altamente redutor. Investigações sobre o papel da GSH na modulação da sinalização apoptótica sugerem que alterações redox no ambiente intracelular induzido por agentes citotóxicos também são modulados pela geração de EROs e pela extrusão de GSH das células (Ghibelli *et al.*, 1995). Vários estudos demonstram uma

diminuição de GSH intracelular concomitante a um aumento em EROs no processo de morte celular por apoptose (Oda *et al.*, 1999; Xu & Thornalley, 2001).

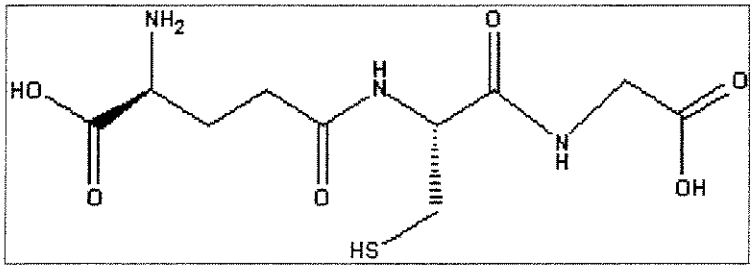

Figura 7. Estrutura química da glutationa (GSH).

A esse respeito, Slater e colaboradores (1995) postularam que a perda de GSH citoplasmático seria um dos eventos característicos de morte celular por apoptose, através da influência na capacidade redox tamponante da célula, tornando-a intolerante à presença de agentes oxidantes. Quando há uma diminuição também nos níveis de GSH mitocondrial, a produção de energia é afetada e a célula incha e sofre "necrose secundária" (apoptose tardia). Estudos demostraram que a morte celular apoptótica pode sofrer uma transição para a necrótica durante estresse oxidativo por dois mecanismos possíveis. O primeiro é desencadeado pela inativação de caspases devido à oxidação do grupo tiol de seus sítios ativos por agentes oxidantes ou S-nitrosilação. No segundo mecanismo ocorre uma redução nos níveis de ATP, portanto, na produção de energia, devido à diminuição de função mitocondrial causada pela ação de agentes oxidantes, levando à liberação de citocrotomo c e alteração de permeabilidade de membrana mitocondrial (McConkey, 1998; Chandra *et al.*, 2000).

4. Cultura de Células da Leucemia Promielocítica Humana (HL60) e Diferenciação Celular

A leucemogênese é um fenômeno complexo caracterizado por anomalias de proliferação e diferenciação resultando em bloqueio de maturação e expansão clonal de células leucêmicas (Uzunoglu *et al.*, 1999). A leucemia mielóide aguda (LMA) é caracterizada pela proliferação anômala dos precursores granulocíticos-macrofágicos da medula óssea. O crescimento acelerado que as células da leucemia mielóide apresentam *in vivo,* em relação às células normais, está relacionado à incapacidade funcional para maturação e diferenciação celular e não a um aumento na taxa de crescimento. A habilidade de responder a fatores exógenos de diferenciação apresenta-se diminuída em células da leucemia, podendo ocorrer alteração na expressão de produtos gênicos específicos obrigatórios para a diferenciação celular (Breitman, 1990).

A leucemia promielocítica aguda (LPA) é um subtipo muito peculiar de LMA com várias características distintas. Esta doença oferece oportunidade de se estudar a patofisiologia molecular da leucemia humana, representando um modelo para o entendimento da origem da leucemia no nível de desenvolvimento e genética molecular. Desta maneira, novas técnicas terapêuticas, diferentes da quimioterapia citotóxica convencional poderão ser desenvolvidas. Dados clínicos e biológicos obtidos na última década despertaram o interesse de pesquisadores neste subtipo de leucemia com o intuito de desenvolver novas técnicas terapêuticas (Grignani *et al.*, 1993; Drexler *et al.*, 1995; 2000).

O diagnóstico de LPA segue uma avaliação citológica padrão com base na morfologia hipergranular típica (M3 conforme a classificação do grupo cooperativo French-American-British (FAB)), imunofenotipagem e a presença de rearranjos citogenéticos específicos (translocações cromossômicas t(15;17)). Alguns problemas associados a

culturas primárias de células da leucemia promielocítica aguda dificultam os estudos com amostras de pacientes. As linhagens celulares reproduzem as características biológicas de LPA humana e oferecem muitas vantagens experimentais relativas à facilidade de manipulação e replicação, permitindo a investigação de questões que não podem ser analisadas em culturas primárias. Grande parte dos conhecimentos atuais sobre a biologia celular da LPA adveio de estudos realizados em linhagens celulares estabelecidas a partir de pacientes com LPA: HL60, NB4 e PL21 (Lübert & Koeffler, 1988; Drexler *et al.*, 1995; 2000).

A linhagem celular da leucemia promielocítica aguda humana HL60, foi estabelecida em 1976 a partir do sangue periférico de pacientes com LPA e foi a primeira e única linhagem de células de leucemia mielóide aguda disponível por vários anos (Collins, 1987; Dalton *et al.*, 1988; Drexler *et al.*, 1995). Estas células proliferam continuamente em culturas em suspensão, consistindo morfológica e histoquimicamente, predominantemente em promielócitos, dos quais cerca de 4-15% apresentam características morfológicas de células mielóides mais maduras: mielócitos, metamielócitos e leucócitos polimorfonucleares (PMN) (Collins *et al.*, 1978; Newburger *et al.*, 1979; Breitman, 1990).

As células da leucemia mielóide humana constituem um modelo apropriado para estudos de regulação da proliferação e diferenciação celular visto que, algumas destas linhagens de células são induzidas *in vitro* à diferenciação terminal por vários compostos químicos (Collins *et al.*, 1977). A diferenciação a células com características morfológicas de granulócitos maduros é induzida por uma ampla variedade de compostos tais como dimetilsulfóxido (DMSO), hipoxantina, dimetilaformamida, actinomicina e ácido retinóico (Collins *et al.*, 1978; Honma *et al.*, 1980, Breitman *et al.*, 1980; Breitman, 1990; Kizaki *et al.*, 1993, 1994; Ohguchi *et al.*, 1999; Kim & Kim, 2002). A indução de diferenciação a

células com características morfológicas de monócitos/macrófagos pode ser promovida por acetato de tetradecanoilforbol (TPA), 1,25-dihidroxivitamina D3 e butirato de sódio (Rovera *et al.*, 1979; McCarthy *et al.*, 1983; Boyd & Metcalf., 1984; Breitman, 1990; Verstuyf *et al.*, 1995; Zheng *et al.*, 2002). Muitas dessas células diferenciadas preservam características funcionais de granulócitos e monócitos/macrófagos de sangue periférico normal tais como fagocitose, liberação de enzimas lisossomais, quimiotaxia, atividade hexose monofosfatase, geração de ânion superóxido e habilidade de reduzir o NBT (nitro blue tetrazólio) (Koeffler, 1983; Verlinden *et al.*, 1997).

Essa estratégia de indução de diferenciação terminal a células com capacidade não-replicativa e posterior morte celular por apoptose indicam que o estado maligno pode ser reversível. Assim, alguns tipos de câncer podem eventualmente ser tratados com agentes indutores de diferenciação celular, reduzindo a morbidade que ocorre em tratamentos com agentes citodestrutivos (Sokoloski & Sartorelli, 1997).

Devido à proliferação desregulada e à inibição de apoptose serem comuns no desenvolvimento de tumores em geral, estes dois aspectos são alvos de intervenção terapêutica em todos os tipos de câncer. Há inúmeros mecanismos pelos quais estes dois defeitos ocorrem e o sucesso da terapia depende, em grande parte, das características individuais de cada tumor (Evan & Vousden, 2001).

Vários estudos demonstram a indução de apoptose em células de leucemia humana constituindo uma vantagem em relação a compostos quimioterápicos com ação estritamente citotóxica (Bertrand *et al.*, 1991; Dive & Hickmann, 1991; Gorczyca *et al.*, 1993; Kayfmann & Earsnshaw, 2000; Bertram, 2001; Reed, 2003). Em adição, uma grande desvantagem da quimioterapia convencional para o tratamento de doenças malígnas, incluindo a leucemia mielocítica, é a alta toxicidade envolvendo mielossupressão e

imunodeficiência. Assim, a indução de apoptose nas células tumorais sem afetar outras células do sistema imunológico seria consideravelmente útil na quimioterapia do câncer (Raffray & Cohen, 1997).

Estudos epidemiológicos sugerem a ingestão de vegetais e frutas na prevenção de câncer, atribuindo tal efeito terapêutico a presença de carotenóides e flavonóides nos alimentos. Hirano e colaboradores (1995), estudaram os efeitos de flavonóides da tangeretina, compostos extraídos de plantas, em células HL60. Neste trabalho observou-se alta toxicidade para estes compostos em células HL60 e reduzida citotoxicidade em linfócitos humanos. Além disso, indução eficiente de apoptose foi demonstrada por alterações morfológicas típicas de células apoptóticas e pela fragmentação do DNA. Da mesma forma, a desidrocrotonina inibe parcialmente o crescimento das células HL60 e induz apoptose, com reduzida toxicidade para linfócitos normais, conforme verificado recentemente (Araújo *et al.*, 2000; Freire *et al.*, 2001; 2003b).

II - OBJETIVOS

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Estudos anteriores revelaram que a desidrocrotonina (DHC) apresenta atividade anti-tumoral comprovada (Grynberg *et al.*, 1999). Recentemente, em nosso laboratório foi verificado que a DHC possui efeito citotóxico sobre as células da leucemia mielóide HL60 (Freire *et al.*, 2003a). Em trabalhos anteriores foi verificado que a abertura do anel lactônico da DHC resultou no derivado dimetilamida-crotonina (DCR), que manteve a atividade anti-ulcerogênica, com diminuição concomitante da citotoxicidade em hepatócitos e em células V79 (Melo, 2000; Melo *et al.*, 2001, 2002). Neste sentido, a investigação dos efeitos citotóxicos da DCR sobre células leucêmicas é de particular relevância para caracterização de suas atividades biológicas.

Com o intuito de identificar a toxicidade e a indução de diferenciação e/ou apoptose pela DCR em células de leucemia promielocítica humana (linhagem HL60), definimos como objetivos:

1- Avaliar a toxicidade da DCR sobre estas células, utilizando diferentes parâmetros de viabilidade celular: redução do MTT, atividade fosfatásica e conteúdo de proteínas;

2- Investigar se a DCR induz diferenciação morfológico-funcional em células HL60, utilizando a capacidade de redução do NBT como teste indicativo;

3- Avaliar a citotoxicidade da DCR em cultura primária de linfócitos, utilizando os mesmos parâmetros de viabilidade utilizados para células HL60;

4- Verificar a indução de apoptose pela DCR como mecanismo de citotoxicidade em células HL60 e comparar com a atividade do composto original, DHC;

5- Avaliar o envolvimento de estresse oxidativo no mecanismo de ação dos compostos DHC e DCR, pela determinação dos níveis de glutationa total (GSH) e de peroxidação lipídica;

III- METODOLOGIAS E RESULTADOS

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Comparative cytotoxicity of dimethylamide-crotonin in the promyelocytic leukemia cell line (HL60) and human peripheral blood mononuclear cells

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Abstract

Dehydrocrotonin (DHC) is a diterpene lactone obtained from *Croton cajucara* (Sacaca). Dimethylamide-crotonin (DCR), a DHC derivative, has a similar inhibitory effect on leukemic HL60 cells than its parent compound evaluated by different endpoints of cytotoxicity. No cytotoxicity or morphological alterations associated with apoptosis were detected in human peripheral blood mononuclear cells (PBMC) after treatment with up to 400 μM DCR in presence of phytohemaglutinin (5 μg/ml). Based on morphological changes and the pattern of DNA fragmentation, DHC and DCR were found to induce apoptosis and terminal differentiation (assessed by nitro blue tetrazolium reduction) in HL60 cells, but these compounds did not show any toxic effect in PBMC. Thus, DCR and DHC inhibit HL60 cell growth in vitro partly by inducing apoptosis and cell differentiation, but does not cause serious damage to immune cells according to our experimental conditions.

Keywords: Apoptosis; Cell differentiation; Dehydrocrotonin; Dimethylamide-crotonin; HL60 cells; Human peripheral blood mononuclear

Abbreviations: ATRA, all-trans retinoic acid; DCR, dimethylamide-crotonin; DHC, dehydrocrotonin; DMSO, dimethyl sulfoxide; DPA, diphenylamine; FBS, fetal bovine serum; HL60, promyelocytic leukemia cell line; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide; NBT, nitro blue tetrazolium; PBMC, human peripheral blood mononuclear cells; PHA, phytohemaglutinin; PBS, phosphate buffer saline; PMA, phorbol 12-myristate-13-acetate; PMN, polymorphonuclear leukocytes; pNPP, p-nitrophenyl phosphate; TAE buffer, Tris 0.4 M pH 8.0, EDTA-Na2 0.01 M, glacial acetic acid 1 M; TCA, Trichloroacetic acid; TE buffer, TrisCl 100 mM, pH 7.5, EDTA 1 mM, pH 8.0; TEX buffer, TE buffer pH 7.4 with 0.2% Triton X-100; TNF, tumor necrosis factor; TPA, tetradecanoyl phorbol acetate.

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1. Introduction

The inability of most cancer cells to mature into non-replicating adult cells means they remain in a highly proliferative state and outgrow their normal cellular counterparts. One approach to control this proliferation is to use antineoplastic agents, which can kill the cells by inducing terminal differentiation. Thus, cells exposed to chemical or biological agents are driven into a differentiation pathway that results in end-stage adult cells with no replicative capacity and which triggered apoptosis (Uzunoglu et al., 1999).

The HL60 cells can be induced to undergo terminal differentiation and apoptosis by a variety of chemical and biological agents. The finding that neoplastic cells can be induced indicates that the malignant state is not irreversible and suggests that certain cancers may eventually be treated with agents that initiate terminal maturation, presumably with a lower morbidity than that produced by cytodestructive agents (Sokoloski and Sartorelli, 1997). The induction of apoptosis in proliferative tumor cells may thus be an efficient strategy for cancer chemotherapy (Wyllie et al., 1980; Israels and Israels, 1999).

Apoptosis is a form of self-controlled cell death characterized by several morphological changes which differ from those of necrosis and include cell shrinkage, chromatin condensation, membrane blebs and internucleosomal cleavage of DNA (Kerr et al., 1972). Many tumors result from alterations in the homeostatic control of cell differentiation and apoptosis. However, the relationship between differentiation and apoptosis is still unclear (Kohroki et al., 1998). Apoptosis involves an active participation of the affected cells in a cascade of self-destruction cascade that culminates in DNA degradation via endonuclease activation, nuclear disintegration and the formation of 'apoptotic bodies' that involves the cell remnants (Raffray and Cohen, 1997; Rich et al., 2000). Recent studies have demonstrated that some anticancer agents can induce apoptosis in human leukemic cells, although these agents show serious cytotoxicity not only to malignant cells but also to normal tissues, including myelocytes and cells of the immune system (Raffray and Cohen, 1997).

Leukemogenesis is a complex phenomenon characterized by anomalies in proliferation and differentiation that result in the inhibition of maturation and clonal expansion. Certain cell lines are used as models in leukemia research (Uzunoglu et al., 1999). The human myelogenous leukemia cell line HL60 was established from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia. These cells proliferate continuously in suspension culture and consist predominantly of promyelocytes ($>90\%$), although 4–15% of them show morphological characteristics of more mature myeloid cells; including myelocytes, metamyelocytes, band forms, and polymorphonuclear leukocytes. This cell line displays a distinct morphological and histochemical commitment towards myeloid differentiation (Collins et al., 1978), and has played a pivotal role in studies on the differentiation and growth of human leukemias (Takahashi et al., 1998). Differentiation in HL60 cells is accompanied by a change in the expression of surface antigens and the acquisition of a number of functions such as the generation of superoxide and the attachment of cells to the walls of culture flasks. Various compounds induce the differentiation of HL60 cells into granulocyte-like (neutrophils) or macrophage-like (monocytes) cells but can also inhibit cell growth, HL60 cells can release superoxide immediately after stimulation with phorbol 12-myristate-13-acetate and thus, provide a good model for studying reactive oxygen radicals and oxidative stress (Ujihara et al., 1998). Since differentiated cells lose their ability to proliferate and their immortality, inducers of differentiation may be useful for treating leukemia (Kohroki et al., 1998). Some inducers such as all-trans retinoic acid, tetradecanoyl phorbol acetate (TPA), 1α,25-dihydroxyvitamin D_3 and tumor necrosis factor also cause apoptosis in leukemia cells (Martin et al., 1990; Terui et al., 1995; Kohroki et al., 1998).

The bark and leaves of *Croton cajucara* Benth. (Euphorbiaceae), an Amazonian medicinal plant known as 'sacaca', are commonly used as an infusion in a powdered or dried pill form to treat a large number of gastrointestinal, renal and

hepatic disorders (Maciel et al., 2000). A clerodane *nor*-diterpene lactone, *trans*-dehydrocrotonin (DHC), is an important bioactive compound of *C. cajucara* and has hypoglycaemic, antigenotoxic (Agner et al., 2001); antiulcer (Souza-Brito et al., 1998; Hiruma-Lima et al., 1999), antiinflammatory and antitumor (Grynberg et al., 1999) effects. Recently our group (Freire et al., 2001, 2003; Freire et al., in press) showed that DHC was cytotoxic to human leukemic cells (HL60). To improve our understanding of the biological effects of DHC, we have now studied the cytotoxic effects of dimethylamide-crotonin (DCR), a DHC derivative, in HL60 cells and compared the results with those for DHC. We also investigated the cytotoxicity of DCR in human peripheral blood mononuclear cells (PBMC).

2. Material and methods

2.1. DHC and DCR

DHC was obtained from *C. cajucara* bark as described by Souza-Brito et al. (1998). The DHC molecule was opened to yield DCR using the dimethylamine reaction described by Cromwell and Cook (1958) and modified by Melo et al. (2001). The compounds are shown in Fig. 1. The purity of DHC and DCR was over 99% assessed by NMR (nuclear magnetic resonance), UV (ultraviolet), IR (infrared) and MS (mass spectroscopy) techniques according to Melo et al. (2001, 2002).

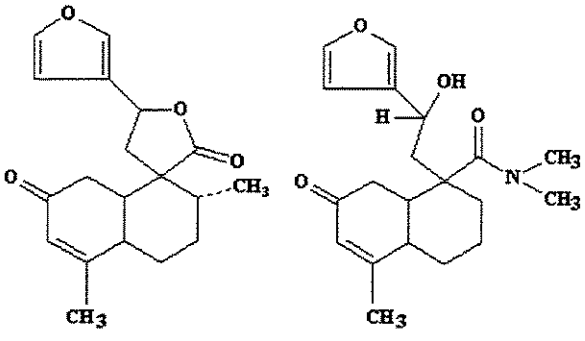

Fig. 1. Dehydrocrotonin and dimethylamide-crotonin.

2.2. Cell culture

The HL60 cells, derived from a patient with acute promyelocytic leukemia, were of the laboratory of cellular metabolism and regulation kindly provided by Dr Rui Curi, Department of Physiology and Biophysic, Institute of Biomedical Sciences (ICB), USP, São Paulo, Brazil. All HL60 experiments were performed on passages 12–26. The leukemia cells (HL60) were cultured in suspension at 37 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU of penicillin/ml and 100 μg of streptomycin/ml in a humidified atmosphere containing 5% CO_2 in air. The cells were passaged twice a week and were used in the exponential growth phase. To assess viability, the cells were seeded (3×10^5 cells/ml) in 96 wells plates and incubated with different concentrations of DCR for 72 h. Cell viability was determined by 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) reduction, protein content and phosphatase activity (Aoyama et al., 2000; Melo et al., 2001). For trypan blue (TB) exclusion, nitro blue tetrazolium (NBT) reduction and the diphenylamine (DPA) assays the cells were cultured in bottles (3×10^5 cells/ml) and incubated with different concentrations (25, 50 and 75 μM) of DCR and DHC for 24, 48 and 72 h. The compounds were firstly dissolved in dimethyl sulfoxide (DMSO) and then in supplemented medium. The final concentration of DMSO in the test medium and controls was 0.1%.

2.2.1. MTT

The tetrazolium reduction assay was done as described by Denizot and Lang (1986). Briefly, 0.1 ml of serum-free medium containing MTT (1 mg/ml) was added to each well. After incubation for 4 h, the supernatant was removed and the blue formazan product obtained was dissolved in 1 ml of ethanol with stirring for 15 min on a microplate shaker after which the absorbance at 570 nm was read.

2.2.2. Protein content

The protein content of the cells was assayed using a modification of the Lowry method, as

described by Hartree (1972), with albumin bovine serum as the standard.

2.2.3. Phosphatase activity

After incubation for 72 h with DCR, the phosphatase activity of HL60 cells was assayed as described by Aoyama et al. (2000). The culture medium was carefully removed from the wells and p-nitrophenyl phosphate dissolved in 1 M acetate buffer, pH 5.5 to a final concentration of 75 mM was added. After incubation 30 min on a microplate shaker at room temperature, the reaction was stopped by adding NaOH. The resulting absorbance was read at 405 nm.

2.2.4. Trypan blue exclusion

The TB exclusion assay was done as described by Renzi et al. (1993). Cells grown in culture bottles (3×10^5 cells/ml) were treated with DCR and DHC (25, 50 and 75 μM) for 24, 48 and 72 h. At the end of each period, the cultures were inspected for morphological alterations. TB solution (0.08% in phosphate buffer saline, PBS) and cell suspension were mixed in equal volumes (0.1 ml) and the number of cells was estimated using a hemocytometer. Cells stained blue were scored as dead.

2.3. Lymphocyte culture

PBMC isolated from the venous blood of healthy volunteers as described previously (Hirano et al., 1989) were treated with different concentrations of DCR for 72 h. The lymphocytes (1×10^6 cells/ml) were cultured in suspension in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU of penicillin/ml, 100 μg of streptomycin/ml and phytohemaglutinin (PHA) (5μg/ml) in a humidified atmosphere with 5% CO_2 in air at 37 °C. Cell viability was determined by MTT reduction, protein content and phosphatase activity, as described elsewhere (Aoyama et al., 2000).

2.4. Assay of NBT reducing activity

Cells (3×10^5 cells/ml) were cultured with various concentrations of DCR and DHC in RPMI-1640 medium containing 10% FBS during 24, 48 and 72 h, after which the NBT reducing activity was determinated by the method of Sakashita et al. (1991) and Kohroki et al. (1998), with slight modifications. Briefly, the cells (2×10^6) were harvested by centrifugation and resuspended in 1 ml of RPMI 1640 medium containing 20% FBS. After the addition of 500 μl of a TPA (200 μg/ml)-NBT (1 mg/ml) solution, the cells were incubated at 37 °C in 5% CO_2 for 25–30 min. After centrifugation, 600 μl of ethanol were added to the cell pellets to solubilize the formazan deposits. The amount of formazan formed was determinated by reading the absorbance at 560 nm.

2.5. Assessment of apoptosis

To induce apoptosis, HL60 cells (3×10^5 cells/ml in 50 ml bottles) were treated with DCR and DHC at a concentration corresponding to the IC_{50} (150 μM) during 72 h to Feulgen reaction and to agarose gel electrophoresis. To quantification of DNA fragmentation the cells was treated at the same conditions that was done to measurement of NBT reducing activity. The cell viability after this incubation was assessed by TB as described above. Several reports have been showed that some flavonoids induce apoptosis in HL60 cells (Zhao et al., 1997; Xiao et al., 1997; Jakubowicz-Gil et al., 2002; Liesveld et al., 2003). Therefore, the bioflavonoid quercetin was used as a positive control in the electrophoresis agarose gel for assessment of apoptosis.

2.5.1. Feulgen reaction

Apoptosis was evaluated by morphological analysis, as previously described (Melo et al., 2000). After treatment, the cells were fixed in a mixture of absolute ethanol–acetic acid (3:1) for 1–5 min, rinsed in ethanol for 5 min, and air-dried at room temperature. The preparations were subjected to the Feulgen reaction, with hydrolysis in 4 M HCl for 1.5 h. The preparations were mounted in Canada balsam ($n_D = 1.54$) and the slides were viewed with an Olympus microscope. Apoptotic cells were defined as those exhibiting characteristic features such as cell shrinkage,

nuclear condensation, and the formation of membrane-bound apoptotic bodies.

2.5.2. Quantification of DNA fragmentation

The amount of fragmented DNA was measured by the DPA method, as described by Zhu et al. (1998) with some modifications. Briefly, 5×10^6 cells were collected and lysed in TE with vigorous stirring. After separation of the fragmented DNA from the intact chromatin, the supernatant was transferred into new test tube. The pellet was dissolved in TEX buffer and 25% trichloroacetic acid (TCA) was added to all tubes. Following incubation overnight at 4 °C, the precipitated DNA was collected by centrifuging for 10 min at $20000 \times g$ at 4 °C. The supernatants were discarded and the DNA was hydrolysed by adding 5% TCA to the pellets followed by heating for 15 min at 90 °C in a heating block. A blank with 5% TCA alone was also prepared. Following the addition of freshly prepared DPA solution (15 mg of DPA/ml, 15 μl of concentrated sulfuric acid/ml, and 90 μg of acetaldehyde/ml), the mixture was incubated for about 4 h at 37 °C and resulting absorbance at 600 nm was measured. The percentage of fragmented DNA was calculated as:

Percentage fragmented DNA
= 100×[amount of fragmented DNA in the supernatant/(amount of fragmented DNA in the supernatant + amount of DNA in the pellet)].

2.5.3. Detection of internucleosomal DNA fragmentation by agarose gel electrophoresis

Internucleosomal DNA fragmentation was detected using the TACS™ Apoptotic DNA Laddering Kit purchased from R&D Systems, (Minneapolis, MN). DNA was isolated from 1×10^7 cells which were collected by centrifugation and washed with ice cold 1 × PBS followed to incubation at 18–24 °C for 10 min. The cells were lysed and the DNA stabilized by adding lysis solution. The samples were centrifuged with extraction solution and extraction buffer to obtain an upper (aqueous) layer. After centrifugation, 0.1 volume of sodium acetate and an equal volume of 2-isopropanol were added to the aqueous DNA sample. After centrifugation, the supernatant was discarded, 1 ml of 70% ethanol was added to the DNA pellet and the mixture again centrifuged at $12000 \times g$ for 5 min. Following removal of the supernatant, the pellet was dried a vacuum centrifuge. The DNA pellet was resuspensed in DNase-free water. The DNA was quantified by diluting in water (1:10) and the absorbance was read at 260 nm using water as the blank. The DNA concentration was determinated as: concentration (μg/μl) = $A_{260} \times 9.88$[1]

Following the addition of 2 μl of gel loading buffer, the samples were applied to 1.5% agarose gels in 1 × TAE buffer (Tris 0.4 M pH 8.0, EDTA-Na2 0.01 M, glacial acetic acid 1 M) and run at 100 V for approximately 2 h. The gels were stained for 15 min in 0.5 μg of ethidium bromide/ml (prepared in DNase-free water or 1 × TAE buffer) and the bands then visualized using a UV transluminator.

2.6. Statistical analysis

The cell viability and DNA fragmentation experiments were performed at least three times with four replicates each time. The IC_{50} values (concentration that produced 50% inhibition on the parameter evaluated) were calculated after expressing the results as a percentage of the controls and were determined graphically from the dose-response curves using the computer software package 'Origin, Data Analyses and Technical Graphics-version 6.0' (Copyright Software, Inc). The results are represented as means ± S.D. Statistical analyses were performed using one-way ANOVA or Student's *t*-test, software graphpad Instat™, with the level of significance set at $P < 0.05$.

[1] 9.88 is a multiplication factor that accounts for the 1:200 dilution and the absorbance of the DNA in H_2O.

3. Results

3.1. Effects on HL60 cells and lymphocytes viability

The effect of DCR on the growth and viability of HL60 cells were examined after 72 h in culture (Fig. 2). Cell viability measured by the MTT assay and phosphatase activity was suppressed by DCR in a dose-dependent manner. The IC_{50} values for the two assays were 65 and 90 μM, respectively. The protein content was less affected by DCR at the concentrations tested ($IC_{50} = 280$ μM). For comparison the IC_{50} values for DHC in the MTT assay and phosphatase activity were the same (180 μM) while for protein content the IC_{50} was 150 μM on treated HL60 cells according to the results of Freire et al. (2003). The effects of DCR on the viability of PBMC were also examined. The cells were incubated with PHA (5 μg/ml) as a mitogen and with different concentrations of DCR (up to 400 μM) for 72 h. No cytotoxicity or morphological features of apoptosis (data not shown) were detected in PBMC after treatment with DCR (Fig. 2) in the presence of PHA (> 80% of the cells were still alive after 72 h of treatment, analysed by protein content and phosphatase activity). DHC did not show any toxic effect in PBMC up to 1.5 mM according to the results of Araujo et al. (2000). Thus, DCR and DHC inhibit HL60 cell growth in vitro, but does not cause serious damage to immune cells as showed by the results with PBMC according to our experimental conditions. Cell viability also was evaluated by TB exclusion after 24, 48 and 72 h of exposure to DCR (Fig. 3A) and DHC (Fig. 3B) on HL60 cells. The number of cells counted in this assay was 5×10^5 cells/ml and 6×10^5 cells/ml after 72 h of treatment with DCR and DHC, respectively. Based on the MTT reduction, phosphatase activity and TB exclusion, DCR was more toxic than DHC to HL60 cells.

3.2. Effects on cell differentiation

To examine the ability of DCR and DHC to induce the differentiation, HL60 cells were incubated with various concentrations of these compounds for up to 72 h after which the cells were

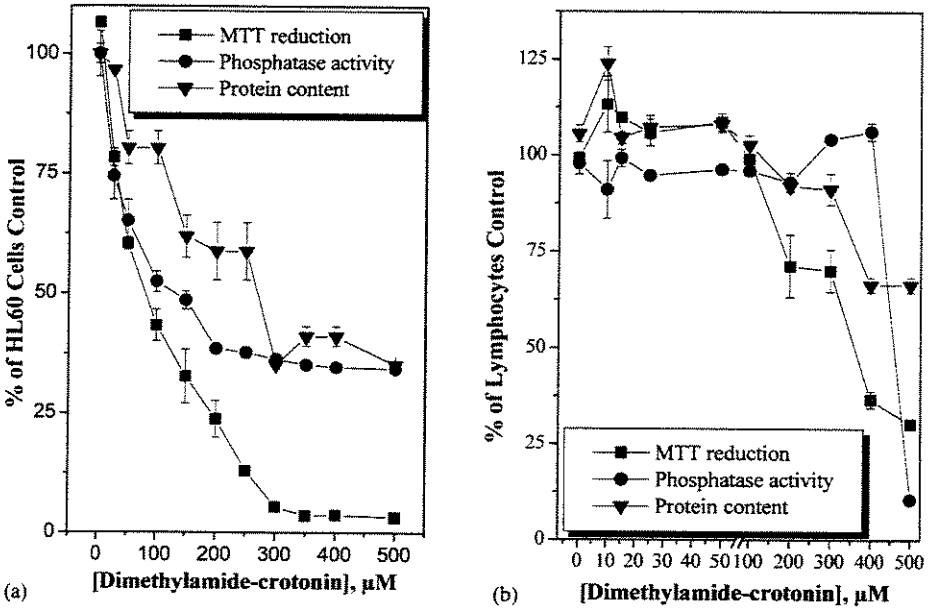

Fig. 2. Viability of cultured HL60 cells (A) and lymphocytes (B) after treatment with DCR for 72 h. The endpoints evaluated were protein content, MTT reduction and phosphatase activity. Each point represents the mean ± S.D. of three experiments in four replicates.

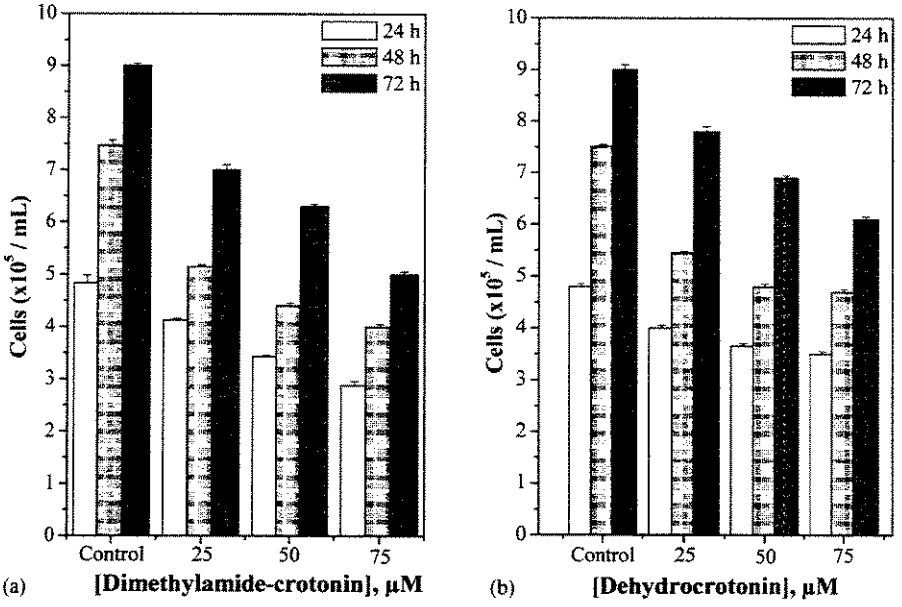

Fig. 3. (A) Cytotoxicity of DCR in cultured HL60 cells treated for 24, 48 and 72 h evaluated by TB exclusion. Each column represents the mean ± S.D. of three experiments in four replicates. (B) Cytotoxicity of DHC in cultured HL60 cells treated for 24, 48 and 72 h and then evaluated by TB exclusion. Each column represents the mean ± S.D. of three experiments in four replicates.

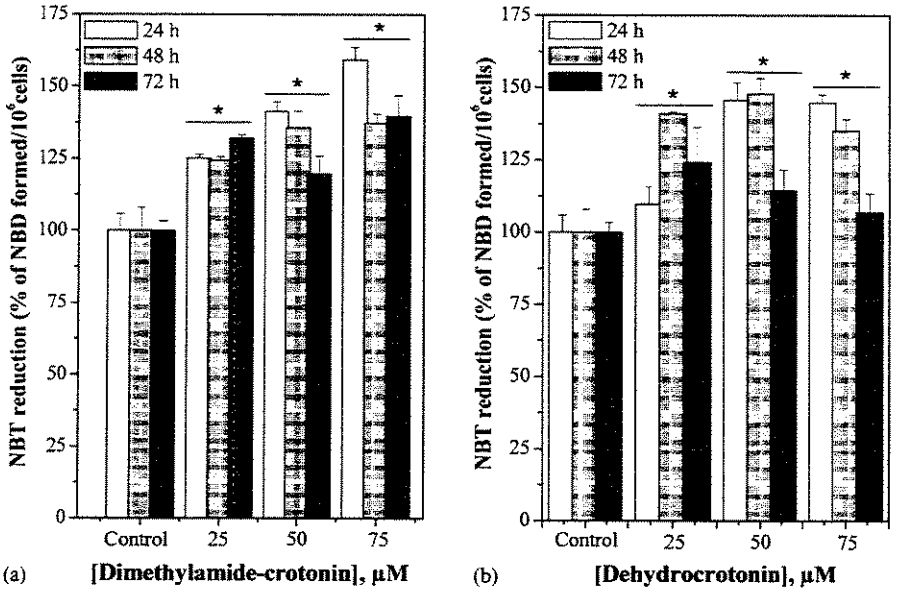

Fig. 4. (A) Differentiation of HL60 cells after treatment with DCR for 24, 48 and 72 h. Cell differentiation was measured by the ability to reduce NBT. Each column represents the mean ± S.D. of three experiments in four replicates. (B) Differentiation of HL60 cells after treatment with DHC for 24, 48 and 72 h. Cell differentiation was measured by the ability to reduce NBT. Each column represents the mean ± S.D. of three experiments in four replicates. (All marked with * are: $P < 0.05$ compared to control cells).

Table 1
Percentage of DNA fragmentation detected by DPA method in HL60 cells treated with different concentrations of DHC or DCR for 24, 48 and 72 h

Time (h)	Control	Dehydrocrotonin			Dimethylamide-crotonin		
		DHC (25 μM)	DHC (50 μm)	DHC (75 μm)	DCR (25 μM)	DCR (50 μm)	DCR (75 μm)
24	5±3	6±2	16±3[a]	18±2[a]	12±4[a]	16±3[a]	20±3[a]
48	6±3	4±2	22±3[a]	20±2[a]	17±3[a]	20±4[a]	25±4[a]
72	8±4	10±2	24±3[a]	17±4[a]	15±4[a]	22+3[a]	27+4[a]

Each value represents the mean±S.D. of three experiments in four replicates.
[a] $P < 0.05$ compared to control cells.

assayed for their reducing activity, a typical marker for the differentiation of myeloid leukemia cells (Theodore, 1990). As shown in Fig. 4A, DCR induced HL60 cell differentiation at concentrations lower that the IC_{50} of 150 μM within 24 h of treatment. DHC produced a lower NBT reducing activity than its derivative (Fig. 4B). According to Fig. 4A, the NBT reduction in HL60 cells treated with 75 μM DCR (half the IC_{50}) increased by about 63% after 24 h. At the same concentration DHC increased of NBD formation by 45%. Thus, DCR and DHC induced HL60 differentiation that was time- and concentration-dependent, with DCR being more effective.

3.3. Apoptosis induction—morphological evidences

Sesquiterpene lactones inhibit the growth of various types of cancer cells (Rodriguez et al., 1976; Grynberg et al., 1999; Cho et al., 2000). At 100 μM, DCR reduced the viability of HL60 cells by approximately 50%, as determinated above by cytotoxicity tests. Since most inducers of differentiation in leukemia cells induce apoptosis (Kohroki et al., 1998), we examined whether the cell death caused by DCR in HL60 was due to apoptosis. Untreated cells showed a typical non-adherent, round morphology after 72 h in culture visualized by microscopy. After a 72 h incubation with 100 μM DHC or 100 μM DCR, some of the cells still appeared normal, whereas others showed dramatic morphological alterations characteristic of apoptosis (Fig. 5). Numerous apoptotic bodies, i. e. membrane-enclosed vesicles derived from a cytoplasmatic extension, were also observed. These apoptotic cells, as well as other intact cells, excluded TB dye, indicating that the cells were not undergoing necrosis. Thus, the DHC derivative had similar inhibitory effect on HL60 cells without the α-methylene-γ-lactone present in the DHC moiety.

3.4. Apoptosis induction—DNA fragmentation

To clarify the mode of cell death caused by DCR and DHC, we examined the effects of these compounds on the internucleosomal DNA 'ladders' in HL60 cells treated for 72 h by agarose gel electrophoresis (Fig. 6). Lane two in Fig. 6 shows the DNA ladder for HL60 cells treated for 72 h with 150 μM DCR and lane three shows that DHC also produced DNA ladder at the same concentration and period of treatment. To confirm and explain biochemical evidence of the induction of apoptosis by the DCR and DHC and also to quantify the degree of DNA fragmentation, we performed the DPA method. As shown in Table 1, the extent of DNA fragmentation in HL60 cells increased dose-dependently with DCR and DHC treatment. However in the concentration of 25 μM, DHC did not induce significantly DNA fragmentation. The cells treated with 50 μM of DCR or DHC showed significant DNA fragmentation about threefold increase in all the times of treatment (Table 1). However, up to this concentration of DHC or DCR, the increase in the degree of DNA fragmentation was little significant compared to the concentration of 50 μM.

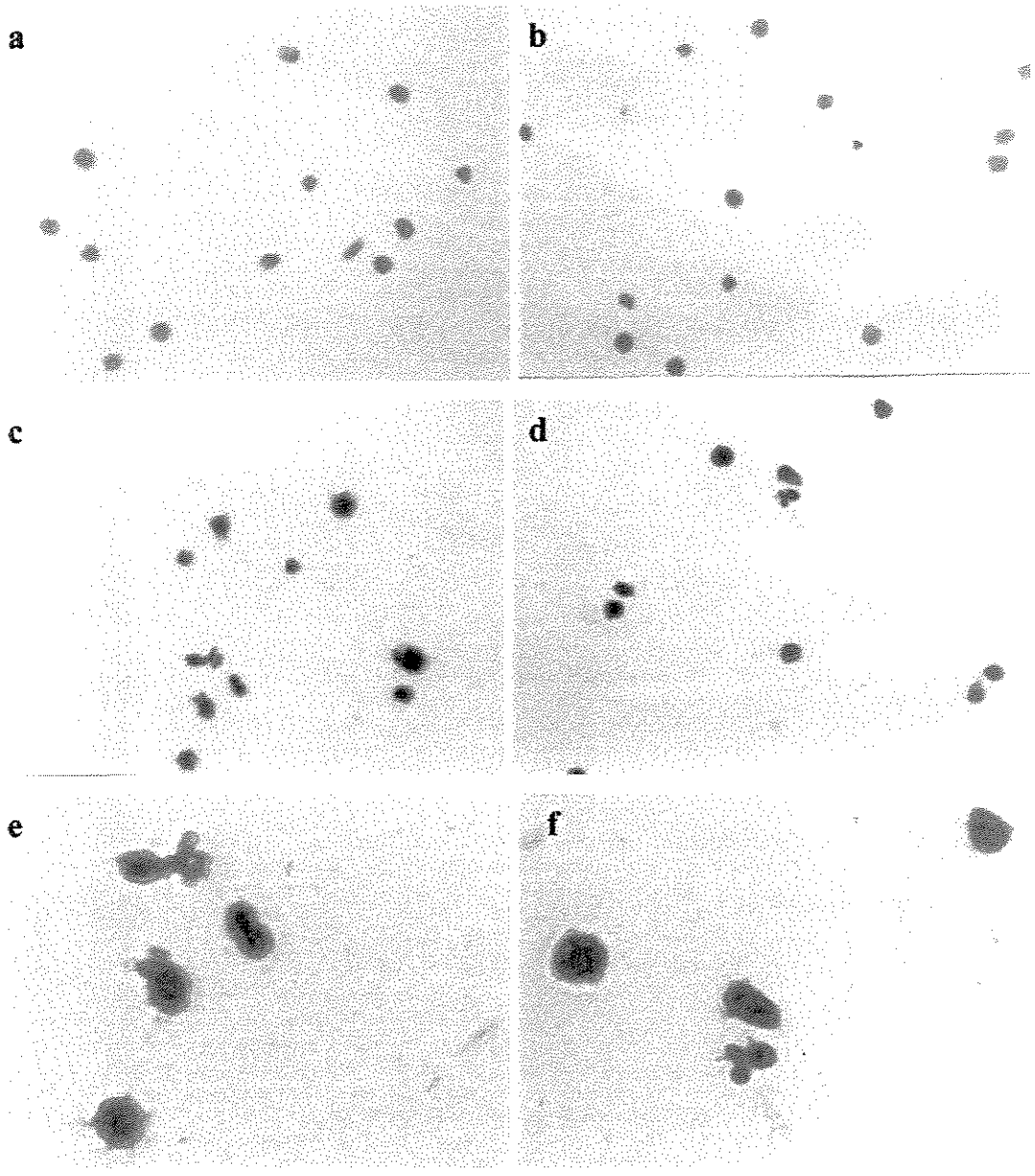

Fig. 5. HL60 cells submitted to the Feulgen reaction. (a, b) control cells, cells-treated with (c, e) 150 μM DHC, (d, f) 150 μM DCR. (a–d) Magnification of 200 ×; (e–f) Magnification of 1000 ×.

4. Discussion

A balance between the therapeutic and toxicological effects of a compound is an important parameter when verifying the compound pharmacological applicability. Many cytotoxic drugs kill malignant cells by inducing apoptosis, a genetically directed form of the cell death, and can induce cell differentiation. Leukemic cells (HL60) provide a model for studying the mechanisms and

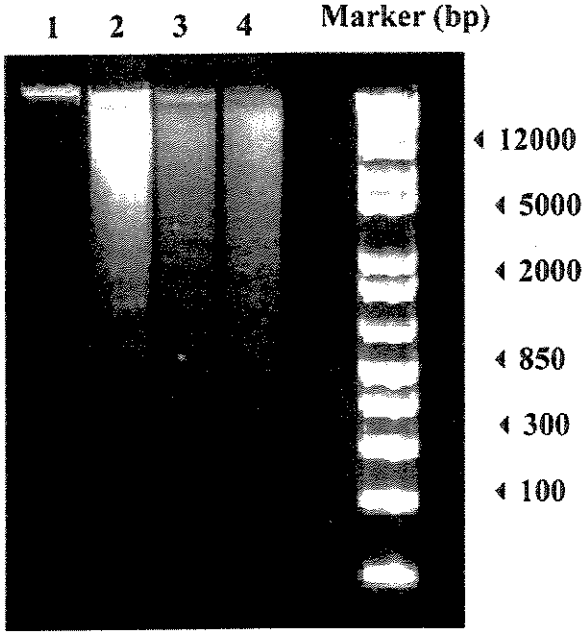

Fig. 6. DNA fragmentation in HL60 cells treated for 72 h with 150 μM DCR (lane two), 150 μM DHC (lane three) and compared to untreated control cells (lane one) and to positive control cells treated with 120 μM quercetin (lane four). DNA fragmentation was detected following electrophoresis in agarose gel and staining with ethidium bromide.

relationships involved in the induction of differentiation and apoptosis in response to antitumor agents.

Cultured mammalian cells provide an important tool for evaluating the cytotoxicity of compounds with potential therapeutic activity (Pailard et al., 1999). The endpoints for these cytotoxic assays often include the integrity of the lysosomal and mitochondrial membranes, which provides information on the susceptibility of cellular organelles and compartments (Loveland et al., 1992; Rodriguez and Haun, 1999). The protein content provides an index of the total cell number based on a determination of the cellular macromolecule content (Forsby et al., 1991; Haun et al., 1992). Phosphatases participate in various cellular processes and the activity of these enzymes can be used to evaluate cell viability (Aoyama et al., 2000). The MTT assay measures cell viability based on endocytosis, a fundamental feature of most living cells. However, factors affecting the endocytosis of MTT, the exocytosis of MTT formazan, and cellular MTT reductase activity can influence the reduction of MTT (Mosmann, 1983; Melo et al., 2001, 2002). The TB exclusion test allows an evaluation of the structural integrity of the cell membrane (Loveland et al., 1992). Using these viability assays DCR was more toxic than DHC to HL60 cells. Interestingly both compounds showed little toxic effect on PBMC. When considering the chemotherapy against dividing normal cells, it is very important to verify whether the drug has a harmful effect against dividing normal cells such as proliferating lymphocytes. Several factors can interfere with chemical cytotoxicity including concentration of chemical agents, length of exposure (T), cell density and type. Duration of exposure (T) and drug concentration (C) is related, although $C \times T$ is not always a slant. Longer exposures can increase sensitivity beyond that predicted by $C \times T$ due to cell cycle effects and cumulative effect from the agents (Melo et al., 2000). Thus, DCR as well DHC inhibits HL60 cell growth in vitro, but does not cause serious damage to immune cells as showed by the results with PBMC according to our experimental conditions that could be influenced by intrinsic characteristics of each type of cell culture evaluated.

Sesquiterpene lactones have functional groups that are responsible for their biological effects. The structure of DHC contains three highly reactive functional groups: $O{=}C{-}C{=}CH_2$, a lactone and cyclopentenone. Since the structure of DHC is not planar, the most available group is $O{=}C{-}C{=}CH_2$, which can function as a Michael acceptor to interact with the SH groups of proteins, GSH and nitrogen bases, mainly guanine, through nuclophilic attack (Jodynis-Liebert et al., 2000; Melo et al., 2002). Other diterpenes isolated from *C. incanus* and *Laetia corymbulosa* also presented cytotoxic effect on several tumor cell lines and the authors suggested that their effect was due to interaction with macromolecules. Furthermore, recently, some authors showed that several sesquiterpene lactones may generate a reactive intermediate capable of damaging cellular constituents (Dimas et al., 1999; Beutler et al., 2000). The oxidative stress provoked by reaction of these

compounds with molecular oxygen results in formation of hydrogen peroxide, altering the intracellular redox equilibrium. These data suggest that oxygen reactive species generated also play an important role in DHC cytotoxicity. Melo et al. (2001) opened the lactone DHC molecule through a dimethylamine reaction to give DCR, as described by Cromwell and Cook (1958). DCR also has a Michael acceptor in the amide moiety so this compound has the same important structural characteristics than DHC to have antitumor activity. Although opening the lactone ring maintained the three essential structural characteristics necessary for antitumor activity, this modification altered the cytotoxicity to other cells, such as V79 fibroblasts and primary cultures of hepatocytes. Previous work suggested that DCR was less toxic than DHC in these cultured cells (Melo et al., 2001, 2002). The IC_{50} values determined in these cells can be used to obtain the lethal dose in vivo. Thus DCR probably will be less toxic to animals than DHC and according to our results is more effective as antitumor compound.

One drawback of drug therapy for the treatment of malignant diseases, including myelocytic leukemia, is serious toxicity involving myelosuppression and immunodeficiency. The induction of apoptosis in cancer cells without affecting cells of the immune system would be of considerable usefulness in cancer chemotherapy.

HL60 is a leukemic cell line blocked at the level of promyelocitic differentiation (Verlinden et al., 1997). Treatment perspectives, therefore, not only include cytotoxic drugs, but more interestingly differentiation-inducing agents. Since differentiated cells loose their proliferative ability and immortality, differentiation inducers may be useful for the treatment of leukemias (Kohroki et al., 1998). The capability to reduce NBT is a first parameter to investigate the functional differentiation. Thus, in the present work, we evaluated the induction of differentiation by DCR and its parent compound using NBT assay. Our results demonstrated that DHC and DCR were able to induce NBT reduction. At the same concentrations DCR improved the induction of cellular differentiation compared to DHC. Thus, both compounds induced HL60 differentiation that was time- and concentration-dependent according to our results, with DCR being more effective. Considering that the NBT assay is rather non-specific, since both monocytes and granulocytes can stain positive, future studies should include a more specific determination of the cell types present in the cultures after treatment (Melo et al., 2003).

Induction of programmed cell death in tumor cells is of generous benefit for cancer chemotherapy. Different compounds may regulate growth of cancer cells and/or tumorigenesis via the induction of apoptosis in malignant cells (Raffray and Cohen, 1997). Our results clearly demonstrated that DHC and DCR induced apoptosis in HL60 cells. As observed in the Feulgen reaction, treatment of these cells with the studied compounds induced morphological alterations which are typical of the apoptotic process. The morphological features of apoptosis showed chromatin condensation in HL60 cells induced by DCR and DHC with 72 h of treatment. Apoptosis was also demonstrated by the increase in the percentage of fragmented DNA quantified by the DPA method. Up to 50 μM of DCR and DHC the increase in the DNA fragmentation remained stationary evaluated by this assay. Until recently, chromatin condensation has been considered to be the consequence of internucleosomal DNA cleavage (Arends et al., 1990). However, Oberhammer et al. (1993) have observed chromatin condensation typical of that occurring in apoptosis without internucleosomal DNA cleavage. Therefore, the results showing chromatin condensation, evaluated by the Feulgen reaction and absence of internucleosomal DNA ladder only in the DHC treated cells indicate different action mechanisms of apoptosis induction by the studied compounds. In a recent report (Anazetti et al., 2002), our group showed that both compounds are associated with activation of a number of aspartate-specific cysteine proteases, the caspases, that are a part of the molecular basis of apoptosis. Interestingly, whereas equal effects on caspase-2 and caspase-6 activities were found after incubation of HL60 cells with DHC and DCR, a different behaviour was observed in the caspase-9 activation, because only DCR significantly activated this caspase in 72 h of treatment. These data confirm that the cell

death in the HL60 cells treated by the studied compounds is triggered by apoptosis but in a different mechanism of activation.

In summary, DCR and DHC inhibited the growth and proliferation of HL60 cells with similar toxicity. Both compounds induced cell differentiation and apoptosis. Although DCR was more effective in its antitumor activity than the parent compound. Moreover, DCR and DHC showed little toxicity towards normal PBMC. These results indicate that those may be considerable differences in the sensitivities of normal and tumor cells to the actions of these compounds.

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To
Dr. John Bennett
Editor of Leukemia Research
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601 Elmwood Avenue, Box 704
Rochester, NY 14642
USA

Campinas may 12, 2003.

Dear Dr. Bennett,

We are sending the manuscript of our work "***Dehydrocrotonin and its derivative, dimethylamide-crotonin, induce apoptosis with lipid peroxidation and activation of caspases-2, -6 and –9 in human leukemia cells HL60***" by **Maristella C. Anazetti, Patrícia S. Melo, Nelson Durán and Marcela Haun**, which we would like to publish in the Leukemia Research. I would be most grateful to you for a prompt and favorable action on this manuscript.
It is important to state at this point that all the authors of this manuscript have participated directly in the planning, execution and analysis of the study and approved the final version submitted. The contents of this manuscript have not been previously published and have not been and will not be submitted or published elsewhere while acceptance by the Journal is under consideration.

We look forward to hearing from you in due course.

Sincerely yours,

Dra. Marcela Haun

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Receipt Manuscript 03-D854 (30/05/2003)

Dear Dr. Haun:

Your manuscript by Anazetti et al. entitled "Dehydrocrotonin and its derivative, dimethylamide-crotonin, induce apoptosis with lipid peroxidation and activation of caspases-2, -6 and -9 in human HL60 leukemia cells" (03-D854) has been received and will be sent for review.

Sincerely,

John M. Bennett, M.D.
Editor, Leukemia Research

JMB: pd

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DEHYDROCROTONIN AND ITS DERIVATIVE, DIMETHYLAMIDE-CROTONIN, INDUCE APOPTOSIS WITH LIPID PEROXIDATION AND ACTIVATION OF CASPASES –2, –6 AND –9 IN HUMAN HL60 LEUKEMIA CELLS

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ABSTRACT

A variety of stimuli can induce cells to undergo apoptosis, with one of the most reproducible inducers being mild oxidative stress following exposure to anticancer agents. Apoptosis involves events mediated by cysteine proteases (caspases), which are classified as initiators (–8, -9 and –12) or executors, (–2, –3, -6 and –7). In this study, we examined the mechanisms of apoptosis induced by dehydrocrotonin (DHC), a diterpene lactone isolated from the Amazonian plant *Croton cajucara*, and its synthetic derivative, dimethylamide-crotonin (DCR), in human HL60 promyelocytic leukemia cells. Flow cytometric analysis of the externalization of phosphatidylserine (PS) using annexin V/propidium iodide after exposure of HL60 cells to DHC and DCR a concentration- and time-dependent increase apoptosis, with maximum cell death at a concentration of 250 μM for both compounds. The DCR and DHC were effective in triggering the activation of caspases–2, –6 and –9. The level of reduced glutathione (GSH), measured by DTNB reaction decreased and the thiobarbituric acid-reactive substance (TBARS) production, quantified by fluorimetry, increased significantly after treatment with both compounds. The cytotoxicity of DHC and DCR was prevented by a high concentration of GSH (15 mM) in the culture medium. These results indicate that DCR and DHC produced apoptosis by oxidative stress-induced lipid peroxidation, which triggered the caspases cascade, that lead to apoptotic cell death in HL60 cells. Based on the pattern of caspases activation, DCR and DHC trigger apoptosis in HL60 cells probably through cytochrome c release and apoptosome formation.

Keywords: apoptosis; caspase activity, dimethylamide-crotonin; dehydrocrotonin; glutathione; HL60 cells; lipid peroxidation.

Abbreviations

DCR – dimethylamide–crotonin; DHC – trans–dehydrocrotonin; FBS – fetal bovine serum; GSH – glutathione; HL60 – promyelocytic leukemia cell line; PS – phosphatidylserine; ROS – reactive oxygen species; TBA – thiobarbituric acid; TBARS – thiobarbituric acid-reactive substance; TCA – trichloroacetic acid

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1. Introduction

Low molecular weight compounds derived from plants are currently being investigated for their ability to regulate apoptosis on programmed cell death, which has received increasing interest because of its importance in cancer therapy [1-3]. In particular, the cytotoxic and antitumor properties of dehydrocrotonin (DHC), obtained from the bark of *Croton cajucara* (sacaca) and its synthetic derivative dimethylamide-crotonin (DCR) have been investigated [4-8]. Wide ranges of anticancer agents, including chemotherapeutic agents, induce apoptosis in malignant cells *in vitro* [9, 10] and apoptosis is important as a mechanism of cell death in the treatment of cancer [2, 11]. Thus, patients with acute leukemia treated with agents such as cytarabine, etoposide, and paclitaxel, show a marked increase in the number of apoptotic blasts. However, the possibility that other cell death processes also play a major role in the regression of solid tumors after treatment cannot be excluded [12].

The generation of reactive oxygen species (ROS) can cause cell death either by apoptosis or necrosis. Necrosis usually occurs in response to severe injury to the cell and is characterized morphologically by cytoplasmic and mitochondrial swelling, plasma membrane rupture and release of the cell contents into the extracellular space. These events are followed by an inflammatory response can cause further injury or even death to neighboring cells [13]. In contrast, apoptosis is a tightly regulated form of cell death in which the cell effectively partakes in its own demise. The execution of the death program is characterized by morphological and biochemical changes which include mitochondrial depolarization, alterations in phospholipid asymmetry, generation of ROS, cell shrinkage, and nucleosomal DNA fragmentation [14]. Many of the morphological changes associated with apoptosis are orchestrated by the activation of a cascade of proteases termed caspases [15, 16]. These cysteine proteases are activated by two distinct pathways: apoptosis–initiated death receptors such as Fas or TNF, which require caspase-8 or –10 in the receptor complex; apoptotic stimuli such as chemotherapeutic agents induce mitochondrial damage to facilitate the release of cytochrome c which, together with dATP, binds to Apaf-1 and changes its conformation. The subsequent addition of caspase–9 to this complex results in activating of the complex through auto processing. Activated caspase–9 then cleaves caspases–2, –6 and –7. Thus, cytochrome c–mediated caspase activation through caspase–9 may serve as an amplification mechanism during apoptosis initiated by the release of cytochrome c [17-19].

A role for oxidative stress in the induction of apoptosis is suggested by the observation that lows levels of ROS induce apoptosis whereas antioxidants such as N-acetylcysteine (NAC) inhibit cell death. Additionally, ROS generation occurs following the treatment of cells with various agents, including chemotherapeutic drug [20]. The ability of oxidative stress to provoke apoptosis through massive cellular damage has been associated with lipid peroxidation and alterations to proteins and nuclei.

Glutathione (GSH) has a pivotal role in numerous cellular functions, including the maintenance of enzyme activity, amino acid transport, protection against harmful oxidative species, the detoxification of xenobiotics, and the suppression of apoptosis [21]. The importance of GSH in modifying the cellular response to several anti-cancer therapies has become clearer with the introduction of agents which can either

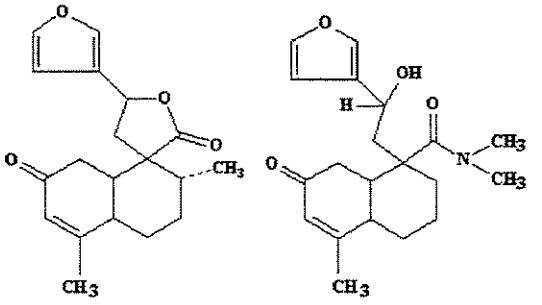
decrease or elevate GSH levels in cells and tissues. In general, GSH depletion enhances the cytotoxicity of several chemotherapy drugs and of nitroimidazole hypoxic cell radiosensitizers [22, 23]. Jacobson et al. [24] presented a model for apoptosis in which a cytoplasmic control system involving a cascade of caspases orchestrates multiple downstream events (such as cell shrinkage and chromatin condensation) that comprises apoptosis. Variations in GSH represent one of these characteristic downstream changes. A loss of cytoplasmic GSH influences the redox buffering capacity of the cell such that it becomes less able to tolerate the presence of oxidants. When mitochondrial GSH is also eventually depleted (this pool is significantly more resistant to depletion than to that in the cytosol), energy production is affected. The cell consequently swells and undergoes "secondary necrosis" [25].

We recently showed that DHC and DCR have cytotoxic effects on the promyelocytic leukemic cell line HL60 [26, 27] and that both compounds induce apoptosis in HL60 cells, as assessed mainly by morphological changes [7, 8]. In this study, we evaluated the activity of caspases (–2, –6 and –9) and assessed phosphatidylserine externalization by flow cytometry in HL60 cells treated with DHC and DCR. Changes in the levels of GSH and oxidative stress-induced lipid were also examined.

2. Material and Methods

2.1. DHC and its derivative DCR

DHC was obtained from *C. cajucara* bark as described by Souza-Brito et al. [28]. The DHC molecule was opened to yield dimethylamide-crotonin by the dimethylamine reaction described by Cromwell and Cook [29] and modified by Melo et al. [5]. The compounds are shown in figure 1. The purity of DHC and DCR was >99%, as confirmed by nuclear magnetic resonance, ultraviolet and infrared spectra and mass spectroscopy [5, 6].



Dehydrocrotonin **Dimethylamide-Crotonin**

Figure 1. Dehydrocrotonin (DHC) and Dimethylamide-Crotonin (DCR).

2.2. Leukemia Cell Culture

Human leukemia cells (HL60) were kindly provided by Dr. Rui Curi, of Laboratory of Cellular Metabolism and Regulation, Department of Physiology and Biophysics, Institute of Biomedical Sciences (ICB), USP, São Paulo, Brazil. The cells were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU of penicillin/mL and 100 μg of streptomycin/mL in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were passaged twice a week and were used in the exponential growth phase at passage 12-26. For all assays, the cells were grown in culture bottles (3 x 10^5 cells/mL) and incubated with different concentrations of DHC or DCR (100, 150 and 200 μM) for 24, 48 or 72 h.

2.2.1. Annexin V-FITC

Apoptotic cells were detected using an ApoDETECT^{TM} Annexin V-FITC kit (Zymed Laboratories Inc., USA). Based on its affinity for phosphatidylserine (PS), annexin V can be used as a sensitive probe for cell surface exposure of PS. HL60 cells (3 x 10^5/mL) were treated with 150 and 250 μM of DCR or DHC

for 12, 24, 48 and 72 h. After washing in PBS, pH 7.4, the cells (1 x 10^6 cells/mL) were resuspended in binding buffer and annexin V-FITC was added. The cells were incubated at room temperature for 10 min and washed with binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). After the addition of 20 µg of propidium iodide/mL, the cells were analyzed by flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) at excitation wavelength of 488 nm (for PI) and 530 nm (for FITC). Data were acquired with CellQuest acquisition software, version 3.3 (BD Biosciences). A total of 10,000 events were collected for each sample and analyzed with WinMDI version 2.8. The cells also were visualized using ZEISS LSM 510 confocal microscope (Carl Zeiss, Jena, Germany) using fluorescence excitation wavelengths of 488 nm and 514 nm (argon ion laser). The images were processed with Adobe PhotoShop (Abacus Inc.).

2.2.2. Caspase –2, –6 and –9 activities

Caspase activities were measured using colorimetric protease kits purchased from R&D Systems (Minneapolis, MN), according to the manufacturer's recommendations, after incubation of the cells with DHC or DCR for 06-72 h. The assays are based on the spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the substrates X-pNA, where X stands for the amino acid sequences VDVAD, VEID and LEHID recognized by caspases-2, -6 and -9, respectively. For the assay, 2 x 10^6 cells were pelleted by centrifugation and lysed on ice. The protein concentration in the lysate was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., USA). Based on the method of Bradford two hundred micrograms of protein were incubated with each X-pNA substrate (200 µM final concentration) at 37°C for 4 h in a microtiter plate after which the absorbance of the samples was measured at 405 nm. The increase in the caspase activity of treated cells was determined by comparing the results with those of untreated control cells after background correction.

2.2.3. Glutathione assay

The GSH concentration was assayed as described by Torsoni et al. [30], with some modifications. HL60 cells (5 x 10^6) were washed with physiological solution and lysed with water (2 mL). To the lysate (2 mL) was added 3 mL of precipitant solution (1.67 g of glacial metaphosphoric acid, 0.2 g of EDTA and 30 g of NaCl in 100 mL of MilliQ water). After 5 min, this mixture was centrifuged and 0.4 mL of the supernatant was added to 1.6 mL of reaction medium (0.2 M Na_2HPO_4 buffer, pH 8.0; containing 0.5 mM DTNB dissolved in 1% sodium citrate). The absorbance of the product (TNB) was measured at 412 nm after 5 min. The GSH concentration was calculated using on extinction coefficient ε = 13.6 $mol^{-1}cm^{-1}$ [30]. The concentration of GSH is given in nmol/10^6 viable cells where cell viability was assessed by trypan blue exclusion. Cells that took up trypan blue were assumed to have lost GSH [31].

2.2.4. Measurement of lipid peroxidation by the TBARS assay

The extent of DCR- and DHC-induced lipid peroxidation was determined by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), a product formed by lipid peroxidation [32]. The assays were done according to Salgo and Pryor [33], with slight modifications. HL60 cells (5 x 10^6) were lysed with 15 mM of Tris/HCl (5 mL) for 60 min. To the lysate were added 2 mL of precipitant

solution (TCA, 40 g/mL) followed by incubation with TBA (0.67 g/mL) for 15 min at 100°C, after which mixture was centrifuged (750 g for 10 min). The relative fluorescence was measured with excitation at 515 nm and emission at 553 nm in a Sim-Aminco (SPF 500) spectrophotofluorimeter. The concentration of TBARS was expressed as equivalents of 1,1,3,3-tetraethoxypropane which was used as the standard.

2.2.5. Cell viability

To assess cell viability and the protective effect of GSH, HL60 cells were seeded ($3x10^5$ cells/mL) in 96 well-plates containing 15 mM of reduced glutathione (GSH) (Sigma®) and incubated with different concentrations of DCR for 72 h. Cell viability was determined by MTT reduction [8]. The tetrazolium reduction assay was done as described by Denizot and Lang [34]. Briefly, 0.1 mL of serum–free medium containing MTT (1 mg/mL) was added to each well. After 4 h of incubation, the supernatant was removed and the blue formazan product obtained was dissolved in 0.1 mL of ethanol with stirring for 15 min on a microplate shaker after which the absorbance at 570 nm was read.

2.3. Statistical analysis

Each experiment was done at least three times (four replicates each) and the results were expressed as percentages (mean ± SD) of the controls. Statistical comparisons were done using one-way ANOVA or Student's t-test as calculated by Origin version 6.0. A probability of $P<0.05$ was considered significant.

3. Results

3.1. Apoptosis induction

In previous work, we showed that DHC and DCR were cytotoxic to HL60 cells, based on the morphological changes characteristic of apoptosis, including chromatin condensation and DNA fragmentation as assessed by the diphenylamine assay, agarose gel electrophoresis and the Feulgen reaction [7, 8, 26]. In contrast, Freire et al. [27] showed that 360 µM DHC induced necrosis in HL60 cells, probably because of the high concentration used. To obtain biochemical evidence for the induction of apoptosis by DHC and its derivative DCR, leukemic cells were treated with DCR and DHC for 12, 24, 48 and 72 h at concentrations of 150 and 250 µM and then labeled with annexin V-FITC and propidium iodide. As shown in figure 2, apoptosis affected approximately 55% of the cells after 48 and 72 h of treatment (figure 2 C, D). Figure 3 shows the percentage of apoptotic cells as determined by flow cytometry. The morphology of apoptotic cells seen in laser scanning confocal microscopy after annexin V labeling with or without simultaneous staining propidium iodide (Ann^+/PI^+; Ann^+/PI^-) is showing in figure 4 A-C.

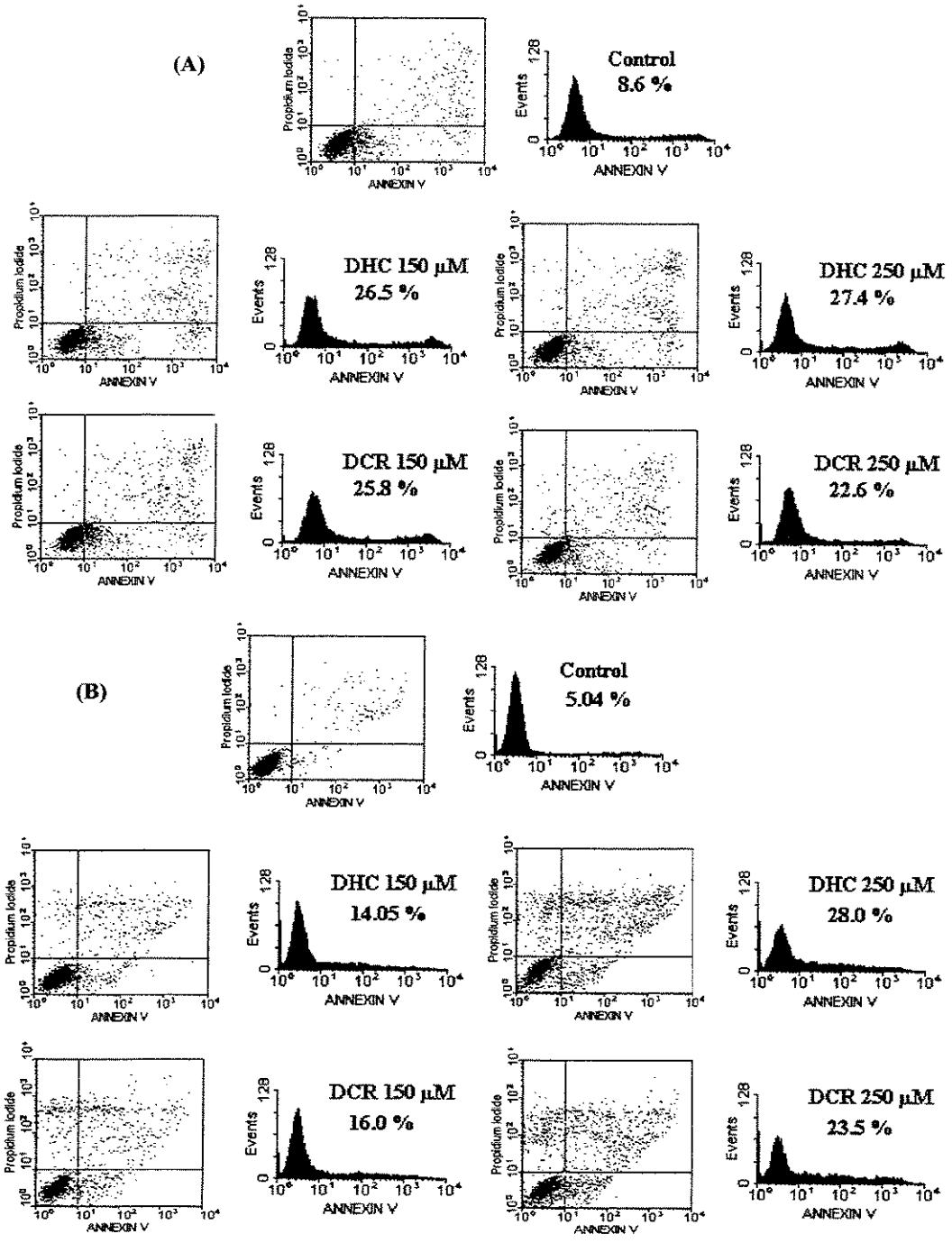
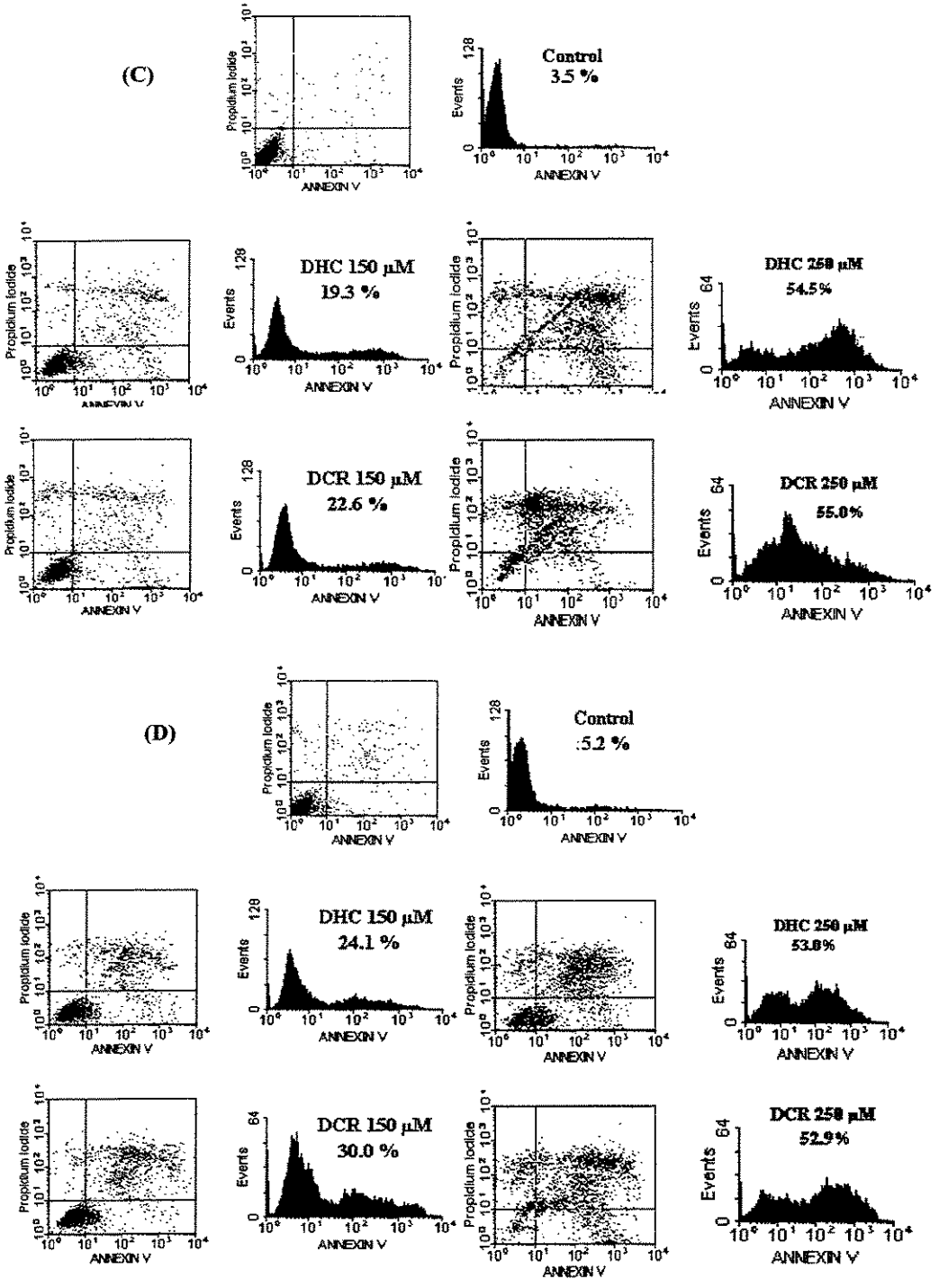


Figure 2. (A-D) Flow cytometric analysis of HL60 cells treated with dehydrocrotonin (DHC) and dimethylamide-crotonin (DCR). Flow cytometry dot plots and their respective histograms for the simultaneous binding of annexin V-FITC and propidium iodide (PI) uptake by following exposure of HL60 cells to DHC and DCR (150 and 250 µM) for **(A)** 12 h, **(B)** 24 h, **(C)** 48 h and **(D)** 72 h. The lower left quadrant of each panel contains the viable cells (annexin V^-/PI^-), the lower right quadrant represents the early apoptotic cells (annexin V^+/PI^-), the upper right quadrant shows the late apoptotic cells (annexin V^+/PI^+) and the upper left quadrant represents necrotic cells.



3.2. Caspase activity

Since most inducers of oxidative stress can also trigger apoptosis by caspase activation, we examined the effects of after a 06 h exposure to both compounds. Whereas caspases–2 and –6 were DHC and DCR on the activity of caspases-2, -6 and –9 in HL60 cells after 06, 12 and 72 h of treatment (figure 5 A, B). Caspase-9 was the first to be activated activated after 12 h of treatment. The kinetics of the activities of caspases –2, -6

and –9 shown that the activation induced by DHC and DCR was significant only after 12 h of exposure. These results suggested that after 12 h of exposure, other downstream effector caspases were activated. A decrease in caspase-2, -6 and –9 activities occurred after 72 h of treatment with both compounds.

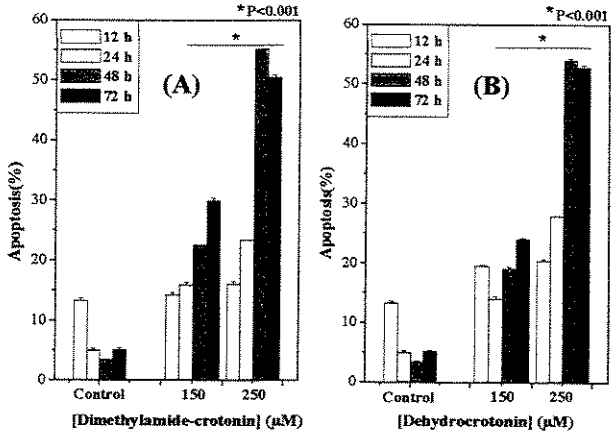


Figure 3. The percentage of apoptosis induced by DCR **(A)** and DHC **(B)** in different times of exposure and concentrations based on data obtained by flow cytometry experiments. (*P<0.001 compared to control)

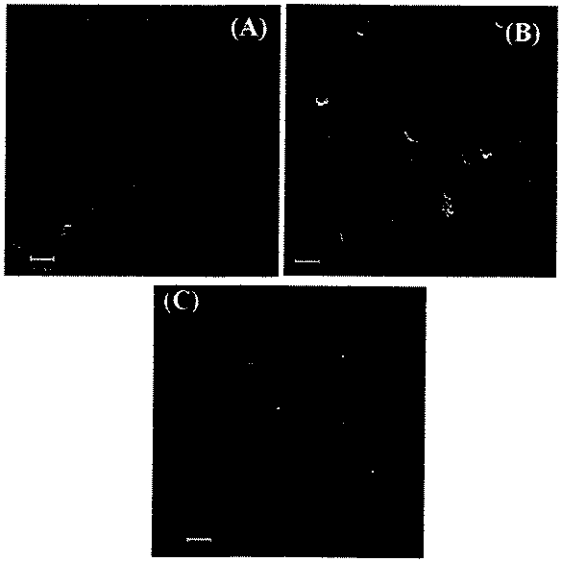


Figure 4. (A-C) Laser scanning confocal photomicrographs of HL60 cells staining with annexin V-FITC/propidium iodide viewed 24 h after the following treatments: Control **(A)**, 100 μM dimethylamide-crotonin **(B)** and 100 μM dehydrocrotonin **(C)**. (Magnification of 2.000x).

3.3. Effects on glutathione levels

As certain forms of sesquiterpene lactones were reported to conjugate with sulphydryl groups intracellular thiols play critical roles for cell survival [35], we hypothesized that dehydrocrotonin induces apoptosis probably by depleting the intracellular GSH.

HL60 GSH levels were initially determined by Xu and Thornalley, [31]. The effects of DCR and DHC on GSH levels were evaluated after 72 h in culture, using concentrations close to the IC_{50} (100 and 150 μM) determined in previous work [8, 27]. DHC and DCR depleted the intracellular GSH levels in HL60 cells, with DCR being the more potent at 100 μM (figure 6 A). However the GSH decreasing provoked by DCR 150 μM was similar to DHC (figure 6 (A)).

3.4. Lipid peroxidation assay

To investigate the oxidative damage triggered by GSH depletion in HL60 cells, we examined the degree of lipid peroxidation after treatment with DHC or DCR. The extent of DCR- and DHC-induced lipid peroxidation was determined by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), a product formed during lipid peroxidation.

The GSH depletion by DHC and DCR caused a significant, dose-dependent increase in TBARS production (figures 4 A, B). Lipid peroxidation increased 3.5- and 4.5-fold in HL60 cells treated with DHC and DCR (both at 150 μM), respectively. DCR, which caused greater GSH depletion at 100 μM, also produced greater lipid peroxidation.

3.5. Effects on cell viability and the protective effect of GSH

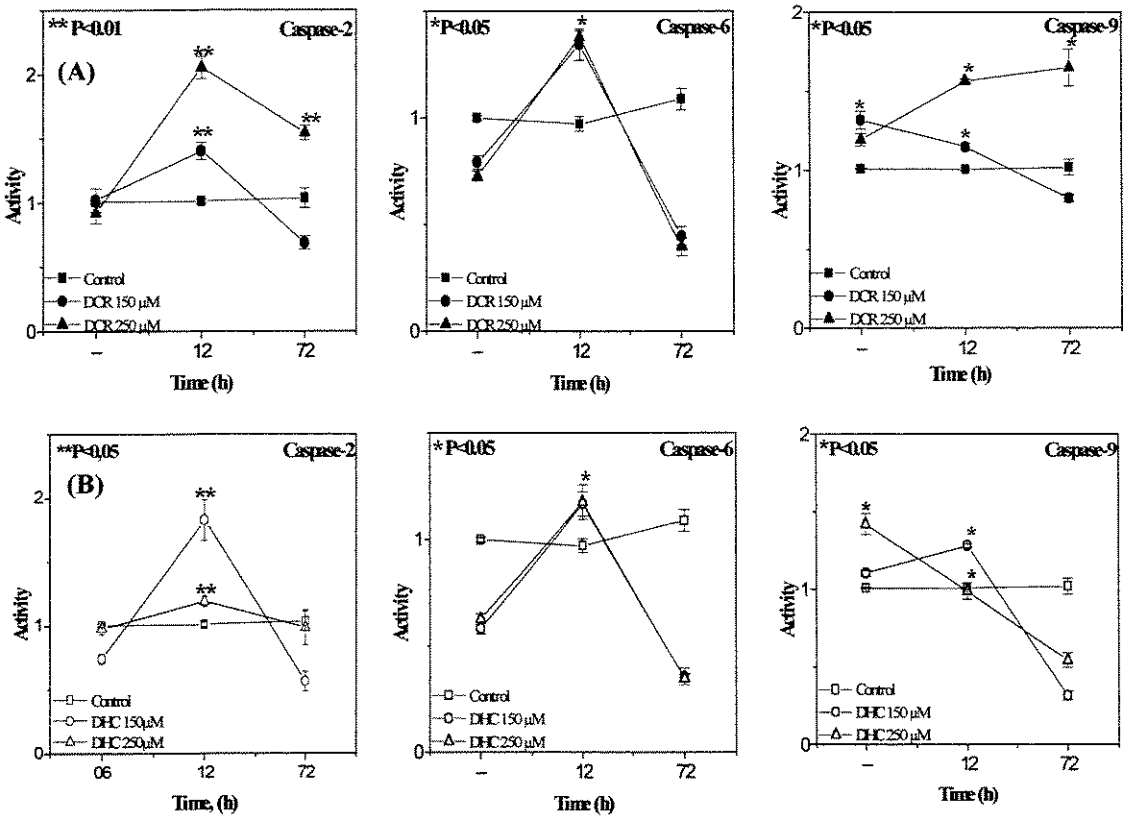


Figure 5. Caspases-2, -6 and –9 activities in HL60 cells treated with dimethyalamide-crotonin **(A)** and dehydrocrotonin **(B)** for 06 h, 12 h and 72 h. The points are means ± SD of four measurements (*P<0.05 and **P<0.01 compared to control).

As shown previously, DHC and DCR are cytotoxic to HL60 cells [7, 8, 27]. The effects of DCR and DHC on the viability of HL60 cells and the protective effect of GSH were assessed after 72 h in culture. Based on the MTT viability assay, the IC_{50} for DCR and DHC were 100 and 180 µM, respectively [8, 27]. To examine the ability of glutathione to protect against the cytotoxic effects of DHC and DCR, HL60 cells were treated simultaneously with reduced glutathione (15 mM) and different concentrations of DHC or DCR (figure 7). Figure 7 A, B shows that the GSH concentration (15 mM) used had no cytotoxic effect on control HL60 cells because GSH occurs in millimolar concentrations in most eukayotic cells [36]. As shown in figure 7 A, B, the GSH present in the culture medium protected cells treated with DHC more efficiently those treated with DCR in concentrations around IC_{50}, 150-200µM (95% and 60% of protection, respectively). The evaluation by trypan blue exclusion confirmed the results obtained by MTT reduction (data not shown). Thus, the addition of GSH (15 mM) to the culture medium did not completely protect HL60 cells from cytotoxic effects of the DCR.

4. Discussion

The importance of apoptosis in many diseases, including cancer, has lead to the use of various compounds that may to inhibit or trigger this fundamental cellular process [2]. In this context, leukemic cells, including HL60, can provide a useful model for studying the induction of apoptosis by antitumor agents [9, 10].

The antiproliferative, and antitumor activity of sesquiterpene lactones has received increasing attention because of the potential usefulness of these

compounds in suppressing the growth of preclinical tumors and in reducing the recurrence of cancer during therapy. Sesquiterpene lactones can inhibit the growth of various types of cancer cells [4, 37, 38]. Moreover, some diterpenes isolated from *Cistus incanus* and *Laetia corymbulosa* also have a cytotoxic effect on several tumor cell lines by interaction with macromolecules [39]. These natural compounds may regulate tumorigenesis and/or the growth of cancer cells by inducing apoptosis in malignant cells. Sesquiterpene lactones have functional groups that are responsible for their

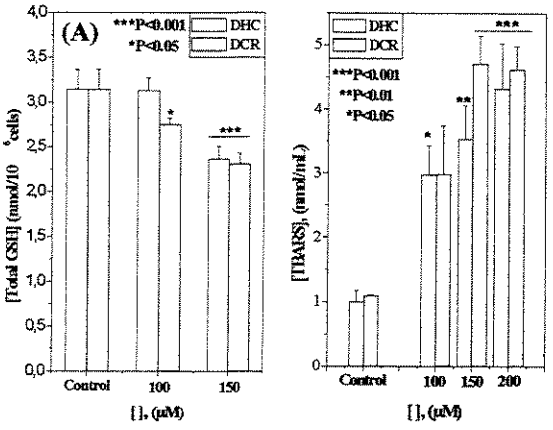


Figure 6. Determination of the glutathione (GSH) activity levels **(A)** and Lipid peroxidation **(B)** in HL60 cells after exposure to DCR and DHC in different concentrations for 72 h. The data are mean ± SD from three samples. (*P<0,05; **P< 0,01; ***P<0,001, compared to control cells).

biological effects. The structure of DHC contains three highly reactive functional groups: O=C-C=CH_2, a lactone and cyclopentenone. Since the structure of DHC is not planar [27], the most available group is O=C-C=CH_2, which can function as a Michael acceptor to interact with the SH groups of proteins, GSH and nitrogen bases, mainly guanine, through nucleophilic attack [5]. DCR has the same reactive functional groups as DHC except for the lactone, and also has another Michael acceptor (O=C-N-$(CH_3)_2$) in its structure.

The biological effects of sesquiterpene lactones have been attributed to these compounds ability to deactivate enzymes and other essential proteins by forming covalent bonds with sulphydryl groups. Several sesquiterpene lactones may generate a reactive intermediate capable of damaging cellular constituents [39, 40].

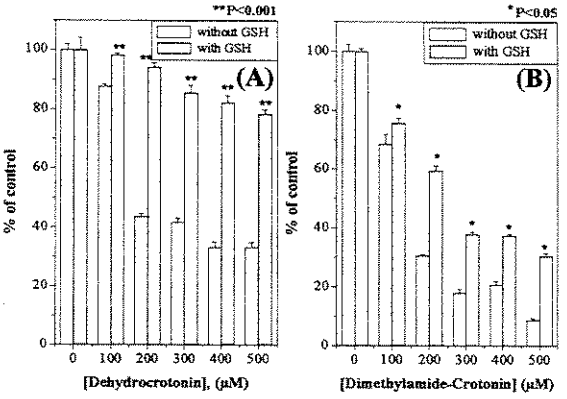


Figure 7. Protective effect of GSH (15 mM) on the viability of HL60 cells treated with DCR (A) or DHC (B) for 72 h as evaluated by MTT reduction. Each column represents the mean ± SD of three experiments in six replicates. *P<0,05 and **P<0,001 compared to the groups without GSH.

The oxidative stress provoked by reaction of these compounds with molecular oxygen results in the formation of hydrogen peroxide, which alters the intracellular redox equilibrium. These data suggest that reactive oxygen species play an important role in the cytotoxicity of DHC and DCR as also confirmed by the depletion of GSH and lipid peroxidation assays.

HL60 cells treated with DCR and DHC showed morphological changes characteristic of apoptosis, including chromatin condensation and DNA fragmentation [7, 8]. As shown here, FITC-labeled annexin V stained HL60 cells with and without simultaneous staining with propidium iodide staining (Ann^+/PI^+ and Ann^+/PI^-, respectively).

When done simultaneously with a dye exclusion test such as propidium iodide, the measurement of annexin V binding effectively discriminates between apoptotic and necrotic cells. The flow cytometry and confocal microscopy results confirmed that DHC and DCR induced apoptosis with similar efficiencies, thus corroborating our previous results [8]. The cells with double marking represented the final stages of apoptosis (secondary necrosis).

The involvement of caspases in the apoptosis induced by DCR and DHC was verified by measuring the activities of caspases-2, -6 and –9. Caspases–2 and -9 are key activators of the caspases cascade *in vitro* and were important for the apoptotic responses in HL60 cells. The activation of caspases-2 and -9 in the apoptosis induced by both compounds indicated involvement of the mitochondrial pathway. However, additional studies are required to elucidate the involvement of other caspases and of the mitochondrial pathway in this process. The activation of caspase-9 suggested that cell death by apoptosis was associated with the perturbation of mithochondrial function through increased generation of ROS. DCR and DHC showed similar efficiencies in inducing apoptosis, but DCR was more effective than DHC in inducing differentiation [8].

The relationship between GSH and carcinogenesis has attracted attention because GSH plays a key role in protecting cells from toxicity by maintaining the intracellular redox status, through conjugation with electrophilic xenobiotics and free radicals to detoxifying reactive peroxides. Thus, GSH depletion is showed enhance the activity of cytotoxic anticancer agents by reducing in the protection against oxidative stress. The depletion of GSH by DHC and DCR could contribute to the significant dose-dependent increase seen in TBARS production. DCR was more potent than DHC in causing lipid peroxidation. Analyzing the spectra (UV/Vis) of DHC or DCR in the presence of GSH (15 mM) we noted that these compounds form conjugates with GSH. The GSH depletion detected in the treated cells probably was due to the rapid binding of $O=C-C=CH_2$ system of DHC and DCR with GSH.

To verify the indirect involvement of oxidative stress in HL60 cell viability after treatment with DHC and DCR, due to conjugation between GSH and these compounds, we investigated the effect of GSH supplementation on the cytotoxicity of these two compounds. GSH (15 mM) fully protected HL60 cells the effects of DHC, but was only partially effect in protecting against DCR. In the later case, the amount of GSH (15 mM) added to culture medium may have been insufficient to completely protect the HL60 cells from the cytotoxic effects of DCR. Xu and Thornalley [31] also showed that the inhibition of HL60 cell growth by phenethyl isothiocyanate was totally prevented by the simultaneous incubation with 15 mM GSH. Thus, maintenance of the intracellular concentration of GSH may prevent phenethyl isothiocyanate-induced apoptosis in an also similar manner to that seen with DHC- and DCR-induced cytotoxicity.

In conclusion, DHC and DCR induced apoptosis in the HL60 cells with the possible indirect involvement of oxidative stress due to conjugation between the compounds and intracellular GSH leading to glutathione depletion and the simultaneous increasing in lipid peroxidation. Annexin V labeling of HL60 cells treated with DHC and DCR confirmed the translocation of PS to the extern surface of the plasma membrane, an indicator of apoptosis. Despite, the

chemical changes in the original moiety, DCR was still cytotoxic and able to trigger apoptosis to a similar extent to DHC The activation of caspases –2, –6 and –9 suggested involvement of the mithochondrial pathway in the induction of apoptosis, probably via cytochrome c release and apoptosome formation. Reactive oxygen species also appear to play an important role in the apoptosis induced by the DHC and DCR since the addition of GSH culture medium partially protected the cells from the cytotoxic effects of DCR. This supplementation completely protected the cells from the cytotoxic effects of DHC. Thus, the conjugate formation verified by spectroscopic analysis (data not shown) provoked the GSH depletion that could be leading to apoptosis induction.

These results provide basis for the potential therapeutic use of DHC and DCR in cancer therapy. Identification of the receptor(s) for DHC and DCR that initiate the signaling for apoptosis and the induction of phase II enzyme expression may lead to improved design of these agents for therapeutic use.

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IV – DISCUSSÃO GERAL

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(Anazetti, M.C., Melo, P.S., Durán, N. and Haun, M. Comparative Cytotoxicity of Dimethylamide-Crotonin in the Promyelocytic Leukemia Cell Line (HL60) and Human Peripheral Blood Mononuclear Cells. Toxicology. 188, 261-274)

1. Citotoxicidade da Dimetilamida-crotonina (DCR) em Células da Leucemia HL60 e em Linfócitos Humanos

Um balanço entre os efeitos terapêuticos e toxicológicos de um composto é um importante parâmetro quando sua aplicabilidade farmacológica está em estudo. Cultura de células de mamíferos fornecem ferramentas importantes para a avaliação da citotoxicidade de compostos com potencial atividade terapêutica (Pailard et al., 1999).

Os resultados dos testes de citotoxicidade da DCR em células HL60, realizados após 72 h de tratamento, baseados na redução do MTT, atividade fosfatásica e exclusão do azul de tripan, mostram que a DCR foi mais tóxica do que a DHC às células HL60 (Anazetti *et al.*, 2003a). É importante destacar que a DCR afeta mais intensamente a redução do MTT (IC_{50} = 65μM). Este fato pode estar relacionado com os mecanismos de indução de morte celular com envolvimento de geração de espécies reativas de oxigênio (EROs), se comparado com os outros testes de viabilidade celular (média do IC_{50} = 150μM).

Estudos anteriores demonstraram que alguns agentes anticâncer podem induzir morte celular em células da leucemia, porém com alta citotoxicidade em células normais, incluindo células da linhagem mielocítica-macrofágica além de outras células do sistema imunológico (Raffray & Cohen, 1997). Assim, ao avaliarmos a atividade anti-tumoral de um composto deve ser verificado se o mesmo apresenta efeito citotóxico sobre células normais em divisão tais como os linfócitos. A capacidade proliferativa de linfócitos avalia as propriedades funcionais dessas células em resposta à presença de mitógenos *in vitro*,

como a fitohemaglutinina (PHA). Ambos os compostos, DHC e DCR, mostraram pouca citotoxicidade em linfócitos, conforme demonstrado pelos valores de IC_{50} determinados (1,5mM e 450μM, respectivamente) (Araújo *et al.*, 2000; Anazetti *et al.*, 2003a). Assim, DCR e DHC inibem o crescimento de células HL60, mas não afeta a proliferação de linfócitos de sangue periférico humano *in vitro*, quando avaliado o parâmetro de redução do MTT. A diferença de citotoxicidade entre os tipos celulares avaliados (HL60 e linfócitos) pode ser devido a características intrínsecas de cada cultura celular.

Em relação à estrutura-atividade, a DHC contém três grupos funcionais altamente reativos: O=C-C=CH_2, uma lactona e uma ciclopentanona. Além disso, a estrutura da DHC não é planar (Freire *et al.*, 2003a), contribuindo para uma maior interação com outros grupamentos químicos. O grupamento O=C-C=CH_2 pode funcionar como aceptor de Michael e interagir com grupos SH de proteínas, GSH e bases nitrogenadas, principalmente guanina, através de ataque nucleofílico (Jodynis-Liebert *et al.*, 2000; Melo *et al.*, 2002). Tanto a DHC como a DCR possuem características estruturais importantes que podem favorecer a interação química com componentes celulares, apesar da abertura da lactona na DCR. Este derivado apresenta ainda um outro aceptor de Michael no resíduo amida que poderia contribuir para sua atividade anti-tumoral, embora essas alterações estruturais tenham contribuído para reduzir sua citotoxicidade em fibroblastos V79 e hepatócitos (Melo *et al.*, 2001, 2002) e em linfócitos como demonstrado neste trabalho (Anazetti *et al.*, 2003a). Em adição, recentemente, alguns autores mostraram que várias sesquiterpeno lactonas podem gerar intermediários reativos capazes de danificar constituintes celulares (Dimas *et al.*, 1999; Beutler *et al.*, 2000). O estresse oxidativo provocado pela reação desses compostos com oxigênio molecular resulta em formação de peróxido de hidrogênio, alterando o equilíbrio redox intracelular. Estes dados sugerem que espécies reativas de

oxigênio geradas também apresentam papel importante na citotoxicidade da DHC. Este efeito também poderia contribuir para a citotoxicidade da DCR nas células HL60.

2. Diferenciação Celular em Células HL60

A maioria das células tumorais permanece em um estado altamente proliferativo, devido à incapacidade de sofrerem maturação terminal a células adultas com capacidade não-replicativa. Portanto, as perspectivas terapêuticas para leucemias consistem na tentativa de controlar a proliferação pela exposição das células tumorais a agentes antineoplásicos indutores de diferenciação celular terminal. As células da leucemia promielocítica aguda HL60 representam um modelo para estudos dos mecanismos envolvidos na indução de diferenciação e apoptose em resposta a agentes anti-tumorais, por constituírem uma linhagem de células bloqueadas para diferenciação no estágio de promielócitos (Verlinden *et al.*, 1997).

A capacidade de células diferenciadas reduzirem o NBT (nitro azul tetrazólio) é um parâmetro inicial na investigação de diferenciação morfológica - funcional. Portanto, nossos resultados de avaliação de indução de diferenciação pela DCR e pela DHC mostraram que as células são capazes de reduzir o NBT de maneira concentração dependente após os tratamentos, indicando indução de diferenciação morfológica - funcional. Além disso, nas mesmas condições experimentais (75 μM e 72 h de exposição) a indução de diferenciação celular pela DCR foi mais significativa que pela DHC, visto que nesta concentração as porcentagens de diferenciação em relação às células controle foram de 8 e 40% para DHC e DCR, respectivamente. Considerando que a técnica do NBT é inespecífica, monócitos e granulócitos podem responder positivamente. Assim, será necessária a realização de futuros estudos, incluindo a determinação dos tipos celulares

presentes nas culturas após o tratamento, utilizando determinantes antigênicos específicos de células diferenciadas.

3. Apoptose

(Anazetti, M.C., Melo, P.S., Durán, N. and Haun, M. Comparative Cytotoxicity of Dimethylamide-Crotonin in the Promyelocytic Leukemia Cell Line (HL60) and Human Peripheral Blood Mononuclear Cells. Toxicology. 188, 261-274)
(Anazetti, M.C., Melo, P.S., Durán, N. and Haun, M. Dehydrocrotonin and its Derivative, Dimethylamide-Crotonin, Induce Apoptosis with Lipid Peroxidation and Activation of Caspases – 2, –6 and –9 in Human Hl60 Leukemia Cells. Leuk. Res. (submitted) páginas 50-65)

3.1. Detecção de Indução de apoptose

Um dos principais objetivos da quimioterapia atual é a destruição das células tumorais pela indução de morte celular por apoptose (Wyllie *et al.*, 1980; Israels and Israels, 1999). Diferentes compostos podem regular o crescimento de células tumorais e/ou tumorigênese via indução de apoptose (Raffray and Cohen, 1997, Mesner *et al.*, 1997; Kaufmann & Earnshaw, 2000). Sesquiterpeno lactonas inibem o crescimento de vários tipos de células tumorais (Rodriguez *et al.*, 1976; Grynberg *et al.*, 1999; Cho *et al.*, 2000). Como a maioria dos indutores de diferenciação em células da leucemia induzem apoptose (Kohroki *et al.*, 1998), nós verificamos se a citotoxicidade da DCR em HL60 foi devido à apoptose. Nossos resultados mostraram que a DHC e a DCR induzem apoptose em células HL60 (Anazetti *et al.*, 2003a, 2003b). Como observado na reação de Feulgen, o tratamento das células HL60 por 72 h, com os compostos em estudo (150 μM) induziu condensação cromatínica, uma alteração morfológica típica do processo apoptótico. A apoptose também foi demonstrada pelo aumento na porcentagem de DNA fragmentado (17 e 27% respectivamente para DHC e DCR, após 72 h de exposição), quantificado pelo método da difenilamina (DPA) (Anazetti *et al.*, 2002, 2003a). Outra alteração característica de

apoptose, a assimetria de membrana após a inativação de translocases e consequente externalização de fosfatidilserina, foi determinada utilizando a marcação com anexina V e o corante iodeto de propídeo (An^+/IP^-) por citometria de fluxo (Anazetti *et al.*, 2003b). As células que mostraram dupla marcação (An^+/IP^+) representam estágio final de apoptose, também chamada de necrose secundária (Inayat-Hussain *et al.*, 2002). A positividade da membrana para a marcação com anexina V foi igualmente evidente nas células tratadas com DCR e DHC, corroborando com os outros resultados sinalizadores de morte celular por apoptose.

Nas células tratadas por 72 h com os compostos em estudo a DCR foi tão efetiva quanto a DHC na indução de apoptose, visto que, nas concentrações de 150 e 250 μM a porcentagem de células em apoptose foi de 30,0 e 55,0% comparado com 24,1 e 53,0% nas células tratadas com DHC respectivamente. É importante destacar que as concentrações utilizadas neste tratamento foram baseadas nos resultados de citotoxicidade da DHC (Freire *et al.*, 2003a) e DCR obtidos anteriormente (Anazetti *et al.*., 2003a).

3.2. Caspases

A ativação de cisteíno proteases (caspases) é um evento característico do processo apoptótico, constituindo a maquinaria celular especializada no desencadeamento de morte celular. As caspases podem ser ativadas via receptores de morte ou via mitocondrial, que requer ativação de caspase-9 (Drexler, 1997; Thornberry & Lazebnik, 1998; Sartorius *et al.*, 2001). Em geral, estímulos apoptóticos tais como hipóxia, agentes quimioterápicos e irradiação induzem alterações mitocondriais facilitando a liberação de citocromo c e formação de apoptossomo, ativação de caspase-9, que cliva as caspases efetoras subsequentes -2, –6 e -7 (Zou *et al.*, 1999; Slee *et al.*, 1999a.,b; Kuida, 2000; Salvesen &

Denault, 2002). Os resultados de ativação deste ensaio indicaram que a caspase-9 é ativadora chave da cascata de caspases *in vitro,* e é importante para a resposta apoptótica observada nas células HL60. A avaliação da atividade das caspases efetoras –2 e –6 corroboram com a ativação da caspase-9 verificada (Anazetti *et al.*, 2003b). A primeira caspase a ser ativada foi a caspase-9 após 6 h de exposição a ambos os compostos (250 μM), permanecendo ativa até 72 h após o tratamento com a DCR e até 12 h após o tratamento com a DHC. Ambos os compostos induziram um aumento na atividade das caspases-2 e -6 após 12 h de tratamento, com semelhante eficiência de ativação. A ativação de caspase-9 sugere que a morte celular por indução de apoptose por ambos os compostos em células da leucemia pode ser mediada pela via mitocondrial. Além disso, essa alteração na função mitocondrial poderia estar relacionada à geração de EROS (Chandra *et al.*, 2000). No entanto, há necessidade de realizar outros estudos para elucidar o envolvimento de outras caspases e para comparar experimentalmente o comprometimento da via mitocondrial neste processo. Após 12 h de tratamento com a DHC e a DCR, ocorreu uma diminuição na ativação das caspases–2, –6 e –2, –6, –9 respectivamente. Estes resultados sugerem que após 12 h de exposição outras caspases efetoras estejam ativas, enquanto a atividade das caspases-2, -6 e –9 diminui. Além disso, a inativação das caspases após um longo período de exposição (72 h) aos compostos pode estar relacionada ao aumento de necrose e de apoptose tardia, com mais de um evento acontecendo ao mesmo tempo, levando a célula a um colapso geral.

3.3. Estresse oxidativo e apoptose

(Anazetti, M.C., Melo, P.S., Durán, N. and Haun, M. Dehydrocrotonin and its Derivative, Dimethylamide-Crotonin, Induce Apoptosis with Lipid Peroxidation and Activation of Caspases – 2, –6 and –9 in Human Hl60 Leukemia Cells. Leuk. Res. (submitted) páginas 50-65)

A glutationa (GSH), tiol de baixo peso molecular envolvido em reações de detoxificação em tecidos de animais, plantas e de microrganismos (Meister, 1984) possui um importante papel no metabolismo de compostos endógenos e também na inativação de muitos xenobióticos reativos, incluindo certas drogas anticancerígenas (Mitchell *et al.*, 1989; Hakimelahi *et al.*, 2002). Sua versatilidade deve-se às suas propriedades químicas de atuar como nucleófilo ou redutor efetivo, interagindo com numerosos compostos eletrofílicos e oxidantes como $O_2^{\bullet}$, $^{\bullet}OH$ e H_2O_2. A GSH é o principal peptídeo redutor em das células, contribuindo com seu grupo sulfidrila para tamponamento e remoção de radicais livres gerados durante os processos metabólicos normais tais como respiração e estresse oxidativo (Voehringer, 1999). Se a situação de estresse oxidativo persiste por tempo prolongado pode resultar em injúria celular com acúmulo de danos oxidativos, incluindo genotóxicos, críticos a biomoléculas. Nestas condições, a homeostase celular pode ser alterada transitoriamente ou permanentemente, com efeitos biológicos sobre a transdução de sinal e expressão gênica para mitogênese, transformação, mutagênese e morte celular (Hensley *et al.*, 2000).

Os níveis de GSH são modificados sob várias condições fisiológicas tais como envelhecimento e neoplasias. Embora um aumento nos níveis de GSH nas células tumorais esteja associado com resistência à irradiação ou quimioterapia, a redução nos níveis de GSH contribui para aumentar a eficácia deste tipo de tratamento em condições de sensibilidade normal aos agentes citodestrutivos. Estudos mostram que a rápida diminuição

dos níveis de GSH intracelular está associada com peroxidação lipídica e morte celular por apoptose (Maellaro, *et al.*, 1990). Tanto a produção aumentada de EROs quanto a queda em GSH mostraram-se estreitamente associadas com uma série de eventos posteriores do processo apoptótico, incluindo a ativação de caspases e a fragmentação de DNA (Liu, *et al.* 2001).

De fato, inúmeros estudos sugerem que o estresse oxidativo possui um papel importante como mediador de apoptose. A habilidade do estresse oxidativo provocar apoptose como resultado do dano celular massivo pode estar associada tanto à indução de peroxidação lipídica quanto a alterações em proteínas (Anuradha *et al.*, 2001; Piwocka *et al.*, 2001). Em trabalhos anteriores, foram avaliados os efeitos citotóxicos da DHC e da DCR em células HL60 (Freire *et al.*, 2001; Anazetti *et al.*, 2002; Anazetti *et al.*, 2003a). Na primeira parte do trabalho mostramos que estes compostos causam apoptose pela reação de Feulgen e fragmentação de DNA (Anazetti *et al.*, 2003a). Na segunda parte do estudo investigamos o padrão de ativação de caspases, a ocorrência de peroxidação lipídica e o papel da glutationa no mecanismo de citotoxicidade dos compostos em células HL60 (Anazetti *et al.*, 2003b). Ainda com o intuito de elucidar a participação do estresse oxidativo na indução de apoptose e na citotoxicidade provocada pelos compostos em estudo avaliamos os efeitos da DHC e da DCR sobre as células HL60 incubadas em meio de cultura suplementado com GSH.

As relações existentes entre GSH e carcinogênese atraíram a atenção de pesquisadores devido ao papel chave que este tripeptídeo intracelular representa na proteção das células em eventos de toxicidade. Com a diminuição de GSH, espera-se potencializar a atividade citotóxica de agentes anti-tumorais. Tanto a DHC como a DCR induziram uma diminuição de 29% nos níveis de GSH das células tratadas com uma

concentração de 150 μM (IC_{50} médio), durante 72 h. Porém, uma maior redução nos níveis de GSH foi observado após exposição das células à 100 μM de DCR por 72 h, sendo 13% inferior ao nível normal (células controle). Nestas condições nenhum efeito foi verificado para a DHC. Este efeito, além de contribuir no processo apoptótico devido à redução da capacidade tamponante redox da célula e alteração de permeabilidade de membrana mitocondrial, também pode estar associado ao aumento concentração-dependente na produção de TBARS observado em nossos experimentos. A este respeito, ambos os compostos mostraram a mesma eficiência na indução de peroxidação lipídica, havendo uma produção de TBARS 4 vezes maior em relação ao controle em ambos os tratamentos. Estes resultados sugerem que EROs participam dos efeitos citotóxicos da DHC e da DCR e consequentemente da indução de apoptose em células da leucemia, com envolvimento da via mitocondrial e ativação da caspase-9 (Anazetti *et al.*, 2003b). Quando o nível de GSH mitocondrial eventualmente também é diminuído, embora esta fração seja mais resistente a uma redução significativa quando comparada com a fração citosólica, a produção de energia é afetada. Consequentemente, a célula incha, perde a integridade de membranana plasmática e pode sofrer o processo de "necrose secundária" ou "apoptose tardia" (Jacobson *et al.* 1994; Slater *et al.*, 1995). Estes fatos em conjunto explicam os resultados obtidos no ensaio de marcação com anexina V/PI, no qual houve um aumento na porcentagem de células duplamente marcadas (quadrante superior direito nos gráficos de citometria) após 48 e 72 h de tratamento com ambos os compostos. Além disso, a ativação de caspase-9 reforça esta hipótese devido a sua relação com as alterações produzidas na função mitocondrial e com o aumento na geração de EROS.

Ainda considerando o papel do estresse oxidativo na citotoxicidade dos compostos investigou-se o efeito da suplementação do meio de cultura com GSH sobre a viabilidade

das células HL60 tratadas com a DHC e a DCR. Enquanto a presença de GSH 15 mM no meio de cultura foi eficiente em inibir totalmente a citotoxicidade da DHC, apenas uma proteção parcial foi observada no tratamento com a DCR. Estes resultados apontam para um maior efeito oxidante da DCR em comparação com a DHC. Outros estudos demonstraram que a formação de conjugado entre parte da molécula de um terpeno e GSH conferiu à célula maior susceptibilidade à ação de EROs, culminando na indução de apoptose (Schmidt, 1997; Choi *et al.*, 2002). Segundo estes autores, a conjugação ocorre em locais da molécula duplas conjugadas, sejam endo ou exo. Analisando os espectros (UV/Vis) da DHC e da DCR na presença de GSH 15 mM, nós observamos deslocamentos na absorção de luz, sugerindo que esses compostos formam conjugados com o GSH. Provavelmente, a diminuição nos níveis de GSH obtida nos tratamentos foi devido à rápida ligação entre o sistema O=C–C=CH_2 da DHC e da DCR com a GSH, porém outros ensaios são necessários para confirmar esta hipótese (Anazetti *et al.*, 2003b).

Em resumo, nossos resultados sugerem que a DHC e a DCR induzem apoptose em células HL60 com envolvimento indireto de estresse oxidativo, devido à conjugação entre os compostos e GSH intracelular, levando à diminuição dos níveis de glutationa e aumento simultâneo na peroxidação lipídica. Mesmo com as alterações químicas na molécula original, a DCR manteve a citotoxicidade e a capacidade de desencadear apoptose da molécula original, a DHC. Além disso, a ativação das caspases–2, –6 e –9 indicam o envolvimento da via mitocondrial no mecanismo de indução de apoptose (Anazetti *et al.*, 2003b).

V - CONCLUSÕES

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Os ensaios experimentais *in vitro* realizados com a dimetilamida-crotonina (DCR) e com a desidrocrotonina (DHC), mostraram que:

- A DHC e a DCR inibem o crescimento de células HL60 com semelhante toxicidade, apesar da DCR não possuir o anel α-metileno-γ-lactona, envolvido na atividade biológica de terpeno lactonas, em sua estrutura.
- Ambos os compostos, DHC e DCR, induzem diferenciação morfológica e apoptose em células da leucemia HL60 em concentrações próximas ao IC_{50} (150 μM). No entanto, a DCR é mais efetiva na indução de diferenciação celular do que o composto original.
- A DCR, assim como o seu composto original, apresenta baixa toxicidade aos linfócitos humanos normais. Portanto, a DCR é um composto com maior potencial farmacológico visto que é menos tóxica às células "normais" (fibroblastos V79, hepatócitos e linfócitos) e possui atividade anti-leucêmica similar ao composto original. Os resultados indicam que a toxicidade destes compostos, em células HL60, seja devido à indução de apoptose, sem alterar atividade funcional de células do sistema imunológico.
- A DCR e a DHC provocam redução nos níveis de GSH, provavelmente devido à conjugação entre o grupamento $O=C-C=CH_2$ de ambos os compostos com a GSH contribuindo para a indução de apoptose.

VI - PERSPECTIVAS

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Em função dos resultados obtidos neste trabalho, novos experimentos poderão ser realizados visando:

- Determinar os tipos celulares presentes na cultura de células HL60 após o tratamento com dimetilamida-crotonina (DCR) e desidrocrotonina (DHC), utilizando determinantes antigênicos específicos de células diferenciadas e analisar a expressão diferencial de genes.
- Analisar a proliferação das células HL60 após o tratamento com DHC e DCR utilizando a técnica de incorporação de bromodesoxiuridina (BrdU). Além disso, verificar em qual fase do ciclo celular as células encontram-se após o tratamento, através de análises por citometria de fluxo.
- Avaliar a proteção das células HL60 contra a toxicidade da DHC e da DCR utilizando outros antioxidantes, tal como N-acetil cisteína (NAC).
- Estudar a relação entre a diminuição dos níveis de GSH e a expressão de proteínas anti-apoptóticas da família Bcl-2.
- Determinar experimentalmente a ocorrência de liberação de citocromo c e alteração de permeabilidade de membrana mitocondrial ($\Delta\psi$).
- Avaliar a ativação de outras caspases que podem estar involvidas no mecanismo de ação dos compostos (DHC e DCR) utilizando inibidores e/ou anticorpos específicos para caspases tais como, -3, -8 e -10 (western blotting).

Com o intuito de melhorar a eficácia terapêutica (atividade anti-tumoral) e estabilidade da DHC, assim como, reduzir os efeitos colaterais (diminuir sua citotoxicidade a células não alvo), no trabalho de doutorado, será desenvolvido um sistema polimérico de

liberação controlada de drogas para a desidrocrotonina, constituído por micro/nanoesferas de PLGA (copolímero poli (L-láctico-co-glicólico)). Os novos métodos para a administração de drogas se baseiam no encapsulamento das drogas em micro/nanoesferas poliméricas. Alguns fármacos já são utilizados há décadas nos mais variados tratamentos terapêuticos através da administração em sistemas de liberação controlada de drogas, compreendendo as formulações clássicas como soluções, suspensões e comprimidos. Atualmente, muitos estudos visam otimizar a resposta terapêutica. Uma das alternativas para isso é controlar a ação dos fármacos, seja como pró-fármaco, seja promovendo alterações nas propriedades físico-químicas do composto e na sua interação com os sistemas biológicos (Rennó, 2001). Uma alternativa para a obtenção de sistemas de liberação controlada de drogas é a utilização de carreadores poliméricos que possibilitam uma ação mais seletiva e um controle da taxa de liberação dos compostos. Um exemplo é a incorporação do Taxol® em nanoesferas de PLGA, que levou a um aumento de 100 vezes na sua eficácia como anti-tumoral (Feng & Huang, 2001).

Portanto, será avaliada comparativamente a atividade anti-tumoral da DHC, na forma livre e encapsulada, utilizando duas linhagens de células de leucemia mielóide humana: HL60 e U937 e uma linhagem de células de leucemia eritróide: K562. Para isso serão utilizadas metodologias para investigação da indução de diferenciação celular e de apoptose. Utilizaremos também a cultura primária de linfócitos humanos, como parâmetro de avaliação da citotoxicidade das formulações em estudo sobre células do sistema imunológico. Este trabalho já foi iniciado utilizando como polímero a poli-caprolactona e, de acordo com os resultados preliminares, a encapsulação da DHC resultou em um aumento

do efeito anti-leucêmico (anexo III: *Cytotoxicity of ε-caprolactone nanospheres incorporating dehydrocrotonin on leukemic cells, páginas 113-117*).

VII – REFERÊNCIAS BIBLIOGRÁFICAS

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VIII - ANEXO

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I- ATIVIDADES DESENVOLVIDAS

1. Cursos Extra-Curriculares

1- "Apoptosis in the Immune System", Prof. Dr. Thomas Brunner, from Pathology Institute of Bern University (Switzerland), realizado na Universidade de São Paulo – USP, de 6 a 10/05/2002.

2- "Citometria de Fluxo", ministrado pelo Prof. Dr. Moisés Bauer (PUCRS) e Profa. Dra. Denise Cantarelli Machado (PUCRS), durante o XI Congresso de Biologia Celular, em Porto Alegre – RS, de 16 a 19/07/2002, duração de 16 h.

3- "First Brazilian Winter School on Nanobitoechnology", organizada pela Rede Nanobiotech, em Campinas, São Carlos e Ribeirão Preto – SP, de 12 a 23/08/2002.

4- "Curso de Microscopia Eletrônica de Varredura", organizado pelos professores Elliot Watanabe Kitajima e Breno Leite, no NAP/MEPA, ESALQ/USP, em Piracicaba - SP, de 27 a 29 de janeiro de 2003.

5- Especialização (*lato sensu*) em Jornalismo Científico, no Laboratório de Estudos Avançados em Jornalismo da UNICAMP, sob coordenação do Prof. Dr. Carlos Vogt. Ingresso em março de 2003 e término previsto para julho de 2004.

2. Congressos Nacionais

1- **XXVIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular-SBBq**, trabalho: "*Study of effects of the dexametasone and hydrogen peroxide on HL60 cells enhancing cytotoxicity, differentiation and apoptosis*", Fabrin, J.B., **Anazetti, M.C.**, Melo, P.S., Ferreira, C.V., Haun, M., Durán, N., em Caxambu - MG, de 19 a 22/05/2001.

2- **XXVI Meeting of the Brazilian Society of Immunology**, trabalho: "Dimethylamide-crotonin inhibits HL60 cell growth with less cytotoxicity on normal lymphocytes" **Anazetti, M.C.**; Melo, P.S.; Durán, N.; Haun, M., em Campos de Jordão – SP, de 07 a 10/10/2001.

3- **III Simpósio Temático sobre Citoesqueleto & Diferenciação Celular**, na Universidade de São Paulo – USP, nos dias 29 e 30/11/2001.

4- **XXIX Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular-SBBq**, trabalho: "*Dimethylamide-Crotonin triggers apoptosis and decreases glutathione levels on human leukaemic cells*", **Anazetti, M.C.**; Melo, P.S.; Durán, N.; Haun, M., em Caxambu - MG, de 18 a 22/05/2002.

5- **XI Congresso de Biologia Celular**, trabalho: "*Dimetilamida-crotonin induz diferenciação em células da leucemia promielocítica (HL60)*" **Anazetti, M.C.**; Melo, P.S.; Durán, N.; Haun, M., em Porto Alegre – RS, de 16 a 19/07/2002.

6- **First Brazilian winter school on nanobiotechnology**, trabalho: "*Cytotoxicity of ε-caprolactone nanospheres incorporating dehydrocrotonin on leukemic cells*", **Anazetti, M.C.**; Melo, P.S.; Azevedo, M.M.M.; Durán, N.; Haun, M., em Campinas, Ribeirão Preto e São Carlos, de 12 a 23/08/2002.

3. Congresso Internacional

1- **6th Latin American Immunology Congress – ALAI,** trabalho: "Treatment of leukemic cells (HL60) with dimethylamide-crotonin triggers apoptosis dependent of caspases–2, –9, and –6 activation", **Anazetti, M.C.**; Melo, P.S.; Durán, N.; Haun, M., em Havana – Cuba, de 9 a 13 de dezembro de 2002.

4. Publicações Científicas

1- **Anazetti, M.C.**; Melo, P.S.; Durán, N.; Haun, M. (2001) "Dimethylamide-crotonin inhibits HL60 cells growth with less cytotoxicity on normal lymphocytes". *Revista da Sociedade Brasileira de Medicina Tropical.* Prata, A. (Editor).34: supl.II pp 252-253.

2- **Anazetti, M.C.**; Melo, P.S.; Azevedo, M.M.M.; Durán, N.; Haun, M. (2002). "Cytotoxicity of ε-polycaprolactone nanospheres incorporating dehydrocrotonin on leukemic cells" Proceeding in Brazil, First Brazilian Winter School on Nanobitoechnology, pp. 175-178.

3- **Anazetti, M.C.**; Melo, P.M.; Durán, N.; Haun, M. (2003). "Comparative cytotoxicity of dimethylamide-crotonin in the promyelocytic leukemia cell line (HL60) and human peripheral blood mononuclear cells". *Toxicology.* 188, 261-274.

4- **Anazetti, M.C.**; Melo, P.M.; Durán, N.; Haun, M. (2003). "Dimethylamide-crotonin induces apoptosis with lipidic peroxidation and dependence of caspases–9, –2 and –6 activation on leukemic cells". *Leuk. Res.* (submetido).

5. Patente

1- M.B.M., De Azevedo; J.B., Fabrin-Neto; M., Haun; M.A.T., Zullo; M.C., Anazetti; P.S., Melo (2002). "Processo de aplicação de precursores sintéticos esteroídicos de brassinosteróides, seus análogos espirostânicos e sues respsctivos compostos de inclusão em ciclodextrinas como agentes citotóxicos e antitumorais" Protocolo nº 300.183-0 (INPI – Instituto Nacional de Propriedade Industrial) em 28/01/2003.

6. Premiação

1- **WELLCOME TRUST/PAHO FELLOWSHIPS**: "Treatment of leukemic cells (HL60) with dimethylamide-crotonin triggers apoptosis dependent of caspases –2 and –9,

but not caspase –6 activation". pelo 6th Latin American Immunology Congress – ALAI, em Havana – Cuba, de 9 a 13 de dezembro de 2002.

7. Experiência didática

Programa de Estágio Docente no módulo BS-111: A Célula, ministrado ao primeiro semestre do curso de Medicina, sob coordenação de um grupo gestor, composto pelos docentes: Prof. Dr. Hernandes F. Carvalho (Biologia Celular - IB) e Profa. Dra. Christine Hackel (Genética Médica – FCM). Período: 1°semestre/2002.

II- Dimethylamide-crotonin inhibits HL60 cell growth with less cytotoxicity on normal lymphocytes. Proceeding in Brazil, XXVI Meeting of the Brazilian Society of Immunology.

III- Cytotoxicity of ε-Polycaprolactone Nanospheres Incorporating Dehydrocrotonin on Leukemic Cells. Proceeding in Brazil, First Winter School on Nanobiotechnology.

Revista da Sociedade Brasileira de Medicina Tropical
34 (Suplemento II), 2001

interaction of ATP with other cytotoxic mechanisms used by T lymphocytes and NK cells is also under investigation.

Support: CNPq, PRONEX, FAPERJ, FUJB-UFRJ.

LA – 06 CONTROL OF LYMPHOCYTE CELL CYCLE BY A MECHANISM INVOLVING NFAT1

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1Divisão de Medicina Experimental, INCA; 2Departamento de Fisiologia e Farmacodinâmica, FIOCRUZ, Rio de Janeiro, Brazil.

NFAT family of transcription factors plays a central role in control of gene expression during the immune response. NFAT1-/- mice showed an increased immune response, including a lymphocyte hyperproliferation. The aim of this work was to further characterize the hyperproliferation phenotype presented by NFAT1-/- mice-derived lymphocytes. In proliferation kinetics, NFAT1-/- cells stimulated in vitro with OVA, presented a striking increase in proliferation when compared to NFAT1+/+ cells. The analysis of lymphocyte subpopulation after OVA in vitro stimulation showed an increased number of CD3+ cells in NFAT1-/- mice. In an apoptosis assay, NFAT1-/- mice shown an increase of apoptotic cells when compared with NFAT1+/+ mice, suggesting that the phenotype is not related with a defect in apoptosis. However, cell cycle analysis after OVA in vitro stimulation, showed that NFAT1-/- cells presented an increased cell number in S-G2/M cell cycle phases when compared with NFAT1+/+ cells. Furthermore, analysis of gene expression demonstrated an overexpression of cyclins A2, B1, E and F; and an overexpression of c-myc in NFAT1-/- cells, which could be related with an enhanced apoptosis rate and with a cell cycle deregulation in the NFAT1-/- lymphocyte. Taken together, these results suggest that NFAT1 transcription factor could play an central role in control of cell cycle during T cell activation probably by a mechanism involving c-Myc.

LA – 07 DEXAMETHASONE-INDUCED EFFECTS ON EXPRESSION OF CD45RA/RO AND CD95

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Human aging has been associated with increased circulating cortisol levels and higher proportions of peripheral lymphocytes with memory phenotype. Cells with memory phenotype may accumulate in the elderly due to repeated antigenic exposure and/or altered sensitivity to glucocorticoids. In this study, we investigated whether memory/naive cells from young adults present different sensitivities to glucocorticoids. We also tested if this could be related to CD95 (Fas) expression. Mononuclear cells were isolated from healthy young adults and incubated in RPMI-1640 medium with dexamethasone (DEX, 10-6M and 10-5M) for 48h at 37°C, 5% CO2. Immunophenotyping was performed by two-color staining with fluorescein (FITC) and phycoerythrin (PE) labeled antibodies. Data was analyzed using a FACScalibur. Prior incubation, there was a higher proportion of CD45RO+CD95+ cells (16.5%) compared to CD45RA+CD95+ cells (8.8%). Following incubation with DEX, we found decreased expression and proportions of CD45RO+ cells (45% vs. 71%, compared to medium) while CD45RA+ cells remained unchanged. DEX also downregulated Fas expression on both CD45RA+ or CD45RO+ cells, with greater effect in the latter. Our data suggest that lymphocytes with memory/naive phenotype are differentially sensitive to DEX and this effect may be coupled to expression of Fas. We are currently investigating whether changes in glucocorticoid sensitivity reflect the accumulation of memory cells in the elderly.

Support: FAPERGS.

LA – 08 DIMETHYLAMIDE-CROTONIN INHIBITS HL60 CELL GROWTH WITH LESS CYTOTOXICITY ON NORMAL LYMPHOCYTES

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Revista da Sociedade Brasileira de Medicina Tropical
34 (Suplemento II), 2001

Croton cajucara is a plant found in the Amazonian region, where local population as a medicinal plant uses it. A clerodane nor-diterpene lactone, trans-dehydrocrotonin is an important bioactive compound of Croton cajucara (Sacaca). These activities include hypoglycaemic, anti-ulcer, anti-inflammatory and anti-tumour effects. Certain anti-cancer agents are known to induce apoptosis in human tumour lines. However, these agents are intrinsically cytotoxic against cells of normal tissue origin, including myelocytes and immunocytes. Recent studies show that dehydrocrotonin inhibits leukaemic HL60 cell growth partially through induction of apoptosis with less cytotoxicity on normal lymphocytes. In an attempt to expand our knowledge of the biological effects of dehydrocrotonin, we studied the cytotoxic effects of a dehydrocrotonin derivative (dimethylamide-crotonin) in HL60 cells comparing the structure-activity relationship. The dehydrocrotonin derivative has similar inhibitory effects on HL60 cells even without the alpha-methylene-gama-lactone in its moiety (IC50 = 100 uM). In order to obtain more information about diterpenes in biological activities, in the present study we show the evaluation of in vitro lymphocyte viability treated with the dehydrocrotonin derivative. Human peripheral blood mononuclear cells (PBMCs) were separated from venous blood of healthy volunteers as described previously (In Vitro Cell Dev. Biol. 27: 307, 1996) and treated with dehydrocrotonin derivative at different concentrations. Different viability endpoints were evaluated: MTT reduction, protein content and acid phosphatase activity. No cytotoxicity or morphological features of apoptosis were detected in PBMCs after treatment with dehydrocrotonin derivative (until 300 uM) either in the absence or presence of phytohemaglutinin (5 ug/mL). More than 80% of the cells were alive after 3 days of treatment. These results suggest that dehydrocrotonin derivative inhibits growth of HL60 cells in vitro, partially through induction of apoptosis, without causing serious side effects on immune cells.

Financial Support: CAPES, FAPESP.

LA – 9 EFFECT OF CURCUMIN AND ITS ANALOGUES ON MURINE B LYMPHOCYTES IN VIVO AND IN VITRO

Authors: Chagas, KKF1, Ricardo-de-Lima1, DD, Alegrio, L2 and Peçanha, LMT1
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INTRODUCTION: The Curcumin is a yellow pigment isolated from the rhizome of turmeric, Curcuma longa, a native plant from India. This substance is used in traditional medicine for the treatment of anorexia, diabetic wounds, hepatic disorders, cough, rheumatism, sinusitis, among other disorders. Moreover, it can exhibit a variety of pharmacological effects such as anti-mitotic, anti-tumoral, anti-viral and anti-inflammatory effects.
OBJECTIVES: In the present study we examined the effect of Curcumin and two structural analogues on the function of B lymphocytes. We evaluated the mitogen-induced proliferative response of B-lymphocytes in vitro and the production of anti-TNP-Ficoll IgM antibodies in vivo.
METHODS: Balb/c mice were immunized with the type 2 antigen TNP-ficoll and they were treated with Curcumin (by i.p. injection) or left untreated. The serum of the animals was obtained at various days after treatment and TNP-Ficoll specific IgM was measured by the ELISA method. We also obtained splenic B cells from normal mice. Those purified cells were stimulated in vitro with bacterial LPS and the type 2 activator anti-IgD-dextran. B cell proliferation in the absence or presence of Curcumin was measured.
RESULTS: Curcumin-treated, TNP-Ficoll-immunized animals showed a significantly higher level of serum anti-TNP-Ficoll IgM antibodies. Also, the analysis of B cell cultures showed that the Curcumin and its analogues induced a decrease in B cell proliferation stimulated by LPS. However, these substances decreased the response induced by anti-delta-dextran only when used in height doses.
CONCLUSIONS: These results suggest that Curcumin and analogues modulate the B cell response both in vivo and in vitro. Also, the response induced by type 2 activators (like TNP-Ficoll and anti-IgD-dextran) seemed to be more resistant to the inhibition mediated by Curcumin and its analogues.

LA – 10 EFFECT OF METHYLENE BLUE IN LYMPHOCYTES ACTIVATED WITH PHA

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Cytotoxicity of ε-Polycaprolactone Nanospheres Incorporating Dehydrocrotonin on Leukemic Cells

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INTRODUCTION: Leukemogenesis is a complex phenomenon that is characterised by uncoupling anomalies of proliferation and differentiation resulting in maturation block and leukemic clonal expansion. Certain cell lines are used as models for leukemic research [1]. The human myelogenous leukemia HL60 cell line has been established from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia. These leukemic cells proliferate continuously in suspension culture and consist predominantly of promyelocytes (>90%), but 4 - 15% of them show morphologic characteristics of more mature myeloid cells: myelocytes, metamyelocytes, bands forms, and polymorphonuclear leukocytes (PMN) [2]. This cell line displays distinct morphological and histochemical commitment towards myeloid differentiation [3], therefore HL60 cells has played a pivotal role in the study of differentiation and growth of human leukemias [4]. This cell line provides a model system for studies on the mechanism of cell differentiation or cell growth and apoptosis cell death [5]. Since differentiated cells lose their proliferative ability and immortality, differentiation inducers and compounds that trigger apoptosis process may be useful for the treatment of leukemia [6].

A clerodane *nor*-diterpene lactone, *trans*-dehydrocrotonin (DHC) (Figure 1) is an important bioactive compound of bark and leaves of *Croton ca*jucara (Sacaca), an Amazonian medicinal plant commonly used as an infusion in the powdered or dried pill form to treat a large number of gastrointestinal, kidney and liver disorders [7]. The biological activities include hypoglycaemic, antigenotoxic [8]; antiulcer [9-11], anti-inflammatory and anti-tumour [12] effects. Recently our group showed that dehydrocrotonin has cytotoxic effects on human leukemic cells (HL60) [13].

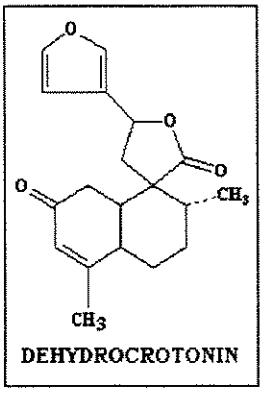

Figure 1. Dehydrocrotonin molecular estruture.

For optimal drug action it is necessary to delivery the drug to the desired site of action in the body in the most efficient way. There have been tremendous efforts to develop systems for site-specific drug delivery [14-15]. Recently a wide variety of polymer particulate carriers has been devised for protecting the active molecules against inactivation by the host and for controlling drug release in body fluids,

e.g., blood, lymph and digestive juice (Kim *et al.*, 1999). Developing intravenously injectable nanoparticulate drug carriers requires first that the polymers used to prepare the particles should be eliminated after use by biodegradation. Recently, the aliphatic polyesters, in particular poly (lactide), poly (glycolide), poly (lactide-co-glycolide), and poly (ε-caprolactone) [14], have been widely studied because they are completely biodegradable to nontoxic metabolites and well tolerated by tissues [16]. Under such circumstances, various methods have been proposed for the preparation of PLGA and PLA nanoparticles. One promising technique is the spontaneous emulsification solvent diffusion (SESD) method, in which nano-sized particles of PGLA or PLA can be effectively obtained by pouring phase with moderate machanical stirring. One technical characteristic of this method is the use of a binary mixture od a water-miscible organic solvent such as acetone and a water-immiscible solvent such as dichloromethne as the solvent of the polymeric solution, and the particles are formed via na emulsification process and a subsequent solvent-evaporation process. However the original SESD method was modified to improve the applicability to large-scale production. The major modification point of the process were that: a mixture of two water- miscible organic solvents was used for the solvent of the polymeric solution instead of the mixture of water-miscible and water-immiscible organic solvents [17].

In an attempt to expand our knowledge of the biological effects of dehydrocrotonin, we studied the cytotoxic effects of ε-polycaprolactone nanospheres incorporating dehydrocrotonin on HL60 cells, comparing to the original compound.

METHODS:

Dehydrocrotonin

DHC was obtained from *C. cajucara* barks as described by Souza-Brito and collaborators [18]. The evaporation technique was used to obtain the incorporation of the DHC moiety into a polymeric matrix of ε-polycaprolactone (PCL) and the system was characterised by the physic-chemical properties.

Preparation of DHC-loaded nanospheres from ε-Polycaprolactone (PCL)

ε-Polycaprolactone nanospheres were prepared according to the solvent evaporation technique associated to spontaneous emulsification diffusion method [17, 19]. The trans-dehydrocrotonin and the PCL were dissolved in the organic solvent mixture consisting of acetone and ethanol in the proportion of 40% (v/v). The polymeric solution obtained above is slowly poured into the emulsifier-containing aqueous phase with agitating by a stirrer at the presence of a surfactant (Tween 40). Nanoparticles are formed via the following steps: when the polymeric solution is added, emulsion droplets are formed in the aqueous phase, acetone quickly diffuses out from each emulsion droplet, drastically reduzing its size to nano-order, and the consequent "solvent-evaporation" process, in which the remaining solvents were removed from the system, makes the droplets solidify to finally form polymeric nanoparticles [17].

Leukemia Cells (HL60) Culture

Human leukemia cells (HL60), that derived from patient with acute promyelocytic leukaemia, was obtained from laboratory of Cellular Metabolism and Regulation kindly given up by Prof Dr Rui Curi from Department of Physiology and Biophysical - Instituto de Ciências Biomédicas (ICB) of Universidade de São Paulo (USP). Human

leukemia cells (HL60) were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml of penicillin and 100 μg/ml of streptomycin in a humidified atmosphere at 37°C containing 5% CO_2 in air. Cells in exponential growth period were used in the experiments. For cell viability assays the cells were seeded (3 X 10^5 cells/ml) in 96 wells plates, incubated with dehydrocrotonin and dehydrocrotonin encapsulated in different concentrations during 72 hours.

Cell viability was determined by MTT reduction, trypan blue exclusion, protein content and phosphatase activity assays, as descibed to follow [20, 21].

MTT Reduction Assay

The tetrazolium reduction assay was performed according to the method of Denizot and Lang [22]. Briefly, was added 0,1-ml serum –free medium countaining MTT (1mg/ml) to each well. After incubation for 4 h, the supernatant was removed and the blue formazan product obtained was dissolved in 1ml ethanol with stirring for 15 min on a microplate shaker and absorbance was read at 570 nm.

Phosphatase Activity Dosage

After the treatment of the HL60 cells during 72 h with dehydrocrotonin and dehydrocrotonin encapsulated, the dosage of the phosphatases activity was carried out as described by Aoyama and collaborators [20] with some modifications. The enzyme was obtained by lysing the cells with acetate buffer, pH 5.5. The reaction mixture (final volume, 0,1mL) contained 100mM acetate buffer, pH 5.5, 5mM p-Nitrophenylphosphate and enzyme (cell extract). After a 30 minutes incubation at 37°C, the reaction was stopped by adding 0,1mL of 1M NaOH. The amount of p-Nitrophenol produced was measured at 405nm.

Trypan Blue Exclusion Assay

The trypan blue (TB) exclusion assay was performed according to the method of Renzi and collaboartors [23]. Cells grown in culture bottles (3 X 10^5 cells/ml) were treated with dehydrocrotonin encapsulated in differents concentrations. At the end of treatment period, cultures were inspected for morphological alterations. Trypan blue solution (0,08% in PBS) and cell suspension, were mixed in equal quantities. The cell number was estimed using a haemocytometer, and (blue) stained cells were scored as dead.

Experiments of cell viability were done three times (four replicates each) separately. To calculate the IC_{50} values (concentration that produces a 50% inhibitory effect on the evaluated parameter) the results were transformed to percentage of controls and the IC_{50} were graphically (Origin version 6.0) obtained from the dose-response curves. Results are expressed as the mean $\pm$ SD.

RESULTS:

The cytotoxicity of DHC incorporated in nanospheres was higher compared to the conventional dehydrocrotonin (Figures 2 and 3), even at the lower concentrations. The HL60 cells were more sensitive to the DHC encapsulated (IC_{50} = 100μM) than to the pure DHC (IC_{50} = 180μM). Thus encapsulated form was about two folds more effective than free DHC on HL60 evaluated by the trypan blue exclusion assay. However HL60 cells showed a different behaviour after treatment with encapsulated DHC related to MTT reduction test presenting stimulus in all concentrations tested (until 400μM).

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DISCUSSION & CONCLUSIONS:

As a drug carrier for dehydrocrotonin, we prepared dehydrocrotonin incorporating in ε-polycaprolactone nanospheres. Drugs with a hydrophobic character can be easily incorporated into the core by a covalent or non-covalent bonding through hydrophobic interactions [14]. According to our data described above, nanospheres containing the hydrophobic drug dehydrocrotonin in their inner core were more toxic than DHC pure on leukemic cells. Probably, the encapsulation of DHC provided the adressing and releasing of the compound inside of the HL60 cells with consequent increase of cytotoxicity.

The results of this study show that encapsulated form stimulated the mitochondrial function evaluated by MTT reduction. Some authors have suggested that MTT reduction is a measurement of mitochondrial function, in particular of succinate dehydrogenase activity. Howevwer, detailed studies with liver homogenates have shown that hepatocyte MTT reduction predominantly assesses the cytosolic NAD(P)/NAD(P)H redox balance. The use of inhibitors or inducers of reactions involved in the redox balance has confirmed the involvement of this pathway in the reduction of MTT byV79 cells. The inhibition or activation of MTT reduction induced by different compounds may thus serve as an indicator of a compound's ability to influence the cell's reducing activity [24].

Our results suggest that the stimulus observed by MTT reduction assay in the HL60 cells treated with dehydrocrotonin encapsulated was induced by oxidative stress. This change in the redox balance can be involved on the major cytotoxic effects on HL60 cells provoked by DHC encapsulated compared to DHC free.

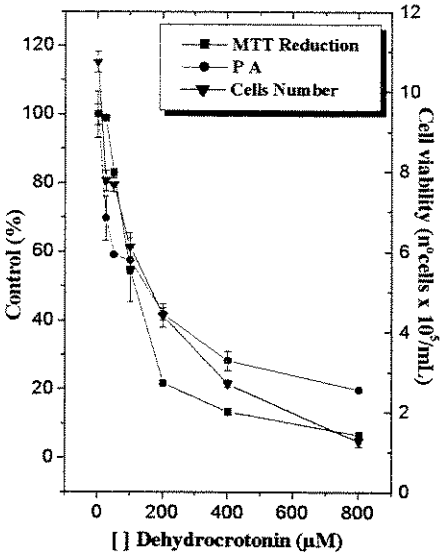

Figure 2. Cytotoxicity of dehydrocrotonin on HL60 cell culture treated for 72h . Each point represents the mean ± SD of three experiments in four replicates.

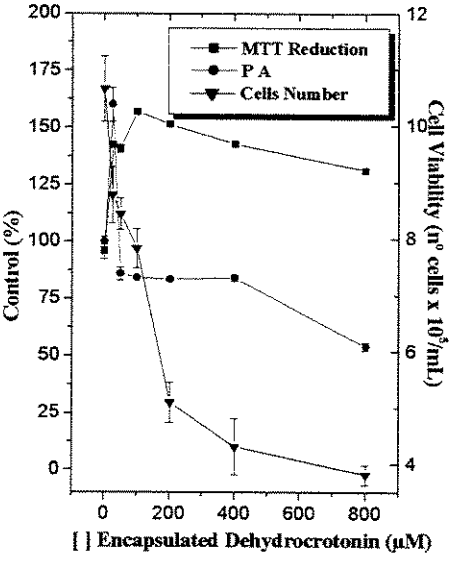

Figure 3. Cytotoxicity of dehydrocrotonin encapsulated on HL60 cell culture treated for 72h . Each point represents the mean ± SD of three experiments in four replicates.

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