Serviço Público Federal
Universidade Federal de Goiás
Instituto de Ciências Biológicas
Programa de Pós-Graduação em Biologia - Mestrado
Área de Concentração - Biologia Celular e Molecular

ALINY PEREIRA DE LIMA

Estudo do potencial genotóxico, citotóxico e antitumoral do composto Cloreto de *cis*-tetraaminodiclororutênio(III) sobre diferentes células tumorais

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular da Universidade Federal de Goiás para obtenção do Título de Mestre em Biologia Celular e Molecular.

Orientadora: Profª. Drª. Elisângela de Paula Silveira Lacerda

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Março/ 2010

Programa de Pós-Graduação em Biologia Celular e Molecular

Universidade Federal de Goiás

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Data: 29/01/2010

“Feliz daquele que transfere o que
sabe e aprende o que ensina”
Cora Coralina

Dedico este trabalho a minha mãe Ana!

AGRADECIMENTOS

A Deus, pelo dom da vida, por todas as oportunidades que me concedeu e pela força para superar as dificuldades. Obrigada pelas minhas conquistas e pelas graças alcançadas!

Aos meus pais Ana e João pelo cuidado e proteção, por todo sacrifício que passaram para que me proporcionassem até chegar neste momento. Obrigada pelo incentivo à minha formação acadêmica e profissional!

A minha querida orientadora, desde a graduação até o mestrado, Dra. Elisângela, a quem tenho grande admiração pela sua competência, profissionalismo e seriedade, exemplo de educadora. Saiba que muito do que aprendi e consquistei até hoje devo a ti. Obrigada por possibilitar essa conquista, pelos ensinamentos, confiança, carinho e acima de tudo pela grande oportunidade que me proporcionou.

Agradeço à Flávia, por termos caminhado sempre juntas, pela dedicação, ajuda, amizade e principalmente pelo companheirismo.

Agradeço ao César, por todo apoio prestado, dedicação, conhecimentos e acima de tudo pela amizade.

Agradeço a Alessandra pela ajuda e amizade durante todo este período.

Agradeço a Manuela pelo apoio, pelas palavras incentivadoras e amizade

A toda equipe do Laboratorio de Genétia Molecular e Citogenética, em especial a Hellem, Francyelly, Lucas, Wanessa e Osmar. Obrigada pelo auxílio durante o Mestrado.

A Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), pela bolsa de mestrado.

A banca examinadora que gentilmente aceitou o convite de participar na defesa desta dissertação.

A todas as pessoas que verdadeiramente torceram por mim, me incentivaram e hoje comemoram comigo esta conquista.

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SIGLAS E ABREVIATURAS

A549: Adenocarcinoma de pulmão humano
Apaf 1: Protease apoptótica ativadora de fator-1
ATCC : Coleção Americana de cultura celular
Bax: Proteína X associada ao Bcl-2
Bcl-2: Proteína anti-apoptótica (Linfoma células B)
Bcl-x: Proteína anti-apoptótica
Bid: Domínio de Morte de Interação com BH3
CD95: Ligante de Receptor de Morte Celular
cis-$[RuCl_2(NH_3)_4]Cl$: Cloreto de *cis*- tetraaminodiclororutênio(III)
DMEM: Meio Eagle modificado por Dulbecco
DNA: Ácido desoxirribonucléico
FITC: Isotiocianato de fluoresceína
G1: Fase G1 do Ciclo Celular
G2: Fase G2 do Ciclo Celular
IC_{50} : Concentração que inibe 50% da viabilidade celular
K562: Linhagem de células de Leucemia Mielóide Crônica humana
KP1019: Indazol *trans*-[tetraclorobis (1*H*-indazol)rutenato(III)]
MRC-5: Fibroblasto de pulmão human0
MTT: 3-(4,5-Dimetiltiazol-2-il)2,5-Difenil Brometo de Tetrazoilium)
NAMI-A: ImH[*trans*-RuCl4(DMSO)Im]
PBMC: Células Mononucleares de Sangue Periférico
PBS: Solução Salina Tamponada com Fosfato
RPMI: Instituto Memorial Park Roswell
S: Fase S do Ciclo Celular
TNF-R: Receptor de Fator de Necrose Tumoral
Trail: Ligante indutor de apoptose relacionado ao fator de necrose tumoral
XTT: (Ácido sulfônico (sódio 3´-[1-(fenilaminocarbonill)- 3,4-tetrasólio]-bis (4-metroxe -6-nitro)
µM: Micromolar

RESUMO

Atualmente drogas inorgânicas como cisplatina e compostos relacionados são amplamente utilizados no tratamento do câncer, no entanto a aplicação destas drogas é limitada devido a severa toxicidade e resistência. Estas limitações têm promovido o desenvolvimento de novos agentes antitumorais baseados em metais que sejam mais eficazes. Dentre os vários complexos a base de metais desenvolvidos, complexos de rutênio (III) representam uma nova família de promissores agentes anticâncer. No presente estudo foi investigado *in vitro* o efeito do composto *cis*-tetraaminodiclororutênio(III) sobre a viabilidade celular, cinética das fases do ciclo celular, mecanismos de indução de apoptose e danos a molécula de DNA. Os resultados de viabilidade celular utilizando os ensaios de redução do MTT e azul de tripano sobre células leucêmicas K-562 demonstrou que o composto reduziu significativamente a viabilidade celular (IC_{50} aproximadamente 10.74 e 73.45 μM), respectivamente, por outro lado, a viabilidade celular de células tumorais de pulmão A549 foi pouco afetada após tratamento com *cis*-tetraaminodiclororutênio(III) (IC_{50} > 383 μM). Adicionalmente, foi observado que *cis*-tetraaminodiclororutênio(III) exibiu uma moderada citotoxicidade sobre células normais de fibroblasto humano MRC-5 (IC_{50} > 383 μM) quando comparado a linhagem tumoral K-562 (10.74 e 73.45 μM) O ensaio clonogênico foi realizado em células tumorais A549, e por meio dos resultados obtidos verificou-se que em baixas concentrações do composto (0,38 e 3,8 μM) houve a diminuição na capacidade das células de formarem colônias e em concentrações elevadas 95 e 383 μM não houve a formação de nenhuma colônia. Na análise do ciclo celular de células tumorais K-562 e S-180, *cis*-tetraaminodiclororutênio(III) alterou a distribuição das fases do ciclo celular de ambas as células, visto que a porcentagem de células nas fases G1, S e G2 diminuiu, o qual correlacionou com uma aumento do número de células em sub-G1 (indicativo de apoptose). Na análise de danos a molécula de DNA de células S180 utilizando o ensaio cometa, pôde-se observar que o composto induziu danos à molécula de DNA para ambos os tratamentos (24 e 48 h) como evidenciado por um aumento do índice de dano. Além disto, o tratamento com *cis*-tetraaminodiclororutênio(III) levou a indução de apoptose nas células S180 como evidenciado pelo aumento no número de células Anexina positiva. Em células K562 a indução de apoptose após tratamento com o composto foi demonstrado pelo aumento no conteúdo de DNA em picos sub-G1 (75,35%) e um aumento na atividade de caspase 3, 8 e 9. Estes dados em conjunto demonstraram que o composto *cis*-tetraaminodiclororutênio(III) apresentou efeito citotóxico frente as linhagens testadas e esta atividade está correlacionada com danos à molécula de DNA, alterações nas fases do ciclo celular e indução de apoptose.

Palavras-chave: Anitumoral, *Cis*-tetraaminodiclororutênio(III), Citotoxicidade, Apoptose, Ciclo Celular.

ABSTRACT

Current inorganic drugs such cisplatin and related compounds widely used in the treatment cancer, however its application is limited by its severe toxicity and drug resistence. These limitations have prompted a search for news metal-based antitumor agents**.** Ruthenium (III) complexes represent a new family of promising metal-based anticancer drugs. In the present study, was investigated *in vitro* the effects of the compound on cell viability, cintetics cell cycle phases, mechanisms of apoptosis and DNA damage on tumors cells. Results of the viability using MTT reduction test and the trypan blue exclusion assay on K-562 cells revealed that this compound significantly reduced the viability of the K-562 tumors cells (IC_{50} approximately 10.74 and 73.45 μM), respectively, moreover viability assays on A549 lung tumor cells showed that *cis*-(dichloro)tetramminеruthenium(III) induced effect moderate this cell lines (IC_{50} > 383 μM). Additionally was observed that this compound exhibits little cytotoxicity towards MRC-5 normal human fibroblast cells (IC_{50} > 383 μM) when compared to K562 tumor cell line (IC_{50} 10.74 μM). Clonogenic Assay was performed on A549 cells, and observed that lower concentrations (0.38 and 3.8 μM) *cis*-(dichloro)tetramminеruthenium(III) diminished colony forming ability and highest concentrations (95 and 383 μM) no colony was observed. In cell cycle analysis on K-562 and S180 tumor cells *cis*-tetramminеruthenium(III) induced change in the distribution the cell cycle phase since that % of cells entering G1, S and G2 was decreased, correlating with increase in the proportion of cells in the sub-G1-peak (indicating apoptosis). In the analysis of damage to the DNA molecule of S180 cells using the comet assay, it was observed that the ruthenium compound induced damage to the DNA molecule to both treatments (24 and 48 h) as evidenced by an increase in damage index. In addition, *cis*-$[RuCl_2(NH3)_4]Cl$ treatment induced apoptosis in cells K-562 as evidenced for increased DNA content in the sub-G1 peak (75.35%) and a significant increase in caspase-3, 8 and 9 activity. In tumor cells S180 the apoptosis was demonstrated for by a increase numbers of Annexin V-positive cells and fragmentation DNA. Taken together, these findings strongly demonstrate that *cis*-$[RuCl_2(NH3)_4]Cl$ exerts antitumor activity and this activity correlates with DNA damage, process apoptotic and change cell cycle.

Keywords: Antitumor, Cytotoxicity, *Cis*-tetraaminodiclororutênio(III), Apoptosis.

1. INTRODUÇÃO GERAL

1.1 Câncer

O câncer é a designação dada ao conjunto de manifestações patológicas que se caracterizam pela perda de controle da proliferação celular e ganho de capacidade de invadir tecidos adjacentes ou de sofrer metástases para tecidos distantes. Esta perda de controle é conseqüência direta de mutações em um ou mais genes que regulam o crescimento celular e a morte celular programada (PINTO & FELZENSZWALB, 2003; NUSSBAUM et al., 2008).

Os genes envolvidos na perda do controle celular são divididos em duas grandes classes: os proto-oncogenes, que estimulam o crescimento celular e impedem a diferenciação, e os genes supressores de tumor, que limitam a proliferação das células. O desequilíbrio na regulação desse sistema, por meio da ativação de proto-oncogenes ou perda da função de genes supressores de tumor, pode levar à proliferação descontrolada de células e ao acúmulo de sucessivas anormalidades genéticas, características das células tumorais (ALBERTS, 2004; LODISH et al.,2005).

De acordo com Karp (2005), as células tumorais apresentam várias características que as distinguem das células normais, tais como: (1) independência de fatores de crescimento, visto que independem dos sinais transmitidos pelos fatores de crescimento aos receptores de superfície; (2) pouca aderência tanto a outras células como a substratos acelulares (esta perda de adesão permite que as células tumorais deixem à massa tumoral e migrem para outros locais no organismo) (3) crescem umas sobre as outras

perdendo a inibição por contato (4) falham ao entrar em processo de apoptose, em conseqüência são imortais e tornam-se resistentes a quimioterápicos.

1.2. Tratamentos do Câncer

Existem três enfoques principais para o tratamento do câncer: excisão cirúrgica, radioterapia e quimioterapia, e o papel de cada uma delas depende do tipo de tumor e de seu estágio de desenvolvimento. A excisão cirúrgica foi a primeira modalidade de tratamento desenvolvida e consiste na retirada física do tecido maligno sendo eficiente somente nos casos em que o tumor é circunscrito (SNELL et al., 2006). Já a radioterapia consiste em expor as células malígnas à radiação ionizante, e é utilizada para tumores localizados que não podem ser extraídos totalmente ou aqueles que apresentaram alta taxa de recidivas locais após a cirurgia (O'SULLIVAN & WALDRON, 2006).

A quimioterapia é um dos métodos mais utilizados e consiste no uso de agentes citotóxicos, sendo que este tipo de tratamento é um dos principais métodos de tratamento de alguns cânceres. (CHABNER et al., 2006; CHU & SARTORELLI, 2001).

1.3. Agentes antitumorais e Mecanismo de Ação

Os agentes farmacológicos utilizados na quimioterapia do câncer incluem uma grande variedade de compostos tais como: agentes alquilantes, antimetabólicos, antimitóticos e antitibióticos que atuam por vários mecanismos. Contudo a maioria desses agentes atua sobre o DNA, impedindo a duplicação celular (BOENTE et al., 1998).

Os agentes alquilantes (mostardas nitrogenadas, etileniminas, alquilsulfonatos, nitrosoréias e triazenos) interferem na replicação e transcrição do DNA, pois alquilam moléculas orgânicas como DNA e RNA levando a apoptose (SIKIC, 1994; CHU & SARTORELLI, 2006).

Os antimetabólicos (análogos das purinas, análogos das pirimidinas e análogos do ácido fólico) incluem um grupo variado de compostos que interferem em diversos processos bloqueando as vias metabólicas normais que operam nas células, o que resulta em interrupção da síntese de ácidos nucléicos (BOENTE et al., 1998).

Os agentes antimitóticos são agentes naturais, derivados de plantas (alcalóides da vinca, epipodofilotatoxinas e taxanos) que agem inibindo etapas de divisão do ciclo celular, através de inibição do fuso celular e inibição de topoisomerases (BOENTE et al., 1998; CHU & SARTORELLI, 2006).

Os antibióticos (antraciclinas, mitoxantrona, dactinomicina, bleomicina e mitomicina C) atuam através da intercalação entre bases específicas do DNA, bloqueando a síntese de DNA, RNA ou de ambos (CHU & SARTORELLI, 2006).

1.4. Ciclo Celular e Agentes Antitumorais

Na quimioterapia antineoplásica muitos fármacos exercem seus efeitos citotóxicos principalmente através de sua atuação sobre o ciclo celular, sendo que a compreensão da cinética do ciclo celular é essencial para o uso apropriado de novas drogas antineoplásicas (CHABNER et al., 2006).

Considerando uma maior atividade em determinadas fases do ciclo celular, as drogas antineoplásicas são classificadas em três categorias: ciclo

celular específicas/fase-específicas, ou no ciclo como um todo, em ciclo celular específica/fase não-específica; ambas necessitando do ciclo celular para exercer seus efeitos citotóxicos. Há ainda aquelas que não agem necessariamente em fases de crescimento, mas em células em repouso (fase G0), sendo denominadas ciclo não específicas (BOENTE et al., 1998).

Partindo-se desta classificação, incluem-se em ciclo celular-específicas/fase específica os agentes antimetábolicos, os quais exercem seus efeitos principalmente por bloquearem bioquimicamente a síntese de DNA, e são portando restritos a fase S do ciclo celular, e os agentes naturais (alcalóides vegetais) que agem através do bloqueio da formação de fuso mitótico e são, portanto, específicos à fase M (ALMEIDA et al., 2005; CHABNER et al., 2006).

Fazem parte dos agentes ciclo-celular/fase não-específica os agentes alquilantes diversos, antibióticos naturais e complexos de coordenação de platina, os quais exercem seu efeito antitumoral na maioria das vezes por meio de ligações cruzadas com as fitas de DNA, podendo, no entanto atuar em diversas fases do ciclo celular G1, S, G2 (Figura 1) (ALMEIDA et al., 2005).

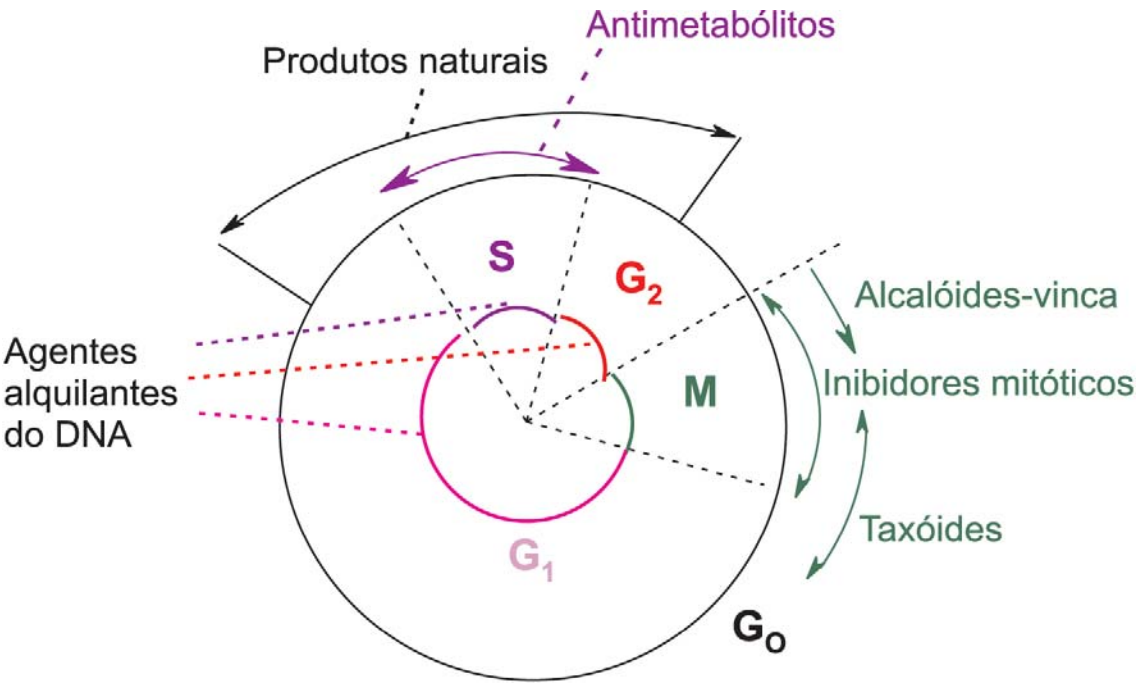


Figura 1. Atuação dos agentes antineoplásicos sobre as fases do ciclo celular. (ALMEIDA et al., 2005).

1.5. Apoptose

Múltiplos sinais modulam a proliferação, sobrevida e morte celular e essa ação coordenada permite que a célula normal cresça e se divida até sua senescência. De modo geral, estímulos tóxicos ou deletérios à célula podem desencadear a morte celular por necrose ou apoptose (Figura 2), as quais são diferenciadas pela morfologia e vias bioquímicas celulares (GIOCONDO, 2007 apud BAYLY et al., 1997).

A necrose é um processo de morte celular acidental induzida por lesões agudas, em que as células liberam seus conteúdos citosólicos no espaço extracelular levando a uma resposta inflamatória (ALBERTS, 2004).

Ao contrário da necrose, a apoptose é um processo altamente regulado que se caracteriza por encolhimento celular, fragmentação do DNA

internucleossomal, condensação da cromatina e formação de corpos apoptóticos, os quais são rapidamente fagocitados por macrófagos sem causar processo inflamatório (COOPER, 2005).

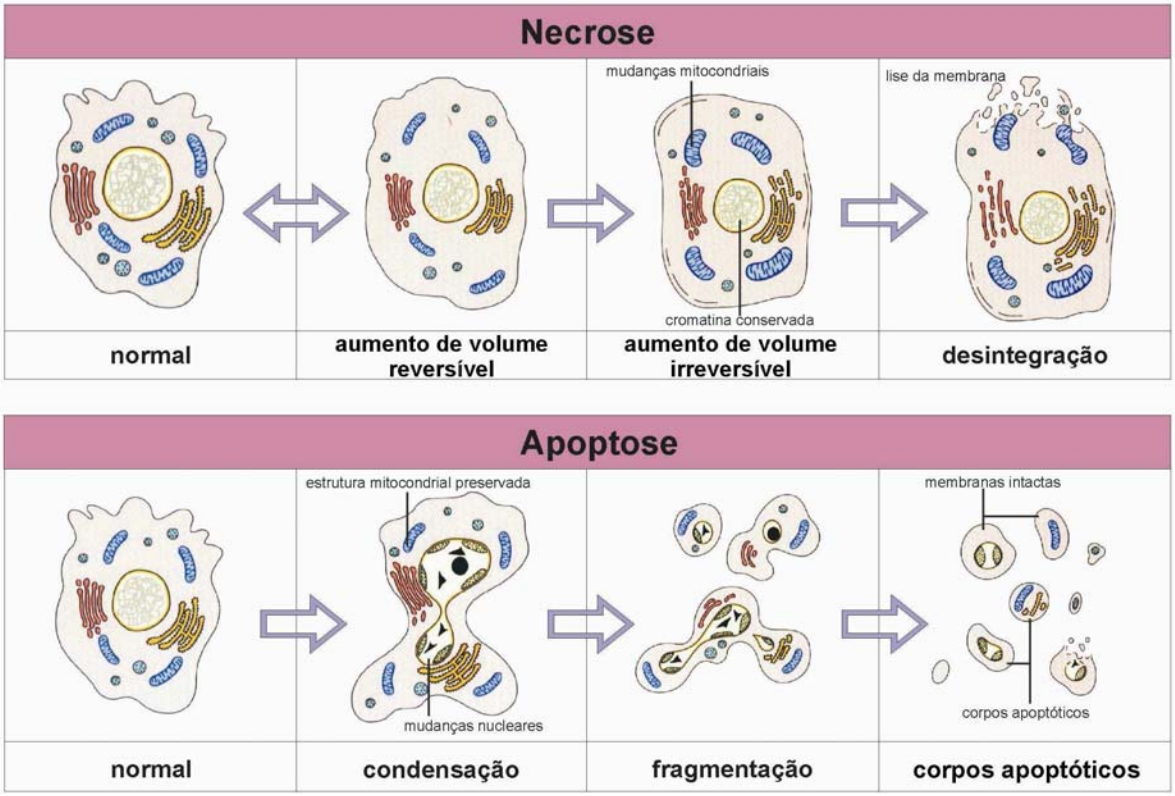


Figura 2. Mecanismo de morte celular: Necrose e Apoptose. (ANAZETTI & MELLO, 2007).

A morte celular por apoptose é mediada por meio de duas vias principais (Figura 3), a via extrínseca a qual é acionada por estímulos externos, através da atividade de receptores específicos presentes na superfície celular chamado receptores da morte célular (TNF), e a via intrínseca ou mitocondrial que ocorre por meio de estresse intracelular. Estas vias culminam com a ativação de proteases conhecidas como caspases executoras. (COHEN, 1997; KAUFMANN & EARNSHAW, 2000; KAUFMANN & HENGARTNER, 2001).

A via apoptótica extrínseca é desencadeada pela interação entre sinais de morte celular TNF (Fator de Necrose Tumoral), CD95 (Ligante de Receptor de Morte Celular), e TRAIL (Ligante Indutor de Apoptose Relacionado ao Fator

de Necrose Tumoral) e receptores da família TNF-R (Receptor do Fator de Necrose Tumoral). Quando ocorre esta interação os receptores formam agregados que, na forma de trímeros, ligam-se à proteína adaptadora FADD (Domínio de morte associada ao CD95) presente no citoplasma. Ocorre, então, a ligação dessas moléculas à pró-caspase-8, resultando na formação de um complexo denominado DISC (Complexo de Sinalização de Indução de Morte), que culmina na auto-clivagem e ativação da caspase 8. A caspase 8 pode assim, diretamente ou pela via mitocondrial, ativar a caspase 3 efetora, o que resulta na apoptose celular (FULDA, 2009).

Por outro lado, a via intrínseca é desencadeada pela presença de sinais de estresse, como exposição à radiação ultravioleta, infecções, alterações metabólicas e redução de fatores de crescimento, que ocasionam a migração de proteínas pró-apoptóticas Bax (Proteína X associada ao Bcl-2) e Bid (Domínio de Morte de Interação com BH3) do citoplasma para a mitocôndria, alterando sua permeabilidade e permitindo a liberação de outras proteínas pró-apoptóticas, como o citocromo C. O citocromo C se liga então a uma proteína adaptadora Apaf-1 (Protease apoptótica ativadora de fator 1) e a pró-caspase 9 levando a formação do apoptossomo e a ativação da caspase 9, a qual irá ativar outras caspases como a pró-caspase 3, culminando assim a apoptose via mitocondrial (KAUFMANN & EARNSHAW, 2000; KAUFMANN & HENGARTNER, 2001).

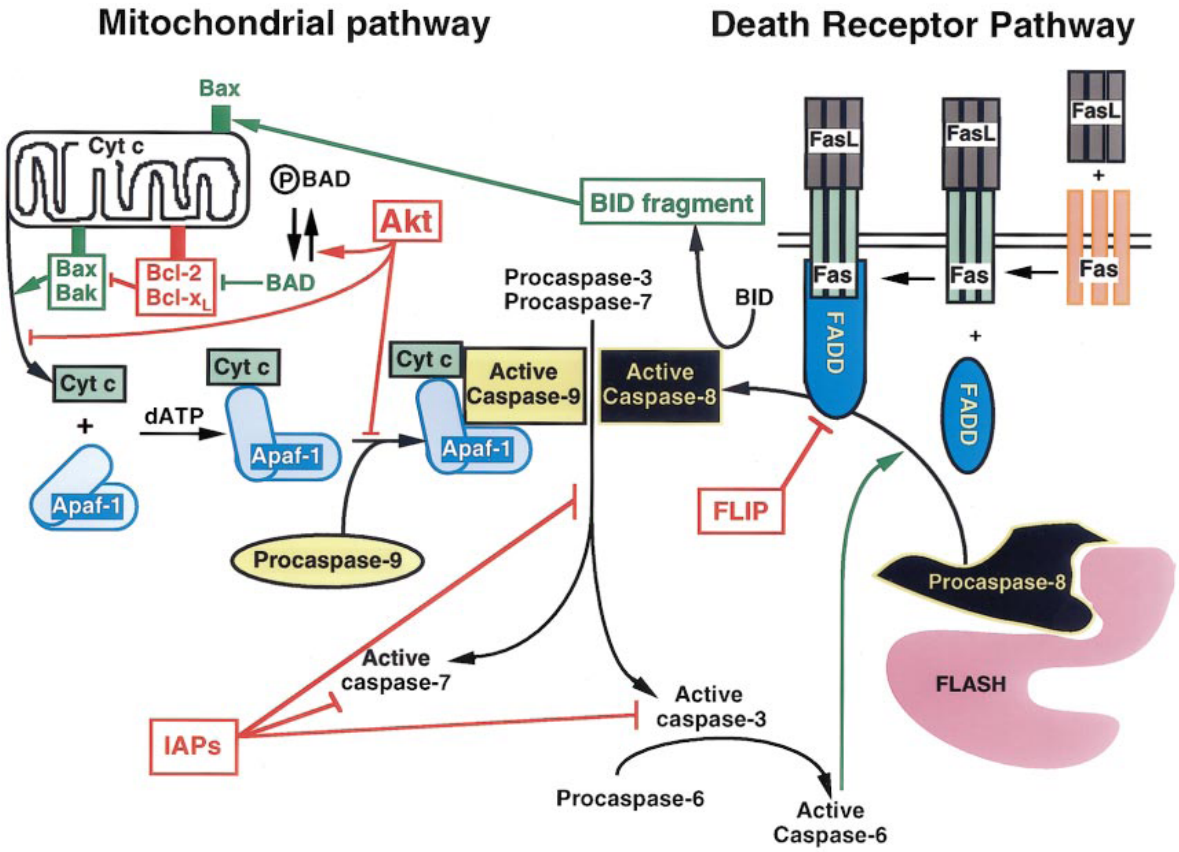


Figura 3. Vias de sinalização intrínseca e extrínseca durante a apoptose. (KAUFMANN & EARNSHAW, 2000).

1.6. Apoptose e Terapia do câncer

Muitos agentes anticâncer atualmente utilizados na quimioterapia foram desenvolvidos a partir de triagens designadas para identificar agentes que seletivamente levam a morte de células tumorais. Entretanto, até recentemente pouco se sabia sobre o mecanismo de ação destas drogas ou até mesmo mecanismos de resistência apresentados pelos tumores (LOWE & LIN, 2000). Apenas recentemente tornou-se claro que os fármacos anticâncer são capazes de induzir apoptose, e que este processo está envolvido com seus efeitos citotóxicos. Além disso, a indução à apoptose foi identificada como um evento comum de diferentes classes de agentes anticâncer, sendo que a interrupção deste mecanismo pode levar à resistência a múltiplas drogas (LOWE & LIN,

2000; FERREIRA et al., 2002).

A partir do conhecimento que agentes anticâncer induzem apoptose e que a interrupção deste processo pode reduzir a sensibilidade ao tratamento, inúmeros trabalhos na literatura têm demonstrado que drogas convencionais tais com flavopiridol, cisplatina, paclitaxel, doxorrubicina induzem apoptose em células tumorais *in vitro* (ACHENBACH et al., 2000; MIZUTANI et al., 2005; BRENES et al., 2007).

Muitos trabalhos demonstram que a morte celular através da indução de apoptose tem sido atualmente um dos principais pilares na descoberta de novos fármacos e uma valiosa ferramenta para o tratamento do câncer (LIU et al., 2006; GE et al., 2009; HYUN et al., 2009).

1.7. Cultura celular e Triagem *in vitro*: uma ferramenta para busca de novos fármacos antitumorais

Apesar das drogas antitumorais convencionais apresentarem diferentes mecanismos de ação, uma propriedade comum à maioria delas é a toxicidade significativa sobre os tecidos normais. Sendo assim, apesar do desenvolvimento dirigido no sentido de torná-los mais seletivos sobre os tecidos neoplásicos, os agentes atualmente disponíveis não apresentam seletividade sobre células tumorais, devendo-se, no entanto, considerar criteriosamente o risco versus benefício quando se usa esses agentes (BOENTE et al., 1998). Além disso, a toxicidade dessas drogas limita seu uso, já que a maiorias dos cânceres humanos apresentam resistência a diversos quimioterápicos e estes podem ainda causar danos ao DNA de células normais (NADIN et al., 2006; HEFFETER et al., 2008). Conseqüentemente, torna-se

evidente a busca de novas drogas menos tóxicas, mais seletivas e eficazes para o tratamento do câncer.

Com o aprimoramento da metodologia de cultura de células foi possível o desenvolvimento de diversas linhagens celulares oriundas de tumores humanos, possibilitando a existência de uma metodologia para triagem *in vitro*. Com esse objetivo, o *Nacional Câncer Institute* (NCI/EUA) desenvolveu um painel de células tumorais que conta com aproximadamente 60 linhagens oriundas de diferentes tipos de tumores sólidos (pulmão, melanoma, mama, rim, cólon, próstata, ovário e cérebro) e do sistema hematopoético (leucemia). Assim, testes *in vitro* utilizando-se cultura de células tumorais são extremamente importantes na pesquisa de novos agentes antitumorais, uma vez que são modelos representativos de diferentes tipos de tecidos tumorais, e podem predizer a atividade de novas substâncias contra um tumor correspondente (BOYD & PAUL, 1995; FRICKER, 1999).

Segundo normas da ANVISA (Agência Nacional de Vigilância Sanitária) para pesquisa de novos fármacos, antes da aplicação de novas moléculas em animais deve ser realizados anteriormente estudos *in vitro*, a fim de identificar potencialidades terapêuticas de uma determinada substância.

Sendo assim, modelos de triagem *in vitro* (ensaios de viabilidade e crescimento celular) como MTT (3-(4,5-Dimetiltiazol-2-il)2,5-Difenil Brometo de Tetrazoilium)) e XTT (Ácido sulfônico (sódio 3´-[1-(fenilaminocarbonill)- 3,4-tetrasólio]-bis (4-metroxe -6-nitro) são de suma importância na determinação da atividade citotóxica e antitumoral de novas substâncias, fornecendo assim, dados que possam subsidiar trabalhos futuros (FRICKER, 1999; PLUMB, 2004).

1.8. Complexos Metálicos e Atividade Antitumoral

Sabe-se que complexos metálicos têm sido usados como agentes terapêuticos desde a antiguidade, sendo que as drogas orgânicas têm dominado a farmacologia desde o início do século 19. No entanto, o reaparecimento de drogas inorgânicas, as quais eram esquecidas, iniciou-se em 1960 com o desenvolvimento e o sucesso da cisplatina como agente antitumoral. Desde então complexos inorgânicos tem sido alvo de pesquisas terapêuticas com numerosas aplicações em diversos ramos da medicina (BAKHTIAR et al., 1999; KELLAND, 2007; REISNER et al., 2008).

Atualmente, a quimioterapia com complexos de platina é um dos principais pilares no tratamento do câncer. Dentre os vários complexos de platina sintetizados e analisados, três deles têm sido aprovados mundialmente para uso clínico: a cisplatina, utilizada para tumores de ovário, testículo, bexiga entre outros; a carboplatina, utilizada para tratamentos de câncer de ovário e por último, a oxaliplatina, utilizada para câncer de coloretal (KELLAND, 2007).

Apesar do sucesso da implementação de compostos de platina, sérios problemas são enfrentados durante a administração de drogas a base platina, como nefro e neurotoxicidade e resistência à droga (KARTALOU & ESSIGMANN, 2001; JIRSOVA et al., 2006). Contudo, estas limitações têm promovido à busca de novos agentes antitumorais baseados em metais que apresentem reduzida toxicidade e melhor efetividade (KOSTOVA, 2006).

Com o propósito de reduzir os efeitos tóxicos, e melhorar o perfil farmacológico e a eficácia terapêutica, uma série de compostos a base de metais não associados à platina tem sido desenvolvidos. Dentre estes compostos metálicos estão os compostos contendo rutênio, gálio, ferro, titânio,

ouro, organotin (JAKUPEC et al., 2008; ALAMA et al, 2009).

Destes compostos a base de metais, os mais promissores são os complexo de rutênio, os quais têm demonstrado atividade antitumoral e antimetastática. Além disso, complexos de rutênio oferecem um maior potencial quando comparado aos complexos de platina, devido à capacidade de apresentar um novo mecanismo de ação, reduzida toxicidade e a possibilidade de resistência não cruzada (SAVA et al., 1995; ALLARDYCE & DYSON, 2001; CLARKE, 2003; KOSTOVA, 2006; BRABEK & NOVÁKOVÁ, 2006).

1.9 Compostos de rutênio e atividade antitumoral

As primeiras investigações sistemáticas de complexos de rutênio e suas propriedades antitumorais foram realizadas no início de 1980 com os compostos *fac*-[$RuCl_3(NH_3)_3$] e *cis*-[$RuCl_2(NH_3)_4$]Cl, precedido pela descoberta que rutênio vermelho apresentava propriedades antitumorais. Mais adiante, estudos levaram ao desenvolvimento de (H2im)[*trans*-$RuCl_4$(Him)(DMSO)] (NAMI-A) e Indazolium trans-[tetrachlorobis(1H-indazole)ruthenate(III) (KP1019) (Figura 4), sendo as primeiras drogas antitumorais a base de rutênio a entrarem em triagens clínicas (RADEMAKER-LAKAHAI et al., 2004; HARTINGER et al, 2006). Em estágios pré-clínicos, NAMI-A demonstrou marcante eficácia contra processos de metástases, enquanto KP1019 apresentou potencial atividade antitumoral contra carcinoma coloretal (PACOR et al., 2004; KAPITZA et al., 2005a).

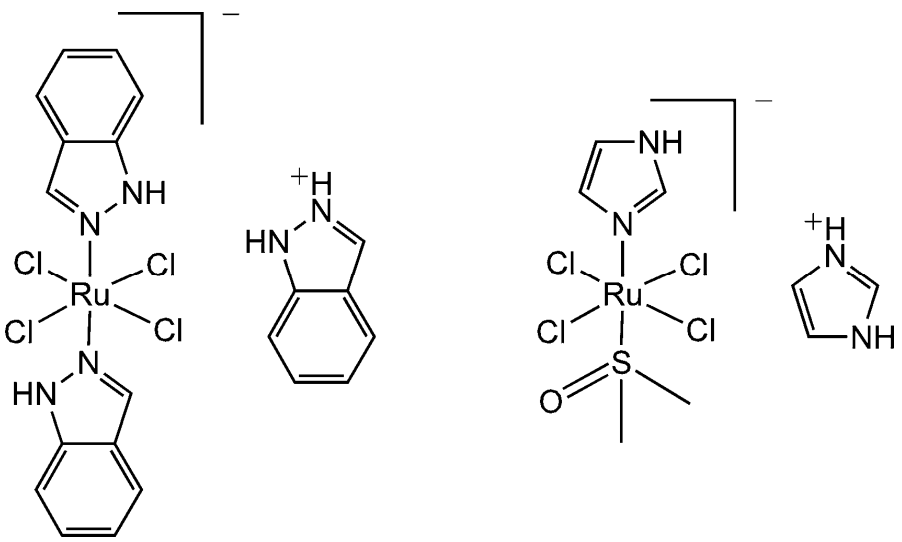


Figura 4. Estrutura química KP1019 e NAMI-A (JAKUPEC et al., 2008).

Além de NAMI-A e KP1019, vários outros complexos de rutênio com diferentes ligantes estão sendo desenvolvidos e testados mundialmente (CLARKE, 2003). Dentre os complexos de rutênios mais estudados para aplicações anticâncer se destacam os complexos heterocíclicos, complexos arenos, complexos amina, dentre outros (KAPITZA et al., 2005b; SCOLARO et al., 2005; SILVEIRA-LACERDA et al., 2009).

Destes complexos de rutênio, os complexos com ligantes aminas cis-tetraaminodiclororutênio (III) e *cis*-tetraaminoxalatorutênio (III) tem recentemente despertado o interesse de nosso grupo de pesquisa para a busca de novos agentes antitumorais.

Recentemente Silveira-Lacerda et al. (2009) estudaram o potencial antitumoral do composto *cis*-tetraaminodiclororutênio (III) sobre diferentes linhagens tumorais *in vitro*, e obtiveram promissores resultados sobre as células tumorais estudadas. Além de apresentar atividade antitumoral *in vitro*, estudos prévios demonstraram que *cis*-tetraaminodiclororutênio (III) apresentou atividade antitumoral *in vivo* sobre tumor Sarcoma 180 (S180) e, além disto,

aumentou o tempo de vida dos animais tratados com este composto (MENEZES et al., 2007).

Baseando-se nos promissores resultados apresentados por este composto, abriram-se novas perspectivas para estudos com este organometálico.

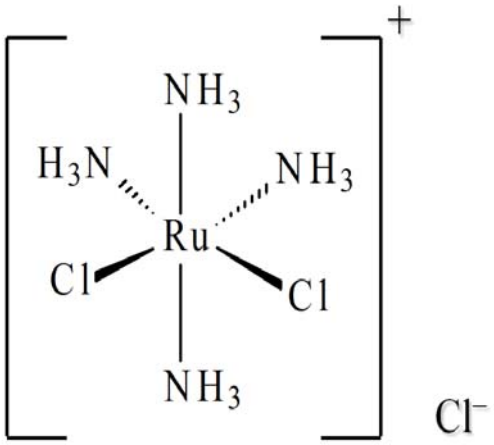


Figura 5. Estrutura química do composto Cloreto *cis*-tetraaminodiclororutênio (III)

1.10 Complexos de rutênio e mecanismo de ação

Apesar da maioria das drogas antitumorais não possuir especificidade às células tumorais, os composto de rutênio têm demonstrado baixa toxicidade sistêmica quando comparado a compostos de platina, e isto pode estar relacionado a um novo mecanismo de ação proposto (Figura 6) (BERGAMO et al., 1999; ALLARDYCE & DYSON, 2001).

A baixa toxicidade de complexos de rutênio (III) comparada a drogas de platina tem sido atribuída à habilidade de compostos de rutênio de acumularem-se especificamente em tecidos tumorais. Sendo assim, a alta especificidade destes compostos a células tumorais pode estar relacionada à

seletiva absorção pelo tumor comparado ao tecido sadio e a ativação por redução para espécies citotóxicas dentro do tumor, no entanto complexos de rutênio (III) servem como pró-droga o qual precisam ser reduzidos em espécies mais reativas para desenvolver seu potencial farmacológico. Essa “ativação por redução” é favorecida por condições de hipóxia e pH levemente ácido encontrado nos tecidos tumorais, o que favorece a redução do estado de oxidação do rutênio (III) para rutênio (II) nessas células (BERGAMO et al., 1999; CLARKE, 2003).

Além disso, algumas propriedades químicas de complexos de rutênio como (1) taxa de substituição de ligante, (2) estados de oxidação acessíveis e (3) habilidade deste elemento de mimetizar o ferro na ligação a várias biomoléculas, incluindo a transferrina e a albumina, faz com que complexos de rutênio sejam melhores disponíveis para aplicações biológicas quando comparados a complexos de platina (ALLARDYCE & DYSON, 2001).

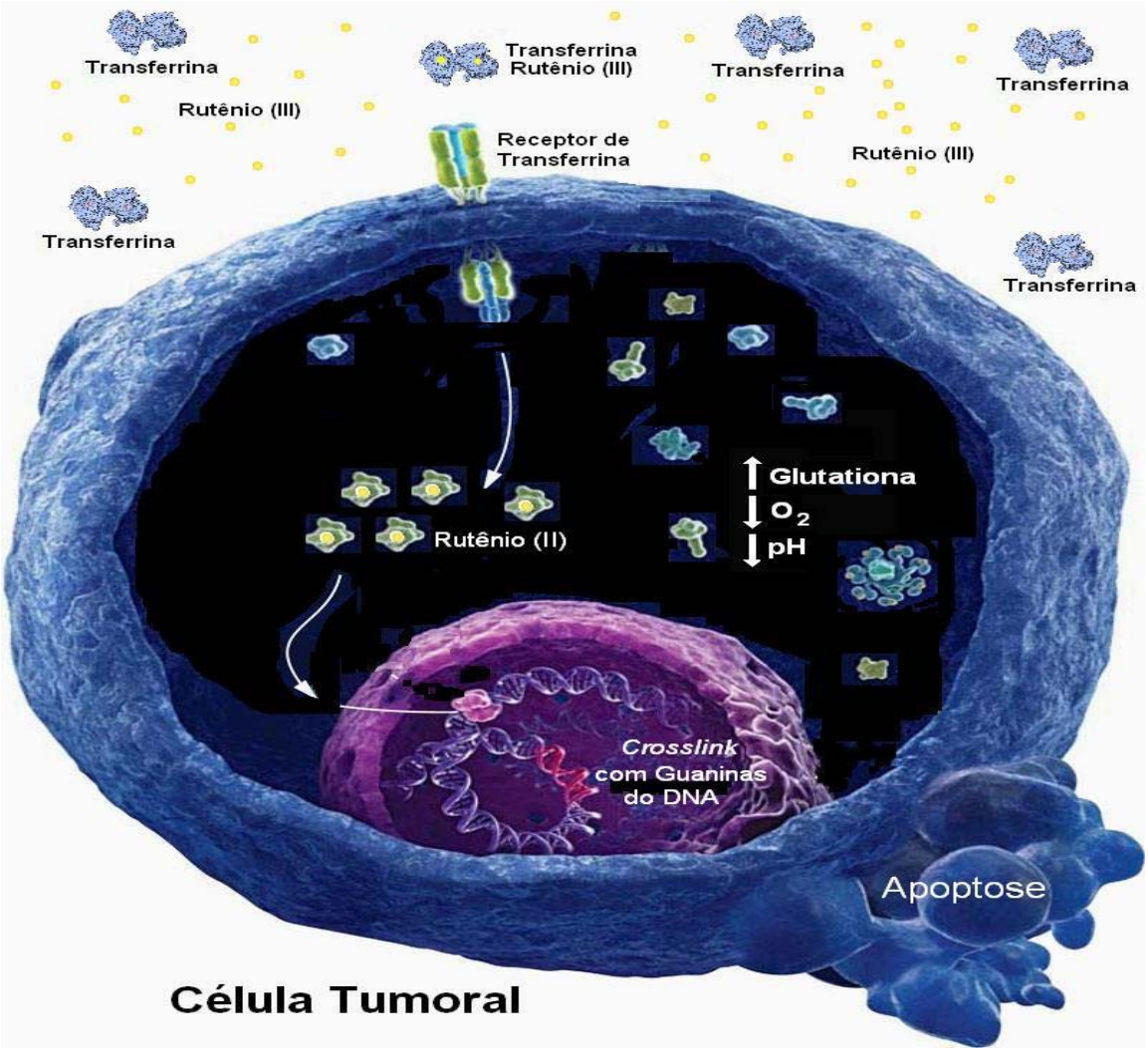


Figura 6. Mecanismo de ação de complexos de rutênio (III) sobre células tumorais (MODIFICADO ALLARDYCE & DYSON, 2001).

1.11. Complexos de Rutênio e principais alvos moleculares

Sabe-se que o principal alvo molecular para muitas drogas a base de platina é o DNA, baseando-se nestas evidências, inúmeros trabalhos com complexos de rutênio tem demonstrado que ligações a molécula de DNA sejam um dos principais responsáveis pelos efeitos antitumorais apresentados por estes compostos. A partir disso, diversos complexos de rutênio foram desenvolvidos e testados quanto à propriedade de ligação à molécula de DNA (PIZARRO et al., 2009; CORRAL et al., 2009).

Estudos sobre o mecanismo pelo qual os complexos de rutênio se ligam ao DNA têm sido investigados, e ao contrário da formação de ligação cruzada intrafita causado por cisplatina, complexos de rutênio tem demonstrado se ligar ao DNA através de ligação cruzada interfita, possivelmente favorecido pelas restrições impostas pela geometria octaédrica destes compostos (ALLARDYCE & DYSON, 2001; PIZARRO et al., 2009). Apesar de cisplatina e complexos de rutênio formarem diferentes tipos de ligação cruzada na molécula de DNA, a interação de ambos os compostos com o DNA ocorre preferencialmente através do N7 da guanina (CLARKE, 2003; PIZARRO, 2009).

A Figura 7 demonstra uma representação esquemática do modo de ação do complexo de rutênio KP1019. Estudo realizado por KAPITZA et al. (2005a), tem demonstrado que este complexo se liga ao DNA causando quebras que, conseqüentemente, levam a um processo de apoptose.

Ao contrário da visão que o principal alvo de complexos de rutênio é o DNA, estudos têm demonstrado que mecanismos independentes do DNA, como inibição de metaloproteases e interferência com processos de adesão e metabolismo do óxido nítrico são responsáveis pelos efeitos antitumorais de alguns complexos de rutênio. Os mecanismos pelos quais complexos de rutênio exercem sua atividade de maneira independente do DNA, têm sido demonstrados através de estudos com o complexo de rutênio NAMI-A, o qual tem demonstrado exercer seu efeito antimetastático, possivelmente pela atuação sobre moléculas de adesão e metabolismo do óxido nítrico (MORBIDELLI et al., 2003; SAVA et al., 2003; PACCOR et al., 2004).

Apesar de alguns estudos demonstrarem que NAMI-A possa interagir com o DNA *in vitro*, a ligação ao DNA não parece contribuir para o seu efeito antimetastático (PLUIM et al., 2004).

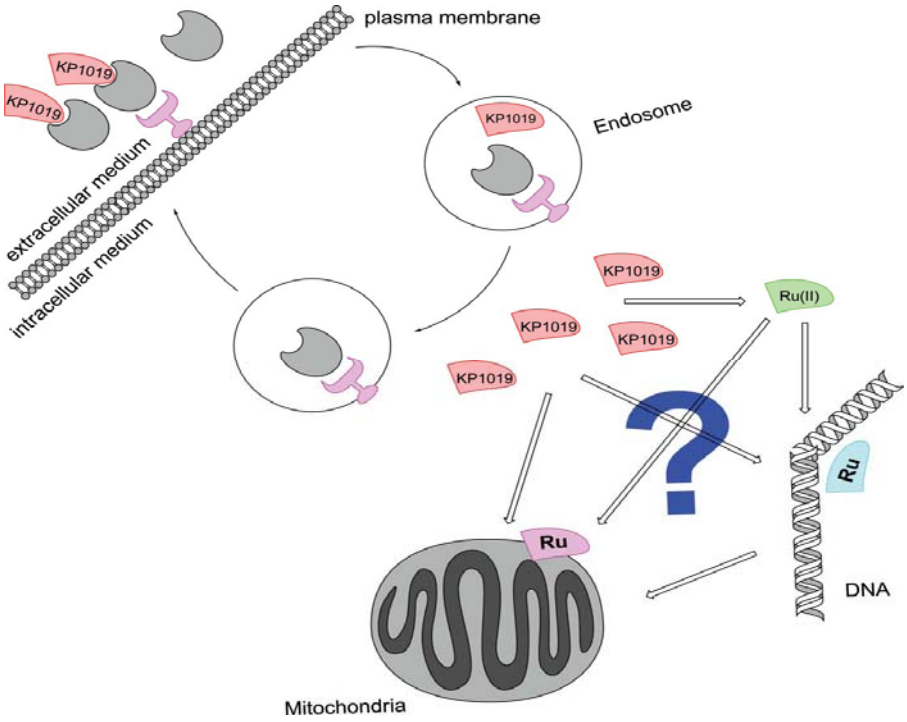


Figura 7. Representação esquemática do modo de ação do complexo de rutênio KP1019. (JAKUPEG et al., 2008).

2. JUSTIFICATICA

Na quimioterapia antineoplásica grandes limitações são ainda enfrentadas, dentre estas se destacam a inespeficidade das drogas quimioterápicas às células tumorais e a grande resistência de alguns tumores aos quimioterápicos. Com o intuito de reduzir os efeitos tóxicos e melhorar o perfil farmacológico e a eficácia terapêutica, uma série de compostos a base de metais têm sido desenvolvidos, e dentre estes uns dos mais promissores são os compostos a base de rutênio, os quais têm demonstrado promissores resultados antitumoras com baixa citotoxicidade sobre tecidos sádios.

Para a descoberta de novos agentes antineoplásicos tem-se a necessidade de um profundo e adequado conhecimento a fim de se evitar tratamentos tóxicos e ineficientes. Para isto são necessárias as realizações de estudos *in vitro* assim como investigações *in vivo* com modelos animais antes da triagem clínica. Desta forma, na busca por drogas mais eficazes e com alta especificidade a células tumorais, e partindo-se dos conhecimentos prévios sobre a atividade antitumoral apresentada pelo composto *cis*-tetraaminodiclororutênio (III), abriram-se novas perspectivas para estudos antitumorais com este composto frente novas linhagens tumorais, a fim de fornecer resultados complementares que possam subsidiar futuros estudos clínicos a serem realizados com este composto.

3. OBJETIVOS

3.1. Objetivo Geral

O presente trabalho teve como objetivo avaliar os possíveis efeitos citotóxico, genotóxico e antitumoral do composto sintético *cis*-tetraaminodiclororutênio (III), utilizando linhagens humanas e de camundongo.

3.2.1 Objetivos Específicos Artigo 1

1. Avaliar a atividade antitumoral do composto cloreto de *cis*-tetraaminodiclororutênio(III) sobre a linhagem tumoral K562 e a atividade citotóxica sobre linhagem normal de fibroblasto humano MCR-5;

2. Analisar o efeito do composto sobre a cinética do ciclo celular de células K562;

3.Verificar se os possíveis efeitos citotóxicos apresentados por *cis*-tetraaminodiclororutênio(III) sobre células K562 estão relacionados com o processo de apoptose;

4. Investigar o envolvimento das caspase 3, 8 e 9 no processo apoptótico de células tumorais K-562 após tratamento com *cis*-tetraaminodiclororutênio(III).

3.2.2 Objetivos Específicos Artigo 2

1. Avaliar o efeito genotóxico do composto cloreto *cis*-tetraaminodiclororutênio(III) sobre linhagem tumoral de camundongo S180;

2. Analisar o efeito do composto sobre a cinética do ciclo celular de

células S180;

3. Verificar se os possíveis efeitos citotóxicos apresentados por *cis*-tetraaminodiclororutênio(III) sobre células S180 estão relacionados com o processo de apoptose.

3.2.3 Objetivos Específicos Artigo 3

1. Avaliar a atividade antitumoral do composto *cis*-tetraaminodiclororutênio(III) sobre a linhagem tumoral A549;

2. Avaliar a capacidade de células A549 de formarem colônias após tratamento com o composto de rutênio;

3. Verificar se os possíveis efeitos citotóxicos apresentados por *cis*-tetraaminodiclororutênio(III) sobre células A549 estão relacionados com processo de apoptose.

4. MATERIAIS E MÉTODOS

4.1. Síntese do Cloreto de *cis*- tetraaminodiclororutênio(III)

O composto Cloreto de *cis*- tetraaminodiclororutênio(III) foi sintetizado no Laboratório de Química do Instituto de Química da Universidade Federal de Uberlândia e encaminhado ao Laboratório de Genética Molecular e Citogenética para realização deste trabalho.

4.2. Preparação do composto de Rutênio e droga controle para ensaios biológicos *in vitro*

Para os ensaios biológicos o composto Cloreto de *cis*-tetraaminodiclororutênio(III) liofilizado foi pesado e dissolvido em meio RPMI 1640 ou DMEM na concentração final de 2 $mg.mL^{-1}$. A partir da solução estoque de 2 $mg.mL^{-1}$ do composto de rutênio em meio de cultura, foram utilizadas as seguintes concentrações para uso experimental, conforme tabela 1. Após preparo da solução estoque do composto de rutênio, esta solução foi submetida à agitação vigorosa para dissolução completa utilizando ultrasom (Ultra Sonic Cleaner Unique), e por fim esterilizada em membranas de 0,22 µm. Como droga controle para os ensaios clonogênico e de viabilidade celular foi utilizado o antitumoral cisplatina (25 e 50 µM) respectivamente, e para o ensaio cometa foi utilizado a droga metrotexato (25 µM), ambos diluídos em meio RPMI ou DMEM.

Cloreto de *cis*- tetraaminodiclororutênio(III)	
mg.mL^{-1}	**µM**
0,01	0,38
0.1	3,8
0.2	19
0.5	95
1	383

Tabela 1: Concentrações do composto Cloreto de *cis*-tetraaminodiclororutênio(III) 0,01 a 1 mg.mL-^{1} (0,38, 3,8, 19, 95, 383 µM) utilizadas para a realização dos ensaios experimentais.

4.3 Linhagens celulares e manutenção do cultivo celular

Para os ensaios biológicos foram utilizadas as linhagens tumorais estabelecidas K-562 (Leucemia mielóide crônica), A549 (Carcinoma alveolar de pulmão humano) e linhagem tumoral de camundongo S180 (Sarcoma 180) e como células normais (controle) foi utilizada a linhagem estabelecida MCR-5 (fibroblasto de pulmão humano). Todas as linhagens celulares foram obtidas do Banco de Células do Rio de Janeiro e mantidas em cultura a 37^{o}C, 5%CO_2 em meio RPMI 1640 ou DMEM e suplementado com 10% de soro bovino fetal, 2 mM L-Gnl, 100IU/mL de penicilina e 100µg/mL de estreptomicina segundo protocolo estabelecido pela *American Type Culture Collection* (ATCC, Rockville, MD, EUA).

4.4. Ensaio de Redução do MTT

Para avaliar a atividade citotóxica e antitumoral do composto *cis*-tetraaminodiclororutênio(III) foi utilizado o método colorimétrico do MTT (3-

(4,5-Dimetiltiazol-2-il)2,5-Difenil Brometo de Tetrazolium). O princípio deste método descrito por Mosman (1983) consiste em medir indiretamente a viabilidade celular pela atividade enzimática mitocondrial das células vivas. Para o teste do MTT, 1 x 10^5 células foram semeadas em microplacas de 96 poços na ausência ou presença do composto *cis*- tetraaminodiclororutênio(III) (0,38 a 383 µM) ou cisplatina (50 µM) e incubadas em estufa a 37^oC com atmosfera contendo 5% de CO_2. Ao final do período de incubação, foi adicionado aos poços de cultivo celular 10 µL de MTT na concentração de 5 $mg.mL^{-1}$, e após 3 h de incubação com o MTT, foram acrescentados 50 µL SDS a 10% diluído em HCL/0,01N. A quantificação da densidade óptica (DO) foi medida em espectrofotômetro (Awareness Technology INE/ Stat Fax 2100). A porcentagem de viabilidade celular foi determinada a partir da seguinte fórmula: **% Viabilidade =** Absorbância do Tratamento/Absorbância do controle negativo*100. O valor de IC_{50} (concentração em µM que inibe 50 % da viabilidade celular) foi determinada por meio da curva dose resposta utilizando o programa estatístico GraphPad Prism 4.02 (GraphPad Software, San Diego, CA, USA).

4.5. Ensaio de viabilidade celular por Azul de Tripano

A viabilidade celular após exposição a drogas pode ser medida pela exclusão de corantes supravitais como o azul de tripano. O princípio deste teste consiste em medir indiretamente a viabilidade celular através da integridade de membrana, onde as células mortas que apresentam a membrana danificada absorvem este corante e consequentemente coram-se em azul, já as células vivas permanecem intactas sem coloração (PERES & CURI, 2005). Para o ensaio de azul de tripano, foram realizados os mesmos

procedimentos de plaqueamento como os realizados no ensaio do MTT. No entanto, ao final do período de incubação, alíquotas de 10 µL de suspensão celular foram colocadas em 40 µL de azul de tripano à 0.4%, e desta solução foi retirada uma alíquota para contagem de células em câmara de neubauer. Para determinar a porcentagem de viabilidade celular foi utilizada a mesma fórmula descrita no item 4.4. Ambos os ensaios MTT e Azul de tripano foram utilizados para calcular o valor da $IC_{50.}$ Para os ensaios foram realizados três experimentos independentes feitos em triplicata.

4.6. Ensaio de sobrevivência clonogênica

Para analisar a capacidade proliferativa de células de formarem colônias após exposição a um determinado agente antiproliferativo utiliza-se o ensaio de sobrevivência clonogênica (PLUMB, 2004). Para verificar a capacidade de células de formarem colônias após tratamento com o composto de rutênio foi utilizado o ensaio de sobrevivência clonogênica, onde 3 x 10^2 células tumorais A549 foram semeadas em frascos de cultura de 25 cm^2 por um período de 24 h para aderência das células; após este período foi retirado todo o meio celular e em seguida as células foram tratadas com diferentes concentrações do composto *cis*- tetraaminodiclororutênio(III) (0,38; 3,8; 95 e 383 µM) por 48 e 72 h. Após os períodos de tratamento as células foram lavadas com solução de PBS para retirada do composto e em seguida foi adicionado as garrafas meio de cultura completo. Posteriormente as células foram incubadas por 10 dias, sendo as culturas observadas diariamente com o auxílio de microscópio invertido (Leica). Ao final do período de incubação, o meio de cultura foi descartado e as células foram lavadas com solução de PBS. Em seguida, as células foram fixadas com uma solução de metanol/ácido acético/água na

proporção de 1:1:8, respectivamente, por 30 min e após período de fixação, as células foram coradas com Giemsa (5%) por 15 min. Para análise densidométrica do crescimento clonogênico os frascos de cultura foram escaneados em equipamento mulifuncional HP e posteriormente foi feito a análise por meio do software Clono-Counter.

4.7. Ensaio cometa: análise de danos à molécula de DNA

O Ensaio Cometa é um método versátil e sensível para a detecção de quebras de fita simples e dupla no DNA. Ele tem sido amplamente utilizado como uma ferramenta para avaliação das respostas celulares aos danos causados ao DNA. O princípio deste teste consiste em isolar células idividualizadas em uma matriz de agarose e submetê-las a uma etapa de lise celular onde restará apenas o conteúdo do DNA nuclear aderido a essa matriz (GONTIJO & TICE, 2003).

Para detecção de danos ao DNA, foi utilizada a versão alcalina do ensaio cometa segundo SINGH et al. (2008), onde 2 x 10^4 de células tumorais S180 foram semeadas na ausência ou presença do composto cloreto de cis-tetraaminodiclororutênio(III) (0,38; 3,8, 19 and 95 μM) ou do controle positivo Metrotexato (25 μM) por 24 e 48 h. Após tratamento, a suspensão de células foram homogeneizadas com 100μL de agarose de baixo ponto de fusão (0,5 %), e então colocadas em lâminas que já continham agarose padrão (1.5%). Estas lâminas foram posteriormente colocadas a 4 °C por 10 min e em seguida colocadas em solução de lise (2,4M NaCl; 100 mM EDTA; 10 mM Tris, 10% DMSO and 1% Triton-X, pH 10) por 24 h. Após etapa de lise as lâminas foram transferidas para cuba de eletroforese contendo tampão (NaOH 300mM, EDTA 1mM, pH ~13) à 4ºC, em corrente de 25V, 300mA. Na etapa seguinte as

lâminas foram retiradas da cuba de eletroforese e posteriormente submetidas a uma solução de neutralização (0,4M Tris–HCl, pH 7,5) por 15 min, após a secagem das laminas elas foram então fixadas com álcool etílico absoluto e posteriormente coradas com brometo de etídio e analisadas por microscopia de fluorescência acoplado a um computador.

A análise dos cometas foi realizada visualmente, de acordo com a migração dos fragmentos conforme classificação proposta por KOBAYASHI et al. (1995) em: classe 0 (nenhum dano), classe 1 (pequeno dano com o comprimento da cauda menor do que o diâmetro da cabeça), classe 2 (médio dano com o comprimento da cauda uma ou duas vezes o diâmetro da cabeça), classe 3 (significante dano comprimento da cauda maior que duas ou três vezes o diâmetro da cabeça), classe 4 (significante dano comprimento da cauda maior que três vezes o diâmetro da cabeça). Foram examinados aleatóriamente 100 nucleóides de cada concentração, assim como do controle negativo e positivo.

Para o cálculo do índice de dano (ID) foi utilizada a seguinte fórmula de acordo com Cavalcanti et al., 2008:

ID= (0 x n0) + (1 x n_1) + (2 x n_2) + (3 x n_3) + (4 x n_4), onde n = número núcleos de cada classe analizada. Desta forma o índice de dano para 100 nucleóides pode variar de zero (completamente sem danos 0 X 100) a 400 (totalmente danificadas 100 X4).

4.8. Análise da Cinética do Ciclo Celular por Citometria de Fluxo

As fases do ciclo celular podem ser caracterizadas por variações no seu conteúdo de DNA, que quando analisado por citometria de fluxo após marcação com iodeto de propídio permite quantificar a percentagem de células

em cada fase do ciclo celular. Para esta análise, 1 x 10^6 células tumorais foram plaqueadas em microplacas de 12 pocos na presença ou ausência do composto cloreto de *cis*- tetraaminodiclororutênio(III). Após exposição das células ao composto de rutênio, estas foram centrifugadas e em seguida lavadas com PBS. Ao final da lavagem o sobrenadante foi desprezado, e o "pellet" celular foi incubado com 2 mL de álcool etílico gelado (70%) por 24 hs a 4°C. Ao final da incubação as células foram lavadas novamente com PBS e em seguida estas foram incubadas por 15 min em uma solução contendo ribonuclease A (RNase A) 0.05% e iodeto de propídio (50 $\mu g.mL^{-1}$). A análise da porcentagem de células em G1, S, G2 e sub-G1 foi realizada em citômetro de fluxo (FACSCanto, BD Biosciences), através do software Diva.

4.9. Ensaio Apoptose (Anexina V-FITC/Iodeto de Propídio)

O procedimento de detecção de apoptose por Anexina V-FITC/Iodeto de Propídio consiste na ligação da anexina V-FITC à fosfatidilserina, na membrana das células que estão iniciando o processo apoptótico, e na ligação do iodeto de propídeo ao DNA das células no processo final da apoptose.

Para detecção de apoptose foi utilizado o Kit de detecção de apoptose Anexina V/Iodeto de Propídio (PI) (Sigma-Aldrich, St. Louis, Mo) de acordo com as instruções do próprio fabricante. Para este ensaio 5 x 10^5 células foram semeadas em microplacas de 12 poços e incubadas na ausência ou presença do composto *cis*-tetraaminodiclororutênio(III). Após tratamento com o composto de rutênio as células foram centrifugadas e posteriormente lavadas com PBS. O sobrenadante foi descartado e ao pellet celular foi adicionado 400 µL de tampão de ligação e em seguida acrescentados 5 µL de Anexina V-FITC e 1 µL de iodeto de propídio. As células foram então incubadas em temperatura

ambiente por 15 min, e posteriormente foi feito à aquisição dos dados em citômetro de fluxo (FACSCanto, BD Biosciences). Para análise dos dados foi utilizado o software Diva.

Foram classificadas como células em apoptose inicial aquelas com marcação somente para Anexina-V (AN+)/(PI-), e como células em apoptose tardia aquelas com dupla marcação de Anexina V e PI (An+)/(PI+), células viáveis não apresentam nenhuma marcação.

4.10. Análises de atividade das caspases

Para investigação da atividade das caspase 3, 8 e 9 foi utilizado o Kit de proteases colorimétrico (R&D Systems) de acordo com as instruções do próprio fabricante. O ensaio de atividade das caspases é baseado na detecção por espectrofotômetro do cromóforo p-nitroanilima (pNA) depois da clivagem do substrato X-pNA, onde X é a seqüência de aminoácidos reconhecida pelas caspases. Neste ensaio, 1 x 10^6 células foram semeadas em microplacas de 12 poços e incubadas por período de 24 h na ausência ou presença do composto *cis*-tetraaminodiclororutênio(III) (95 μM). Após incubação as células foram inicialmente centrifugadas e o pellet de células formado foi incubado com de tampão de lise em banho de gelo por 10 min. Em seguida o lisado celular foi centrifugado e posteriormente coletado. Para a reação foram utilizados 50 μL da amostra, 50μL de tampão de reação e 5 μL do substrato colorimétrico. Posteriormente a microplaca foi incubada a 37 °C por 2 h e a atividade das caspases 3, 8 e 9 foi determinada pela medida de p-Nitroanilina (pNA) liberada pela clivagem dos substratos de caspase 3 (DEVD-pNA), caspase 8 (IETD-pNA) e caspase 9 (LEHD-pNA). A liberação de pNA foi monitorada pelo

espectrofotômetro (Awareness Technology INE/ Stat Fax 2100) utilizando um comprimento de onda de 405 nm. As atividades enzimáticas foram expressas em relação ao controle negativo.

4.11. Análise estatística

Para comparação entre os grupos tratados e controle foi utilizado Anova segundo um único critério e pós-teste Dunnet´s e Tukey (software GraphPad-Prism V4.) Foi considerado como diferença significativa valores de p menores que 0,05 ($p<0.05$).

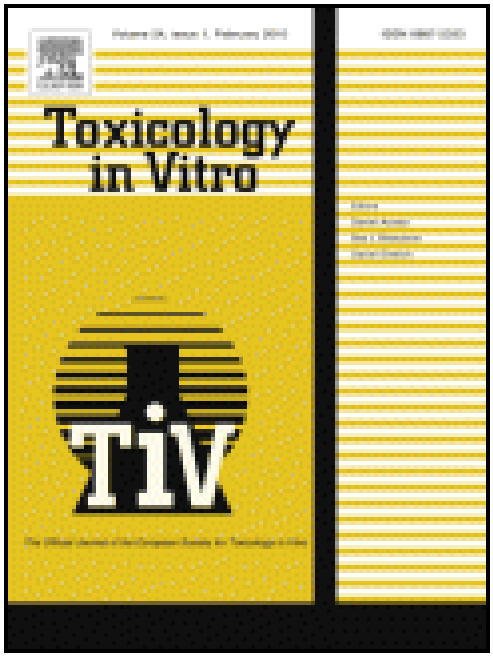
5. PRODUÇÃO CIENTÍFICA

ARTIGO 1 – The organometallic compound *cis*-(dichloro)tetramminеruthenium(III) chloride induces caspase-mediated apoptosis in K562 cells

ARTIGO 2 – The ruthenium complex *cis*-(dichloro)tetramminеruthenium(III) chloride induce apoptosis and damage DNA in murine ascitic sarcoma 180 (S-180) cells

ARTIGO 3 – Human lung carcinoma cells (A549) show resistance to the ruthenium complex *cis*-(dichloro)tetramineruthenium(III) chloride

ARTIGO 1



Artigo Submetido ao periódico **Toxicology in Vitro**

<http://ees.elsevier.com/tiv/>

Ciências Biológicas I: Qualis B1

The organometallic compound *cis*-(dichloro)tetramminеruthenium(III) chloride induces caspase-mediated apoptosis in K562 cells

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ABSTRACT

Ruthenium(III) complexes are increasingly attracting the interest of researchers due to their promising pharmacological properties. In the present study, we investigated the ability of *cis*-(dichloro)tetramminеruthenium(III) chloride to produce lethal effects in human chronic myelogenous leukemia K-562 cells. The MTT tetrazolium reduction test and the trypan blue exclusion assay revealed that the IC_{50} for the compound after 48 h of incubation with K562 cells was approximately 10.74 and 73.45 μM, respectively. Interestingly, it was observed that this compound exhibits mild cytotoxicity towards MRC-5 human fibroblast cells (IC_{50} > 383 μM). Flow cytometric analysis revealed that *cis*-(dichloro)tetramminеruthenium(III) chloride was capable of change cell cycle distribution since the percentage of cells in the G1, S and G2 phases decreased. In addition, treatment with this compound induced apoptotic cell death in K562 cells, demonstrated by increased DNA content in the sub-G1 peak and a significant increase in caspase-3 activity. In summary, *cis*-(dichloro)tetramminеruthenium(III) chloride displayed a significant anti-tumor effect through cell cycle arrest and apoptotic induction in K562 cells, which suggests that *cis*-(dichloro)tetramminеruthenium(III) chloride might have therapeutic potential against leukemia.

Keywords: Apoptosis; *cis*-(dichloro)tetramminеruthenium(III) chloride; Cytotoxicity; K562; MRC-5.

1. INTRODUCTION

Chronic myelogenous leukemia (CML) is a hematological disorder characterized by the Philadelphia chromosome, which is formed by the reciprocal translocation of chromosomes 9 and 22 and results in a constitutively activated fusion oncoprotein (Bcr-Abl) with unregulated tyrosine kinase activity (Deininger et al., 2000). The tyrosine kinase activity of Bcr-Abl, which is the principal driving force behind its oncogenic potential, is responsible for mediating tyrosine phosphorylation of specific cellular proteins and Bcr-Abl itself (Hantschel and Superti-Furga, 2004). The resulting Bcr-Abl protein product is an oncoprotein that confers resistance to apoptosis induced by many anticancer drugs (Fernandez-luna, 2000; le Coutre et al., 2000; Gorre et al., 2001).

Heavy metal coordination complexes such as platinum, gallium and ruthenium have been investigated for their possible antitumor activity (Jakupec et al., 2008; Alama et al., 2009). Among the most studied metal complexes, cisplatin and related platinum-based drugs have drawn much attention. However, the development of these metallo-drugs is hindered by some major problems such as significant toxicity, side effects and major problems with resistance (Kartalou and Essigmann, 2001; Jirsova et al., 2006). In order to find compounds with less toxic side effects as well as compounds active against tumors that do not respond (or that show resistance to) cisplatin, new metal coordination complexes have been studied. In particular, some ruthenium derivatives have been synthesized in recent years and screened for their antineoplastic activity and their cytotoxic effects (Clarke, 2003; Kostova, 2006). Among these ruthenium derivates, ImH[trans-RuCl(DMSO)Im] (NAMI-A) and indazolium trans-[tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019 or FFC14A) are the first ruthenium-based anticancer drugs that have been

transferred into clinical trials (RADEMAKER-LAKAHAI et al., 2004; Hartinger et al., 2006). However, many other compounds that include Ru centers are being developed and tested in academic labs or in small companies in the field (Clarke, 2003).

Ruthenium compounds are well suited for medical applications because they have convenient rates of ligand exchange, a range of accessible oxidation states, and the ability to mimic iron in binding to certain biological molecules. This latter feature is thought to be why some ruthenium complexes have particularly low general toxicity, which allows larger doses of the compounds, and therefore more effective anticancer therapies (Allardyce and Dyson, 2001). This reduced toxicity and the non-cross-resistance of cisplatin-resistant cancer cells are particularly attractive attributes of these complexes (Allardyce and Dyson, 2001; Clarke, 2003; Brabec and Nováková, 2006; Kostova, 2006).

An example of a ruthenium-based complex with potential for anticancer therapy is *cis*-(dichloro)tetrammineruthenium(III) chloride (*cis*-$[RuCl_2(NH_3)_4]Cl$), which has been shown to selectively kill tumor cells without harming normal cells (Silveira-Lacerda et al., 2009a; Silveira-Lacerda et al., 2009b; Ribeiro et al., 2009).

Based on these observations, the aim of the present study was to examine *in vitro* the effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ on cell viability, cell cycle and apoptosis of the chronic myelogenous leukemia cell line K-562; studies have shown that this cell line is particularly resistant to cell death via apoptosis by a variety of different agents (Bedi et al., 1995). Since the toxicity of many drugs used in chemotherapy limits their clinical success, we also analyzed here the cytotoxic activity of the of *cis*-$[RuCl_2(NH_3)_4]Cl$ on normal human lung fibroblast

cells (MRC-5).

2. MATERIALS AND METHODS

2.1. Synthesis of *cis*-$[RuCl_2(NH_3)_4]Cl$

The *cis*-$[RuCl_2(NH_3)_4]Cl$ complex (Figure 1) was prepared as follows. Air-free concentrated ammonium hydroxide (25 mL) was added to $[RuCl(NH_3)_5]Cl_2$ (1.0 g, 3.4×10^{-3} mols), which was then refluxed under argon until a burgundy-colored solution was produced. Sodium dithionate (0.7 g) was added to the hot solution, which was subsequently cooled in an ice bath. An off-yellow-cream solid, $[RuOH(NH_3)_5]S_2O_6$, precipitated and was collected by filtration and air dried. A second crop of crystals was obtained by addition of anhydrous ethanol (50 mL). This material was collected by filtration, washed with ethanol and air-dried. The $[RuOH(NH_3)_5]S_2O_6$ was dissolved in saturated oxalic acid (11 mL) and then refluxed for approximately 10 minutes under argon, and a yellow solid formed. The mixture was then cooled to complete the precipitation. The crystals of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2S_2O_6$ were isolated by filtration, washed with ethanol and air dried. The tetraammineoxalatoruthenium(III) dithionate crystals were dissolved in 5 M HCl (12 mL), and the solution was refluxed for 10 minutes. The hot solution was filtered and absolute ethanol (30 mL) was added to the yellow filtrate. A yellow solid, *cis*-$[RuCl_2(NH_3)_4]Cl$, precipitated. After the mixture had cooled, the *cis*-product was collected by filtration, washed with ethanol and air dried. The yield was 55%. The compound was characterized by electronic spectra at room temperature using quartz cells in an HP 8453 spectrophotometer with a diode

arrangement that interfaced with a compatible PC HP Vectra XM. The electronic spectrum of the *cis*-$[RuCl_2(NH_3)_4]Cl$ complex presented bands at 260, 308 and 350. Briefly, the absorption spectra in aqueous solution were dominated by the ligand-to-metal charge transfer bands with λ_{max} at 350 nm ($\varepsilon = 1.5\times10^3$ M^{-1} cm^{-1}), 308 nm ($\varepsilon = 1.3\times10^3$ M^{-1} cm^{-1}) and 260 nm sh ($\varepsilon = 500^3$ M^{-1} cm^{-1}). Carbon, hydrogen and chloride microanalyses were performed by the staff of the Analytical Center of the Chemical Institute of Universidade de São Paulo. The calculated proportions were: N, 20.3; H, 4.4, Cl, 38.6. The observed proportions were: N, 20.2; H, 4.5, Cl, 38.5.

2.2. Cell culture

K-562 cells obtained from the Rio de Janeiro Cell Bank/RJ, Brazil were cultured in suspension in RPMI 1640 medium (Sigma Chemical Co., MO) supplemented with 10% fetal calf serum, 100 UI/ml penicillin, and 100 $\mu g ml^{-1}$ streptomycin in a humidified atmosphere at 37 °C in 5% CO_2. The normal human fibroblast cell line MRC-5 was also used. MRC-5 cells obtained from the Rio de Janeiro Cell Bank/RJ, Brazil were cultured in DMEM medium supplemented with 100 U mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine, 1.5 g L^{-1} sodium bicarbonate, 4.5 g L^{-1} glucose, 10 mM HEPES and 10% fetal calf serum 1% (w/v) at 37°C under a 5% CO_2 humidified atmosphere. MRC-5 cells were routinely sub-cultured using 2.5 g. L^{-1} trypsin-ethylenedinitrile tetraacetic acid (EDTA) solution (Sigma-Aldrich, St. Louis, MO).

2.3 MTT assay

The effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ on viability of K562 and MRC-5 cells were studied by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described by Mosman, (1983). Briefly, 1×10^5 K562 and MRC-5 cells were plated in 96-well tissue culture plates and treated with different concentrations of *cis*-$[RuCl_2(NH_3)_4]Cl$ (0,01, 0.1, 0,2, 0,5 and 1mg.mL^{-1} that correspond to (0.38, 3.8, 19, 95 and 383 μM) for 48 h. After treatment, 10 μL MTT (5 mg.mL^{-1}) was added to each well and the plates were incubated at 37°C for another 3 h. The purple formazan crystals were dissolved in 50 μL SDS (10%), and absorbance was determined at 545 nm using an Stat Fax 2100 microplate reader (Awareness Technology, Palm City, FL, USA). The cell viability was calculated as follows: Viability (%) = (Absorbance of the treated wells)/(Absorbance of the control wells)*100. Each concentration was tested in three different experiments run in triplicate. The IC_{50} value (compound concentration (μM) that produces a 50 % reduction in cellular viability) was obtained from the dose-response curves using GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA, USA).

2.4 Trypan blue assay

The viability of K-562 cells was also evaluated by the trypan blue exclusion method. The cells at a concentration of 1×10^5 cells/well were treated with different concentrations of *cis*-$[RuCl_2(NH_3)_4]Cl$ (0.38, 3.8, 19, 95 and 383 μM) and incubated for 48 h at 37 °C in a humidified air atmosphere with 5% CO_2. At the end of this period, the medium from each plate was removed by

aspiration. Next, the cells were washed with PBS and centrifuged at 1500 rpm for 10 min, and then PBS was removed by aspiration. Then, 10 µL of suspended cells was incubated with 40 µL 0.4% trypan blue solution (Sigma) and the number of cells was estimated using a hemocytometer. Thereafter, an Olympus BX41 microscope was used to identify nonviable cells, which were dark blue, and viable cells, which excluded the dye. The IC_{50} (compound concentration (µM) that produces a 50 % reduction in cellular viability) was obtained from the dose-response curves using GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA, USA

2.5 Cell cycle analysis

K-562 cells were treated with *cis*-$[RuCl_2(NH_3)_4]Cl$ (95 and 383 µM) for 48 h. Briefly, 1 X 10^6 cells were harvested by centrifugation, washed with PBS, fixed with 70% (v/v) cold aqueous ethanol and stored overnight at -20 ◦C. The fixed cells were washed with PBS and incubated with propidium iodide (50 $\mu g.mL^{-1}$) (PI; Sigma) containing 0.05% RNase. Samples were incubated at 4 ◦C in the dark and analyzed by flow cytometry (FACS Canto, BD Biosciences). The percentage of cells in G1, S, G2 and sub-G1 was analyzed using Diva software.

2.6. Caspase activity

Direct measurements of caspase activity were performed using colorimetric protease kits (R&D Systems, USA) according to the manufacturer's recommendations after the incubation of cells with *cis*-$[RuCl_2(NH_3)_4]Cl$ (95 µM) for 24 h. The caspase activity assay is based on the spectrophotometric

detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the substrates X-*p*NA, where X stands for amino acid sequences specifically recognized by caspase-3, caspase-8 and caspase-9. According to the procedure, 1 x 10^6 K-562 cells were pelleted by centrifugation and lysed on ice. Subsequently, the proteins were incubated with each X-*p*NA substrate at 37ºC in a microtiter plate. The optical density of the samples was measured at 405 nm. The increase in the activities of caspase 3, 8 and 9 was determined by comparing these results with the levels in untreated controls.

2.7 Statistical analysis

Statistical analysis of the results was performed by using one-way ANOVA followed by Dunnett's or Tukey post hoc test for multiple comparisons with a control. All statistical analyses were performed using the statistical software GraphPad Prism, version 4. A probability of 0.05 or less was deemed statistically significant. The following notation was used throughout; *p <0.05, **P<0.01, relative to control.

3. RESULTS

3.1 Effect of *cis*-$[RuCl_2(NH_3)_4]Cl$ on K-562 and MRC-5 cell viability

To investigate the antitumor effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ on K-562 cells, the viability of K-562 cells was studied using MTT and trypan blue exclusion assays. The results of the MTT and trypan blue assays showed that *cis*-$[RuCl_2(NH_3)_4]Cl$ dose-dependently reduced viability of K-562 cells (Fig. 2 and 3). The IC_{50} values in the MTT and trypan blue assays were approximately 10.74 and 73.45 μM after 48 h of exposure, respectively. In contrast to K-562 cells, low cytotoxic effect was observed in normal human MRC-5 cells using assay MTT (IC_{50} > 383 μM) (Fig. 2).

3.2. Cell Cycle Analysis

The cell cycle distribution of K-562 cells treated with 95 and 383 μM *cis*-$[RuCl_2(NH_3)_4]Cl$ for 48 hours as examined by flow cytometry showed a dose-dependent increase in the sub-G1 peak indicating induction of apoptosis. As shown in Fig. 4, treatment with *cis*-$[RuCl_2(NH_3)_4]Cl$ caused an increase in the proportion of cells in the sub-G1-peak correlating with fewer cells in G1, S and G2. The fraction of sub-G1 cells increased from 30.7% in the control to 75.35% and 67.4% in cells treated with 95 and 383 μM of *cis*-$[RuCl_2(NH_3)_4]Cl$, respectively.

3.3 Activation of caspase activity

The effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ (95 μM) on the activities of caspase-3, 8 and 9 were investigated in K-562 cells after 24 h of treatment

(Fig. 5). As it is possible to see from these results, the activities of caspase-8 and -9 changed only modestly in *cis*-$[RuCl_2(NH_3)_4]Cl$-treated cells when compared to untreated control cells. However, the assessment of caspase-3 activity showed a significant enhancement in relation to the control after incubation of K-562 cells with *cis*-$[RuCl_2(NH_3)_4]Cl$ for 24 h.

4. DISCUSSION

Current platinum-based anticancer therapies have been used very successfully in the clinic to combat a range of cancers, but they are not universally applicable. Active research in developing new therapies has led to extensive investigations into novel complexes with alternative metal centers. Some ruthenium complexes have been demonstrated to play a major role in the regression of cancer and in addition, some have surprisingly low general toxicity (Bergamo et al. 1999; Scollaro et al., 2005).

In the search of new anticancer compounds, we tested the effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ on cell line K-562. Measurement of the number of living cells using MTT or similar assays in drug-treated and control cultures is the most commonly used method in cell-based screening experiments. In this study, using the MTT and trypan blue exclusion viability assays, we found that *cis*-$[RuCl_2(NH_3)_4]Cl$ significantly reduced the viability of K-562 cells in a dose-dependent manner relative to untreated cells, with an IC_{50} of approximately 10.74 and 73.45 μM, respectively. These results confirm previous observations that *cis*-$[RuCl_2(NH_3)_4]Cl$ induces cytotoxicity towards tumor cell lines such as A-20, SK-Br-3 and S-180 (Silveira-Lacerda et al., 2009b). Besides being cytotoxic in vitro for tumor cells, a previous report indicated that *cis*-$[RuCl_2(NH_3)_4]Cl$

exhibited antitumor effects *in vivo* on S180 tumor cells, showing tumor volume reduction and increased survival time in treated animals (Menezes et al., 2007). The antitumor activity of ruthenium-based complexes has been evaluated in K-562 cells, and the studies showed either no activity or very mild antitumor action toward this cell line after 72 h of treatment (IC_{50} >200 μM) (Djinovic et al., 2009).

Current studies aimed at developing effective cancer preventive approaches have focused mainly on the utilization of chemical compounds that can induce selective cytotoxicity in cancer cells. Therefore, we examined the cytotoxicity *cis*-$[RuCl_2(NH_3)_4]Cl$ in MRC-5 human fibroblast cells. By comparison, we found that the toxicity of this compound to the tested human cancer cells was much stronger than its toxicity to normal MRC-5 cells (IC_{50} > 383 μM), suggesting that the cytotoxic activity of this compound may be selective to tumor cells (Fig. 2). Recently, Silveira-Lacerda et al., (2009a), demonstrated *in vitro* that *cis*-$[RuCl_2(NH_3)_4]Cl$ showed very low toxicity toward human peripheral blood mononuclear cells (PBMC), corroborating what was found in this study.

The lower general toxicity of ruthenium compounds compared to platinum drugs has been attributed to the ability of ruthenium compounds to specifically accumulate in cancer tissues. The higher specificity of these compounds for their targets may also be linked to their selective uptake by the tumor compared with healthy tissue and to selective activation by reduction to cytotoxic species within the tumor (Bergamo et al., 1999; Allardyce and Dyson, 2001; Clarke, 2003).

Several reports have indicated that ruthenium complexes inhibit the proliferation of cells by inhibiting cell cycle progression and inducing apoptosis

(Capozzi et al., 1998; Kapitza et al., 2005a). As shown in the cell cycle analysis, the sub-G1 population increased dramatically after treatment with *cis*-$[RuCl_2(NH_3)_4]Cl$, which has been suggested to be the apoptotic DNA. Furthermore, the results obtained suggested that *cis*-$[RuCl_2(NH_3)_4]Cl$ caused alterations cell cycle distribution since the percentage of cells in G1, S and G2 phases decreased. Previous studies showed that *cis*-$[RuCl_2(NH_3)_4]Cl$ reduced the number of A-20 cells in G0/G1, but not in S and G2/M; on the other hand, in Jurkat cells this compound slightly diminished the number of cells in G0/G1 and greatly reduced the number of cells in G2/M (Silveira-Lacerda et al., 2009b).

It is well established that cell cycle progression is a tightly ordered and regulated process involving multiple checkpoints. These checkpoints respond to a variety of growth signals, alterations in cell size, and DNA integrity (Alberts, 2004). We postulate that the ruthenium complex may act on these checkpoints, preventing further cell division and subsequently initiating death by apoptosis. However, this theory has not yet been proven experimentally.

Anticancer drugs generally inhibit growth by blocking cells in various phases of the cycle (G1, S, or G2) depending on the drug and the cell type. Cisplatin, a cytotoxic drug that acts by binding to guanine bases of DNA, is known to completely disrupt the distribution of cells among the cell cycle phases at cytotoxic concentrations and to stop the cell cycle at G2-M only at non-cytotoxic concentrations (Bergamo et al., 1999).

Apoptosis is currently a subject of research interest, in part because tumor cells are susceptible to death by apoptosis in response to various drugs used in chemotherapy (Brenes et al., 2007). Some ruthenium-derived organometallic compounds have been reported to mediate their cytotoxicity via induction of apoptosis (Gaiddon et al., 2005). Based on this evidence, we next

examined whether cell viability inhibition caused by *cis*-$[RuCl_2(NH_3)_4]Cl$ was due to apoptosis.

The mechanisms of apoptosis mainly involve two signaling pathways identified according to the initiator caspase: the death receptor pathway involving caspase-8, and the mitochondrial pathway, in which various signals can trigger the release of harmful proteins by mitochondria into the cytoplasm, leading to activation of caspase-9. Both pathways result in the downstream activation of caspase-3. Caspase-3 is able to cleave a variety of substrates, thereby contributing to the typical morphological and biochemical features observed in apoptosis inducing DNA fragmentation (Cohen, 1997; Kaufmann and Earnshaw, 2000; Kaufmann and Hengartner, 2001).

In the present study, our results showed clearly that *cis*-$[RuCl_2(NH_3)_4]Cl$ induced apoptosis in K-562 cells as evidenced by caspase activation and a sub-G1 peak. According to the results shown in Fig. 5, *cis*-$[RuCl_2(NH_3)_4]Cl$ elevated caspase-3 activity significantly in K562 cells relative to the untreated control ($p<0.05$). However, only a slight increase in caspase-8 and 9 activation was observed in K562 *cis*-$[RuCl_2(NH_3)_4]Cl$ treated cells. This result could be related to the fact that caspase-8 and 9 are considered to be an important initiating event in apoptosis, whereas caspase-3 is involved in the effector phase of the apoptotic process (Shi, 2002). Based on this evaluation, it is possible that caspases play an important role in the activation and executioner phase of *cis*-$[RuCl_2(NH_3)_4]Cl$-induced apoptosis. Several studies have shown that ruthenium complexes, such as Nami-A, KP1019, and RAPTA-C, induce apoptosis through caspase activation, particularly caspase 3 (Sana et al., 2002; Kapitza et al., 2005b, Chatterjee et al., 2008). The mechanisms by which ruthenium complexes induce apoptosis are still largely unknown.

However, Ru(III) complexes might interact with DNA to cause crosslinks (like cisplatin) or to induce strand breaks (Vilaplana et al., 2006; Pizarro and Sadler, 2009). On the other hand, ruthenium belongs to the same group of elements as iron (iron-triad), which is reflected by its strong affinity for transferrin and by the necessity of its reductive activation in cells . It can be assumed that Ru(III) can substitute for Fe(III), which induces Fenton type redox processes and intracellular radicals, resulting in cellular damage that induces apoptosis. Hence, induction of oxidative stress seems to be an essential component of the cytotoxic effect of Ru(III) complexes (Frasca et al., 2001; Clarke, 2003; Kapitza et al., 2005b; Brabec and Nováková, 2006).

In summary, the data presented here clearly show that *cis*-$[RuCl_2(NH_3)_4]Cl$ induces apoptosis in human K562 cells, as verified by caspase activation and a sub-G1 peak. Thus, these results lay the groundwork for further research characterizing the antitumor properties of *cis*-$[RuCl_2(NH_3)_4]Cl$.

Acknowledgments

The authors gratefully acknowledge the financial support of Research and Projects Financing (FINEP) (Grant No.01.06.0941.00/CT-Saúde to Elisângela de Paula Silveira-Lacerda). Coordination for the Advancement of Higher Education Staff (CAPES) through fellowship to Aliny Pereira de Lima and Cesar Augusto Sam Tiago Vilanova-Costa, and Brazilian National Counsel of Technological and Scientific Development (CNPq) through fellowship to Flávia de Castro Pereira (Grant No. 131960/2008-3).

Funding sources

There are no financial or personal interests that might be viewed as inappropriate influences on the work presented herein. This manuscript was completely financed by governmental and nonprofit institutions, Brazilian National Counsel of Technological and Scientific Development (CNPq), Research and Projects Financing (FINEP) and Coordination for the Advancement of Higher Education Staff (CAPES).

Ethical Approval

No studies involving humans or experimental animals were conducted in this work. The chronic myelogenous leukemia (K562) and normal lung fibroblast (MRC-5) cells were purchased from Rio de Janeiro Cell Bank/RJ, Brazil and cultured *in vitro*.

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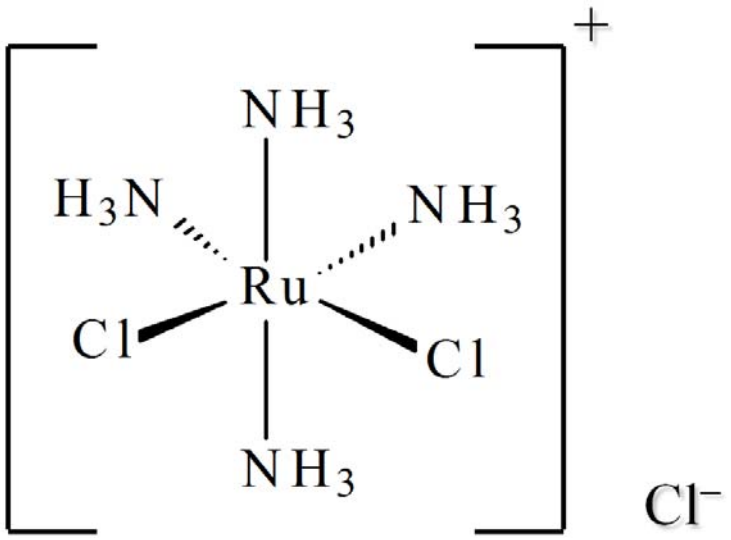


Fig. 1 Chemical structure of *cis*-(dichloro)tetraammineruthenium(III) chloride

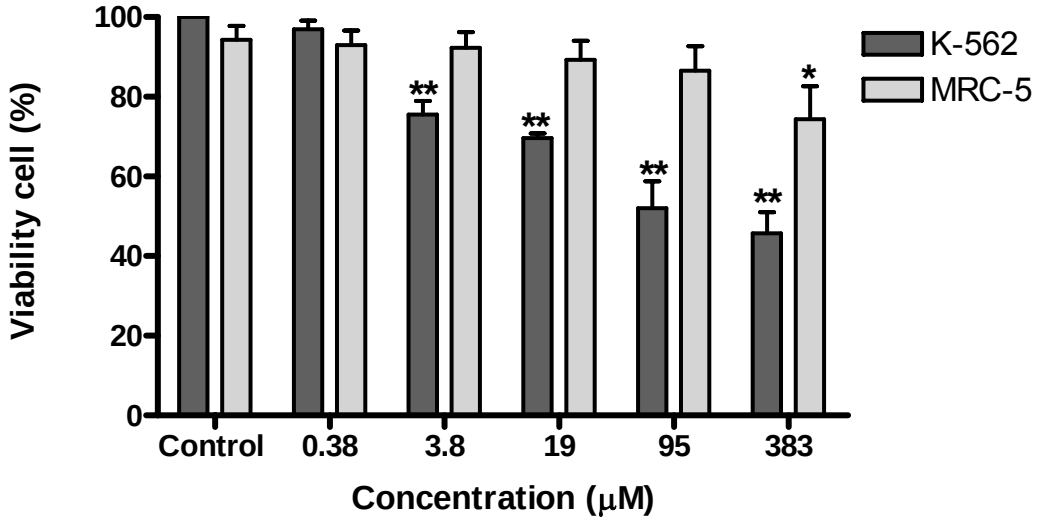


Fig. 2 Effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ on the viability of K562 and MRC-5 cells. Cells were treated for 48 h in the presence of *cis*-$[RuCl_2(NH_3)_4]Cl$. Cell viability was then determined by MTT assay. Data show the mean ± SD of three separate experiments. IC_{50} of 10.74 and >383 μM for K562 and MRC-5 cells, respectively. Significant differences from the untreated control are indicated by *$p < 0.05$; **$p < 0.01$.

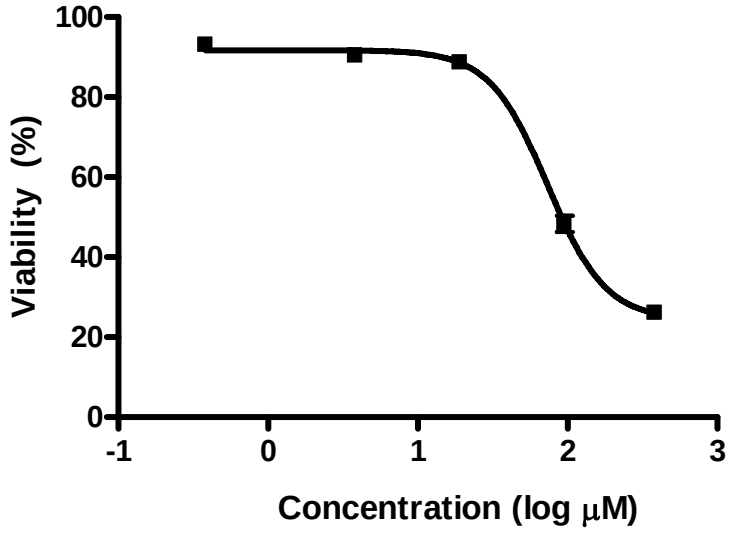


Fig. 3 Dose response curve of K-562 cells treated with *cis*-$[RuCl_2(NH_3)_4]Cl$. K562 cells were treated for 48 h in the presence of *cis*-$[RuCl_2(NH_3)_4]Cl$ and cell viability was then determined by trypan blue assay, IC_{50} 73.45 μM.

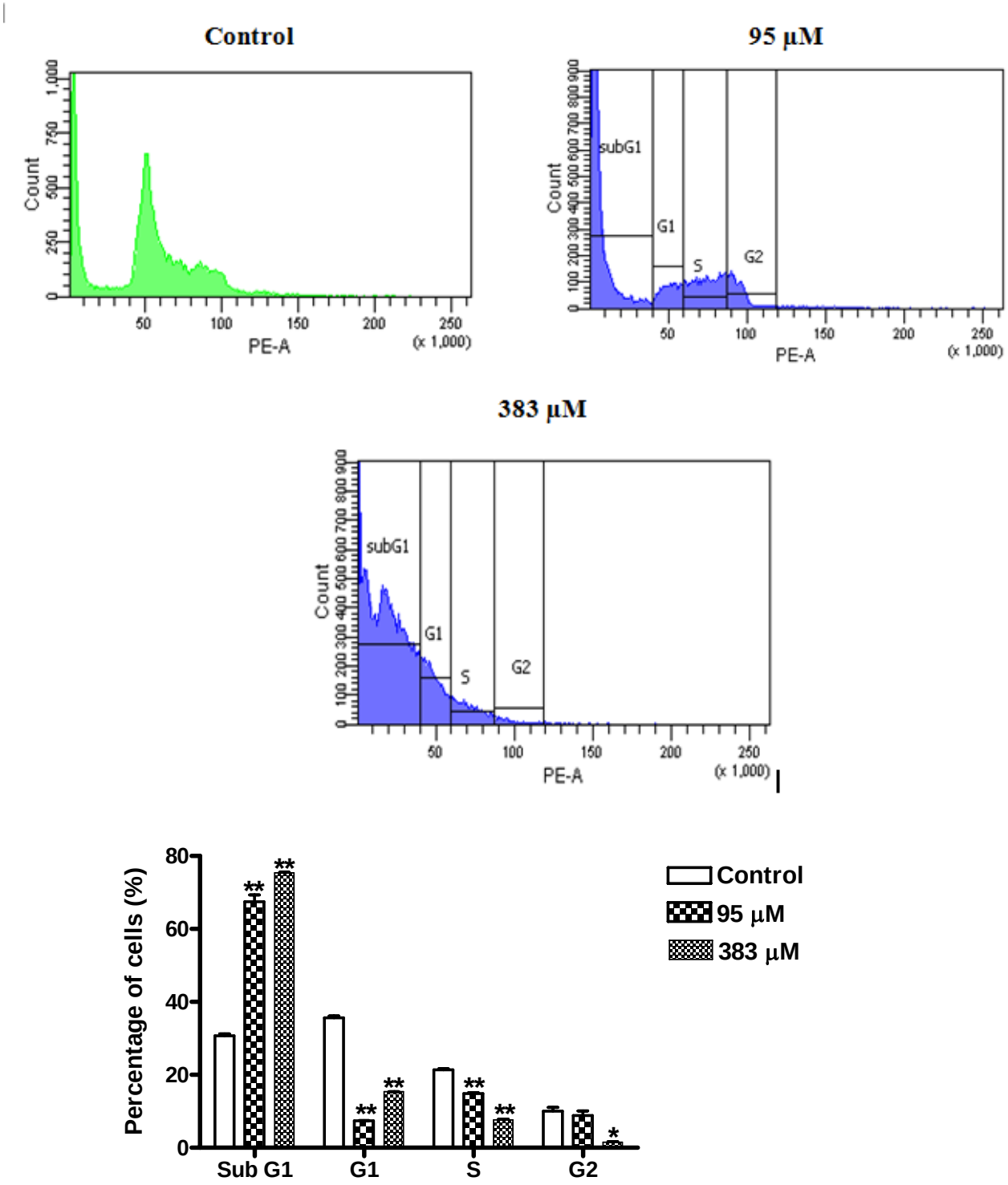


Fig. 4 Effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ on the cell cycle cinetics of K562 cells after 48 h exposure**.** Data show the mean ± SD of two experiments. Significant differences from the untreated control are indicated by *p < 0.05; **p < 0.01.

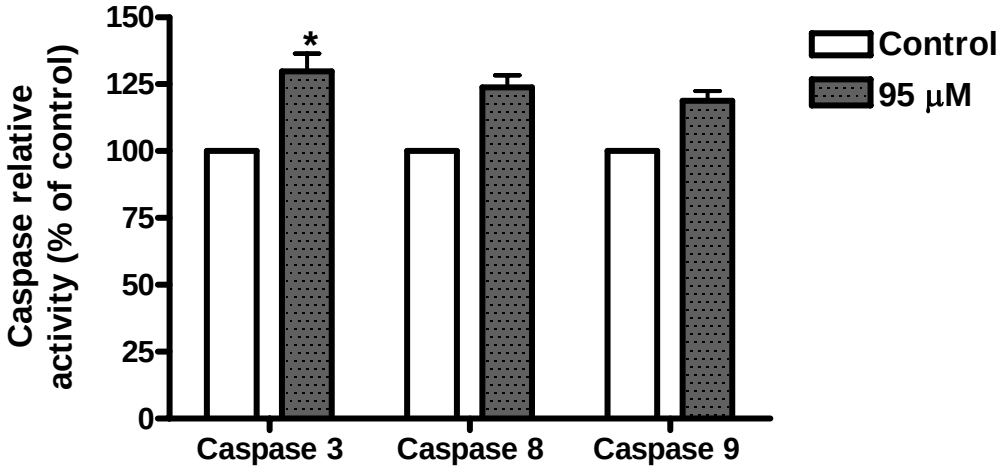
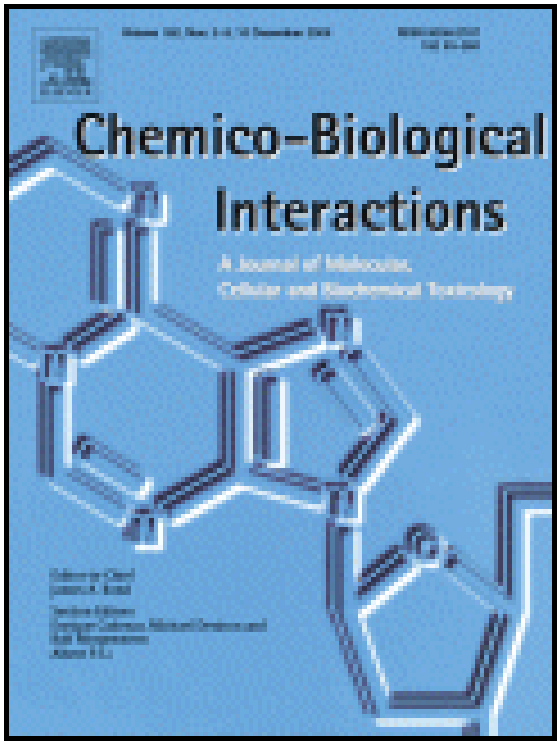


Fig. 5 Activation of caspase -3, -8 and -9 in K-562 cells treated with 95 μM *cis*-$[RuCl_2(NH_3)_4]Cl$ for 24 h. Caspase activity was calculated as fold induction of basal caspase-3, -8 and -9 activities in non-treated K562 samples. Each column represents the mean ± SD (*bars*) of two experiments. * $P < 0.05$ compared to control

ARTIGO 2



Artigo Submetido ao periódico **Chemical-Biological Interactions**

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Ciências Biológicas I: Qualis A2

The ruthenium complex *cis*-(dichloro)tetramminеruthenium(III) chloride induces apoptosis and damages DNA in murine ascitic sarcoma 180 (S-180) cells

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ABSTRACT

Ruthenium(III) complexes are increasingly attracting the interest of researchers due to their promising pharmacological properties. Recently, we reported that the *cis*-(dichloro)tetramminеruthenium(III) chloride compound has cytotoxic effects on murine sarcoma 180 cells (S-180). In an effort to understand the mechanism responsible for their cytotoxicity, the present study investigated the genotoxicity, cell cycle distribution and induction of apoptosis caused by *cis*-(dichloro)tetramminеruthenium(III) chloride in S-180 tumour cells. *cis*-(dichloro)tetramminеruthenium(III) chloride treatment induced significant DNA damage in S-180 cells, as detected by the alkaline comet assay. In the cell cycle analysis, *cis*-(dichloro)tetramminеruthenium(III) chloride caused an increase in the number of cells in G1 phase, accompanied by a decrease in the S and G2 phases after 24 h of treatment. In contrast, the cell cycle distribution of S-180 cells treated with *cis*-(dichloro)tetramminеruthenium(III) chloride for 48 h showed a dose-dependent increase in the sub-G1 phase (indicating apoptosis), with a corresponding decrease in cells in the G1, S and G2 phases. In addition, *cis*-(dichloro)tetramminеruthenium(III) chloride treatment induced apoptosis in a time-dependent manner, as observed by the increased numbers of Annexin V-positive cells. Taken together, these findings strongly demonstrate that DNA damage, cell cycle changes and apoptosis may correlate with the antitumour effects of *cis*-(dichloro)tetramminеruthenium(III) chloride on S-180 cells.

Keywords: Apoptosis, *cis*-(dichloro)tetramminеruthenium(III) chloride, cycle cell, DNA damage, S-180.

1. INTRODUCTION

Apoptosis is an active physiological process that results in cellular self-destruction involving specific morphological and biochemical changes in the nucleus and cytoplasm [1,2]. Agents that suppress the proliferation of malignant cells by inducing apoptosis may represent a useful mechanistic approach to both cancer chemoprevention and chemotherapy. While many anticancer agents have been developed, unfavourable side effects and resistance to apoptosis are serious problems [3,4]. Thus, there is growing interest in the design, synthesis, and pharmacological evaluation of metal-based antitumour agents for the treatment of various cancers and the development of safer and more effective therapeutic agents [5-7].

Ruthenium(III) complexes represent a new family of promising metal-based anticancer drugs that offer the potential of reduced toxicity compared to the antitumour platinum(II) complexes currently used in the clinic, a novel mechanism of action, the prospect of non-cross-resistance and a different spectrum of activity [5,8,9,10]. In addition, some chemical properties, such as the rate of ligand exchange, the range of accessible oxidation states and the ability of ruthenium to mimic iron in binding to certain biological molecules make these compounds well suited for medicinal applications as an alternative to platinum antitumour drugs in the treatment of cancer cells that are resistant to cisplatin and its analogues, thus justifying the further development of this novel and interesting group of metal complexes [8,10].

The two ruthenium compounds: ImH[*trans*-$RuCl_4$(DMSO)Im] (NAMI-A) and indazolium *trans*-[tetrachlorobis (1*H*-indazole)ruthenate(III)] (KP1019) are the first ruthenium-based anticancer drugs to be entered into clinical trials

[11,12], and many other compounds that include ruthenium centres are being developed and tested [9].

Among the ruthenium complexes studied for anticancer application, the *cis*-(dichloro)tetramminеruthenium(III) chloride (*cis*-$[RuCl_2(NH_3)_4]Cl$) complex has shown promising results on tumours cells in humans and mice [13]. Recently, was demonstrated that *cis*-$[RuCl_2(NH_3)_4]Cl$ exerts antitumour activity against both *in vitro* and *in vivo* models of the sarcoma 180 (S-180) cell line [13,14]. However, no studies on the mechanisms of induction of apoptosis and DNA damage have been reported for this cell line. In an effort to understand the mechanisms of *cis*-$[RuCl_2(NH_3)_4]Cl$ on these cells, we investigated the effect of the compound on cell cycle distribution, apoptosis and DNA damage.

2. MATERIALS AND METHODS

2.1. Synthesis of *cis*-$[RuCl_2(NH_3)_4]Cl$

The *cis*-$[RuCl_2(NH_3)_4]Cl$ complex (Figure 1) was prepared as follows. Air-free concentrated ammonium hydroxide (25 mL) was added to $[RuCl(NH_3)_5]Cl_2$ (1.0 g, 3.4×10^{-3} mols), which was then refluxed under argon until a burgundy-coloured solution was produced. Sodium dithionate (0.7 g) was added to the hot solution, which was subsequently cooled in an ice bath. An off-yellow-cream solid, $[RuOH(NH_3)_5]S_2O_6$, precipitated, was collected by filtration and air-dried. A second crop of crystals was obtained by the addition of anhydrous ethanol (50 mL). This material was collected by filtration, washed with ethanol and air-dried. The $[RuOH(NH_3)_5]S_2O_6$ was dissolved in saturated oxalic acid (11 mL) and then refluxed for approximately 10 minutes under argon, forming a yellow solid. The mixture was then cooled to complete the

precipitation. The crystals of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2S_2O_6$ were isolated by filtration, washed with ethanol and air-dried. The tetraammineoxalatoruthenium(III) dithionate crystals were dissolved in 5 M HCl (12 mL), and the solution was refluxed for 10 minutes. The hot solution was filtered, and absolute ethanol (30 mL) was added to the yellow filtrate. A yellow solid, *cis*-$[RuCl_2(NH_3)_4]Cl$, was precipitated. After the mixture had cooled, the *cis*-product was collected by filtration, washed with ethanol and air-dried. The yield was 55%. The compound was characterised by electronic spectra at room temperature using quartz cells in an HP 8453 spectrophotometer with a diode arrangement that interfaced with a compatible PC (HP Vectra XM). The electronic spectrum of the *cis*-$[RuCl_2(NH_3)_4]Cl$ complex presented bands at 260, 308 and 350 nm. Briefly, the absorption spectra in aqueous solution were dominated by the ligand-to-metal charge transfer bands with λ_{max} at 350 nm (ε = $l.5\times10^3$ M^{-1} cm^{-1}), 308 nm (ε = $l.3\times10^3$ M^{-1} cm^{-1}) and 260 nm sh (ε = 500^3 M^{-1} cm^{-1}). Carbon, hydrogen and chloride microanalyses were performed by the staff of the Analytical Center of the Chemical Institute of Universidade de São Paulo. The calculated proportions were N, 20.3; H, 4.4; Cl, 38.6. The observed proportions were N, 20.2; H, 4.5; Cl, 38.5.

2.2. Cell culture

The murine sarcoma 180 cells (S-180) were cultured in suspension in RPMI 1640 medium (Sigma Chemical Co., MO) supplemented with 10% fetal calf serum, 100 UI/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere at 37 °C in 5% CO_2.

2.3. DNA analysis by the COMET assay

The presence of DNA damage was examined by single-cell gel electrophoresis (SCGE) (COMET assay), according to published methods [15]. Briefly, 2 X 10^4 S-180 cells were treated with *cis*-$[RuCl_2(NH_3)_4]Cl$ (0,01, 0.1, 0,2, 0,5 and 1mg.mL^{-1}) that correspond to (0.38, 3.8, 19 and 95 μM) and Metrotexate (25 μM) for 24 and 48 h. After incubation, a S-180 cell suspension was homogenised with 100 μL of a low melting point agarose (0.5%), spread onto microscope slides pre-coated with a normal melting point agarose (1.5%) and covered with a coverslip. After 10 min at 4 °C, the coverslip was removed and the slides were immersed in cold lysis solution (2.4 M NaCl; 100 mM EDTA; 10 mM Tris, 10% DMSO and 1% Triton-X, pH 10) for 24 h. After lysis, the slides were placed in an electrophoresis chamber and covered with electrophoresis buffer (300 mM NaOH per 1 mM EDTA, pH > 13) for 20 min to allow for unwinding of the DNA. The electrophoresis proceeded for 20 min (25 V and 300 mA). Afterwards, the slides were submerged for 15 min in a neutralisation buffer (0.4M Tris–HCl, pH 7.5), dried at room temperature and fixed in 100% ethanol for 5 min. Slide staining was performed immediately before analyzing using ethidium bromide (20 μg mL^{-1}). Slides were prepared in duplicate, and 100 cells were screened per sample (50 cells from each slide) using a fluorescence microscope (Leica, Wetzlar, Germany) interfaced with a computer. The nucleus was classified visually according to the migration of the fragments, as previously proposed [16], as follows: class 0 (no damage); class 1 (little damage with a short tail length smaller than the diameter of the nucleus); class 2 (medium damage with a tail length one or two times the diameter of the nucleus); class 3 (significant damage with a tail length between two and a half to three times the diameter of the nucleus); and class 4 (significant damage with a long tail of

damage greater than three times the diameter of the nucleus). A value (damage index, DI) was assigned to each comet according to its class, according to the following formula:

DI = (0 x n0) + (1 x n_1) + (2 x n_2) + (3 x n_3) + (4 x n_4), where n = number of cells in each class analysed.

DI thus ranged from 0 (completely undamaged: 100 cells x 0) to 400 (with maximum damage; 100 cells x 4).

2.4. Annexin V/PI staining.

Apoptosis-mediated cell death of S-180 tumour cells was examined using a FITC-labelled Annexin V/propidium iodide (PI) Apoptosis Detection Kit (Sigma-Aldrich, St. Louis, Mo) according to the manufacturer's instructions. Briefly, 5 x 10^5 cells were harvested and washed with PBS. Cells were re-suspended in 400 µL binding buffer. Next, 5 µL Annexin V-FITC and 1 µl PI was added. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a flow cytometer (FACSCanto, BD Biosciences) using Diva software. The cells in early stages of apoptosis were Annexin V positive and PI negative, whereas the cells in the late stages of apoptosis were both Annexin V and PI positive.

2.5 Cell cycle analysis

Cell cycle analysis of the S-180 cells was performed using flow cytometry. After treatment with *cis*-[$RuCl_2(NH_3)_4$]Cl (19 and 95 µM) for 24 and 48 h, cells were harvested by centrifugation, washed with PBS, fixed with 70% (v/v) cold aqueous ethanol and stored overnight at -20 ◦C. The fixed cells were washed with PBS and incubated with propidium iodide (50 µg/mL) containing

0.05% RNase. Samples were incubated at 4 ◦C in the dark and analysed by flow cytometry (FACS Canto, BD Biosciences). The percentage of cells in G1, S, G2 and sub-G1 was determined using Diva software.

3. RESULTS

3.1 Induction of DNA damage by *cis*-$[RuCl_2(NH_3)_4]Cl$

To directly determine if *cis*-$[RuCl_2(NH_3)_4]Cl$ induces DNA damage, S-180 cells were treated with different concentrations of *cis*-$[RuCl_2(NH_3)_4]Cl$. Figure 2 shows the effect of *cis*-$[RuCl_2(NH_3)_4]Cl$ on DI in S-180 cells. After 24 and 48 h of exposure, *cis*-$[RuCl_2(NH_3)_4]Cl$ significantly increased the DI in relation to the negative control ($p<0.01$). In addition, the DNA damage occurred in a concentration- and time-dependent manner.

3.2 *cis*-$[RuCl_2(NH_3)_4]Cl$ induces apoptosis in S-180 cells

As shown in Fig. 3, after treatment with 19 and 95 μM *cis*-$[RuCl_2(NH_3)_4]Cl$ for 24 and 48 h, the early apoptotic cells (Annexin V+ and PI-) represented 6.9% and 4.1% (24 h) and 3.7% and 1.7% (48 h) of the total cells, respectively. Meanwhile, the late apoptotic cells (Annexin V+ and PI+) represented 29.6% and 47.1% (24 h) and 82.6% and 59% (48 h) of the total cells, respectively. These results suggest that the induction of apoptosis by *cis*-$[RuCl_2(NH_3)_4]Cl$ accounts for part of its antitumour activity.

3.3 *cis*-$[RuCl_2(NH_3)_4]Cl$ induces the alteration cell cycle distribution in S-180 cells

To test whether *cis*-$[RuCl_2(NH_3)_4]Cl$ affects the cell cycle of S-180 cells,

cells treated with *cis*-$[RuCl_2(NH_3)_4]Cl$ were subjected to flow cytometric analysis. After 24 h, cells treated with 95 μM of *cis*-$[RuCl_2(NH_3)_4]Cl$ showed a statistically significant increase in the G1 fraction (44.2%) accompanied by a decrease in the S (9.5%) and G2 (20.5%) fractions (Fig. 4 A). In contrast, the cell cycle distribution of S-180 cells treated with *cis*-$[RuCl_2(NH_3)_4]Cl$ (19 and 95 μM) for 48 h showed a dose-dependent increase in the sub-G1 peak (49.7% and 50.0%) (indicating apoptosis) with a corresponding decrease in cells in the G1 (15.6% and 27.35%), S (15.25% and 9.1%) and G2 (14% and 8.8%) phases, respectively (Fig. 4 B).

4. DISCUSSION

In the last few years, clinical trials using metal-based drugs for the treatment of tumours have become increasingly widespread in cancer therapy [6,17]. There have been reports that *cis*-$[RuCl_2(NH_3)_4]Cl$ exhibits antitumour effects on S-180 cell lines. However, in an effort to understand the mechanism behind their cytotoxicity, we investigated whether *cis*-$[RuCl_2(NH_3)_4]Cl$ can induce DNA damage, change the cell cycle and induce apoptosis. Previous studies have demonstrated that *cis*-$[RuCl_2(NH_3)_4]Cl$ induces cytotoxicity in S-180 tumour cells (IC_{50} 63 μM). Besides being cytotoxic to tumour cells *in vitro*, it was recently it reported that *cis*-$[RuCl_2(NH_3)_4]Cl$ exhibits antitumour effects on S-180 tumour cells *in vivo* and results in tumour volume reduction and an increased survival time in treated animals [14].

To assess DNA damage caused by *cis*-$[RuCl_2(NH_3)_4]Cl$ in S-180 cells, the comet assay was used. This assay has achieved the status of a standard test in the battery of tests used to assess the safety of novel pharmaceuticals or other chemicals and it is now well established as a sensitive assay for detecting

strand breaks in the DNA of single cells [18].

It is commonly believed that the main target for ruthenium compounds and other antitumour metal complexes is DNA; as shown for platinum drugs, the antitumour action of ruthenium (III) complexes would be the consequence of direct DNA binding and damage [10,19]. The present study clearly showed an increase in DNA damage after treatment with *cis*-$[RuCl_2(NH_3)_4]Cl$, in relation to the negative control. The DI increased from 41 in controls to 237 and 311 in cells treated with 19 and 95 μM *cis*-$[RuCl_2(NH_3)_4]Cl$ for 48 h, respectively. These results demonstrated that DNA lesions induced by the ruthenium complex are difficult to eliminate with cell-repair mechanisms over 24 and 48 h. In addition, when compared to a positive control (Metrotexate), it is important to note that the ruthenium compound causes greater DNA damage when compared to another chemotherapeutic agent after 48 h of treatment (Figure 2). Several studies have shown that ruthenium complexes induce DNA damage in the tumour cells, mainly by intercalant effects [20,21]. A previous report indicated that *cis*-$[RuCl_2(NH_3)_4]Cl$ induces internucleossomal DNA fragmentation in Jurkat leukaemia cells [13]. Unlike the results of DNA damage presented in this study in S-180 cells, genotoxic studies using the alkaline version of the comet assay to evaluate the possible DNA-damaging effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ in human peripheral blood lymphocytes (PBL) showed that this compound does not cause significantly increased DNA damage in normal cells [22], suggesting that the genotoxic activity of this compound could be selective to tumour cells.

Thus, it is possible that the cytotoxicity of *cis*-$[RuCl_2(NH_3)_4]Cl$ to several cancer cell lines *in vitro* [13] is partially due to its DNA damaging effects, as DNA damage is a potent stimulus for apoptotic cell death. This finding led us to

evaluate the nature of cell death involved in the cytotoxicity of *cis*-$[RuCl_2(NH_3)_4]Cl$ in S-180 cells.

Cell apoptosis is important to destroy undesired cells during the development and homeostasis of multicellular organisms, and it is characterised by distinct morphological changes, such as plasma membrane blebbing, cell shrinkage, mitochondrial depolarisation, chromatin condensation and DNA fragmentation [23,24]. Therefore killing tumour cells through the induction of apoptosis has been recognised as a strategy for the identification of antitumour drugs and as a valuable tool for cancer treatment [3,25]. In the present study, our results clearly showed that *cis*-$[RuCl_2(NH_3)_4]Cl$ induced apoptosis in S-180 cells in a time-dependent manner, as evidenced by a significant increase in Annexin V-positive apoptotic cells. Consistent with our results, previous studies have demonstrated that *cis*-$[RuCl_2(NH_3)_4]Cl$ induces increased numbers of Annexin V-positive Jurkat cells [13]. Thus, the mechanisms by which *cis*-$[RuCl_2(NH_3)_4]Cl$ induced apoptosis may be associated with the increase in DNA damage, as demonstrated by the comet assay.

Several reports have indicated that ruthenium complexes inhibit the proliferation of cells by inhibiting cell cycle progression and inducing apoptosis [26,27]. As shown in our cell cycle profile analysis, *cis*-$[RuCl_2(NH_3)_4]Cl$ induced G1 cycle arrest after 24 h of treatment, and after 48 h of treatment, *cis*-$[RuCl_2(NH_3)_4]Cl$ caused a significant increase in the number of cells in the Sub-G1 phase (indicating apoptotic DNA), with a corresponding decrease of cells in the G1, S and G2 phases. The results obtained herein suggest that the complex was capable of preventing cell cycle progression and inducing apoptosis, as the percentages of cells entering the G1, S and G2 phases were decreased, correlating with an increased proportion of cells in the sub-G1-peak, probably as

a consequence of DNA damage generation.

Recently, it was shown that *cis*-[$RuCl_2(NH_3)_4$]Cl reduced the number of murine B-cell lymphoma (A-20) cells in G0/G1 but not in S and G2/M. In contrast, in human T-cell leukaemia (Jurkat) cells, this compound slightly diminished the number of cells in G0/G1 and greatly reduced the number of cells in G2/M [13].

It is well established that cell cycle progression is a tightly ordered and regulated process that involves multiple checkpoints. These checkpoints respond to a variety of growth signals, alterations in cell size and DNA integrity [28]. Thus, it is possible that the ruthenium complex may act on these checkpoints, thereby preventing further cell division and subsequently initiating death via apoptosis. However, this theory has not yet been proven experimentally. Studies with the ruthenium complex RDC (Compound Derived Ruthenium) showed that this compound induced the arrest of cells in G1 and induced high p21 protein levels, an inhibitor of the cell cycle that blocks CDK activity [29].

The mechanisms by which ruthenium complexes induce apoptosis remain largely unknown. However, ruthenium(III) complexes might interact with DNA, causing cross-linking similar to that induced by cisplatin does, or inducing strand break [21,30]. In contrast, ruthenium belongs to the same group of elements as iron (iron-triad), which is reflected by its strong affinity for transferrin and by the necessity of its reductive activation in cells. It can be assumed that ruthenium(III) can substitute for iron(III), which induces Fenton type redox processes and intracellular radicals [9,20,31]. This may result in cellular damage that induces apoptosis.

In conclusion, the results presented herein demonstrated that DNA

damage, apoptosis and changes in the cell cycle may correlate with the antitumour effects exhibited by *cis*-$[RuCl_2(NH_3)_4]Cl$ in S-180 cells.

Acknowledgments

The authors gratefully acknowledge the financial support of Research and Projects Financing (FINEP) (Grant No.01.06.0941.00/CT-Saúde to Elisângela de Paula Silveira-Lacerda), Coordination for the Advancement of Higher Education Staff (CAPES) through fellowship to Aliny Pereira de Lima and Cesar Augusto Sam Tiago Vilanova-Costa and Brazilian National Counsel of Technological and Scientific Development (CNPq) through the fellowship to Flávia de Castro Pereira (Grant No. 131960/2008-3).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Funding sources

There are no financial or personal interests that might be viewed as inappropriate influences on the work presented herein. This manuscript was completely financed by governmental and non-profit institutions, Brazilian National Counsel of Technological and Scientific Development (CNPq), Research and Projects Financing (FINEP) and Coordination for the Advancement of Higher Education Staff (CAPES).

Abbreviations

A-20: Murine B-Cell Lymphoma

cis-$[RuCl_2(NH_3)_4]Cl$: *cis*-(dichloro)tetramminеruthenium(III) chloride

Jurkat: Human T-Cell Leukaemia

KP1019: indazolium *trans*-[tetrachlorobis (1*H*-indazole)ruthenate(III)]

NAMI-A: ImH[*trans*-RuCl4(DMSO)Im]

PBL: Peripheral Blood Lymphocytes

RDC: Ruthenium Derived Compound

SCGE: Single-Cell Gel Electrophoresis

S-180: Murine Sarcoma 180 Cells

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FIGURES

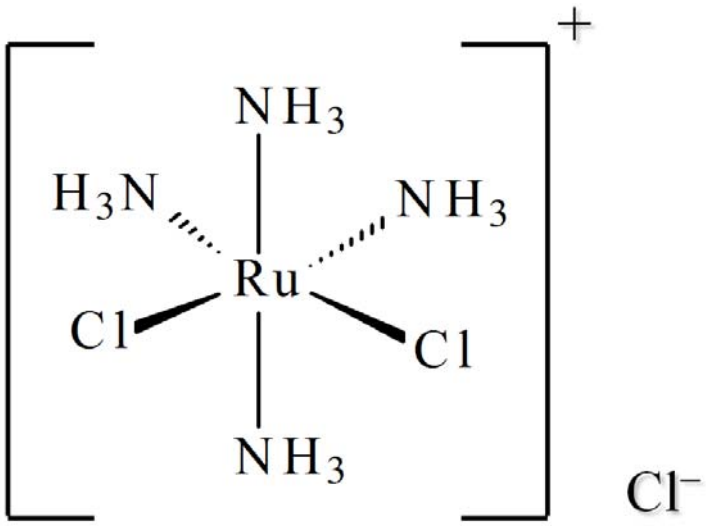
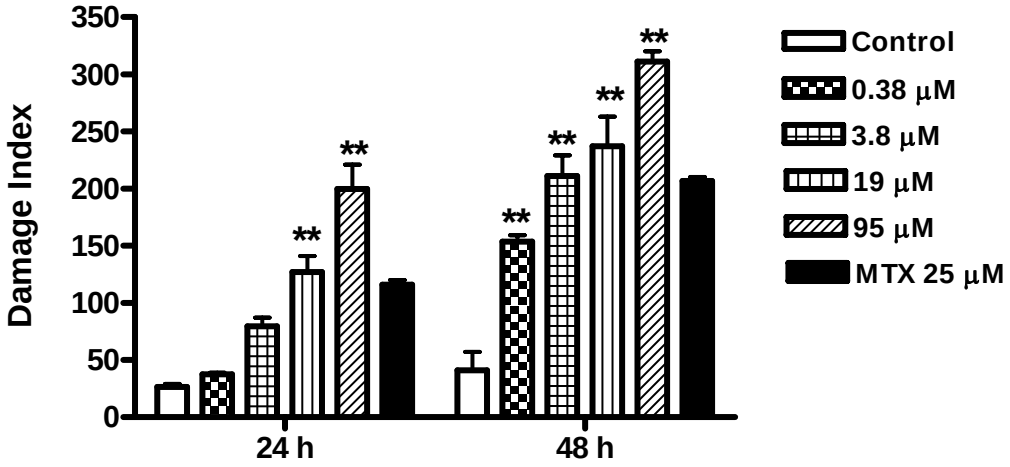


Figure 1. Chemical structure of *cis*-(dichloro)tetraammineruthenium(III) chloride {*cis*-$[RuCl_2(NH_3)_4]Cl$}.

A



B

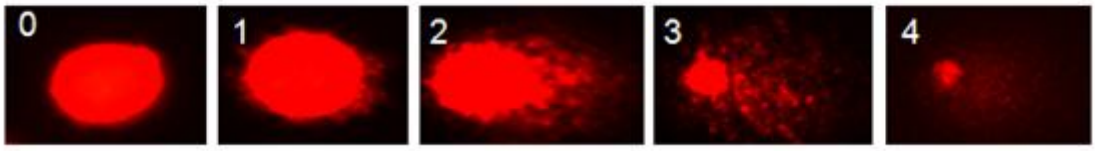
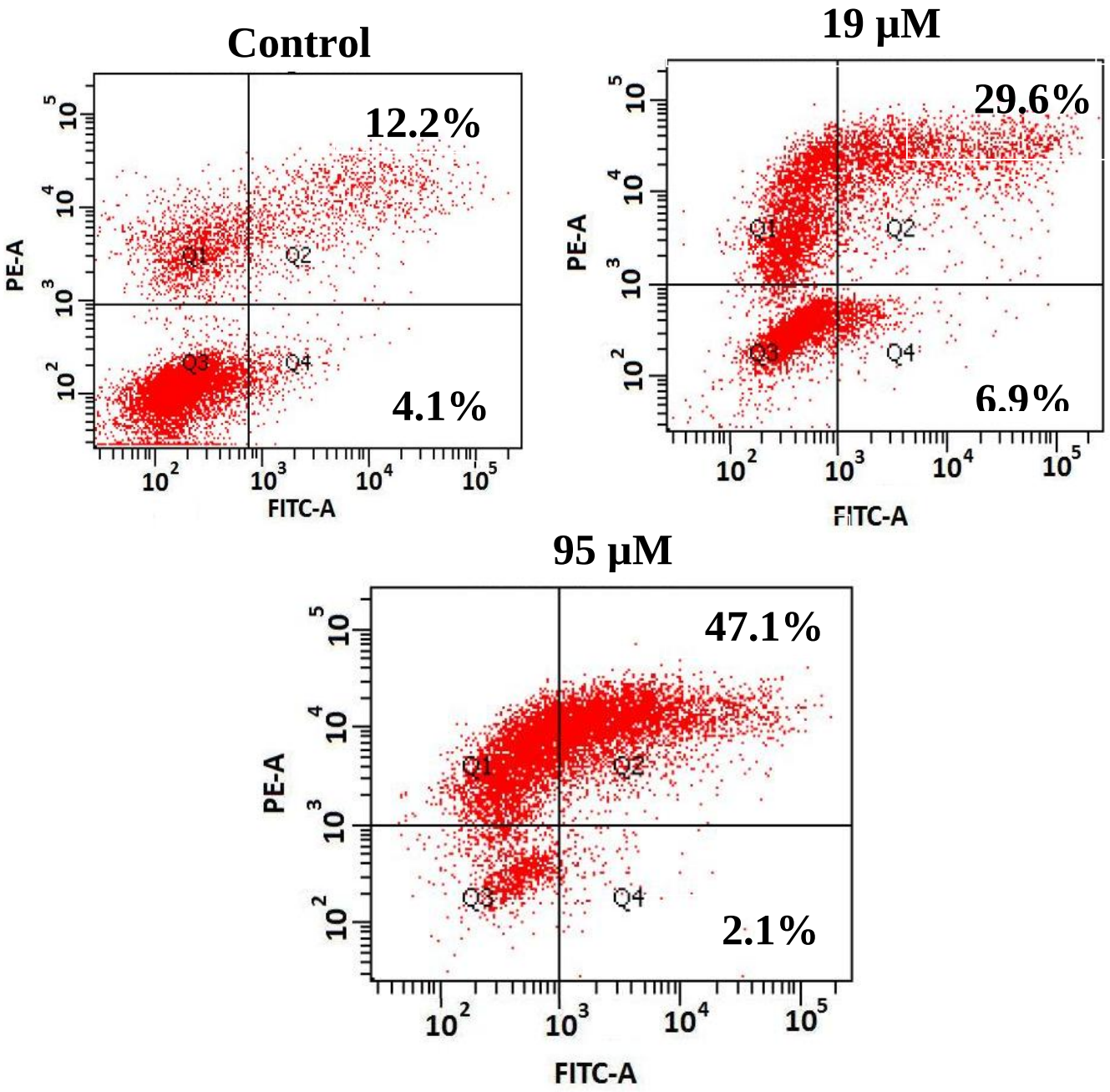


Figure 2. Effect of *cis*-$[RuCl_2(NH_3)_4]Cl$ on the DNA damage index, assessed by the comet assay. S-180 cells were treated with different concentrations of *cis*-$[RuCl_2(NH_3)_4]Cl$ (0.38 to 95 μM) for 24 and 48 h. Data represent the mean ± SD of two experiments. Significant differences compared to the untreated control are indicated by *$p < 0.05$ and **$p < 0.01$ (A). Comet images of S-180 cells treated with *cis*-$[RuCl_2(NH_3)_4]Cl$. The comets illustrate the visual scoring classification: class 0 represents undamaged nucleoids, classes 1-4 represent increasing DNA damage reflected by the increasing length of the tail (B).

A



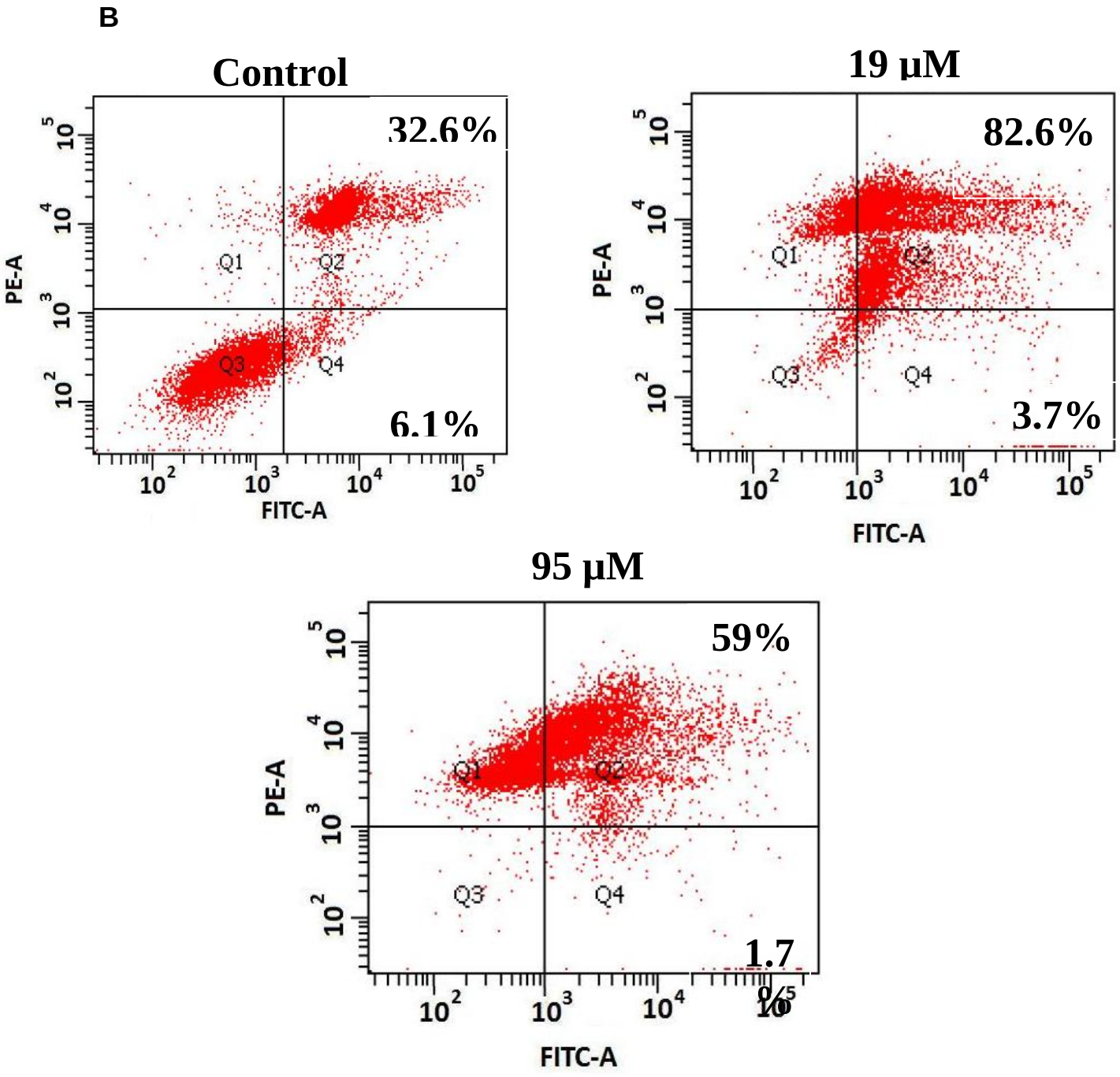
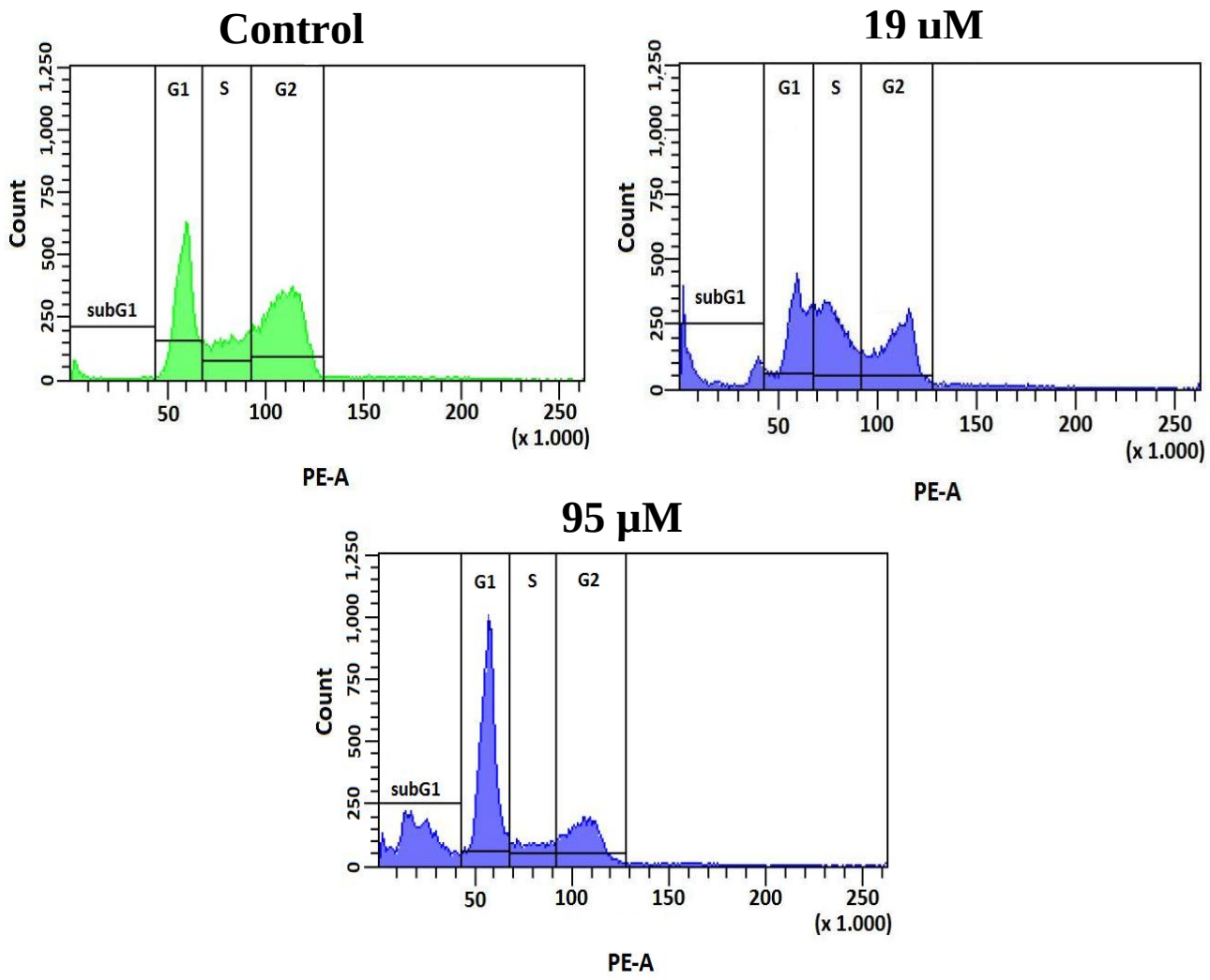
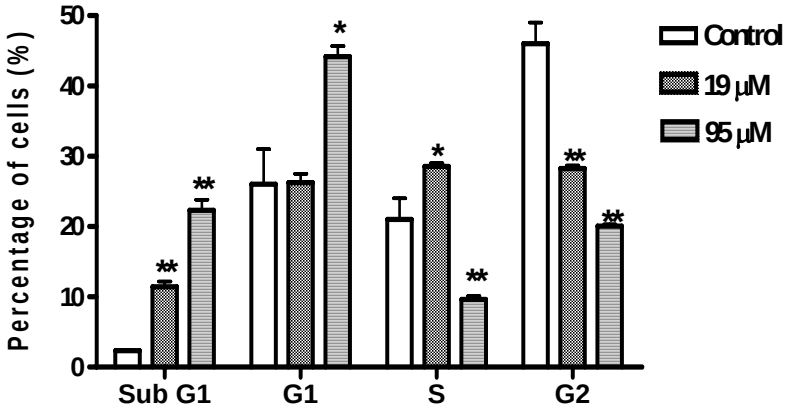


Figure 3. Flow cytometric analysis of phosphatidylserine externalisation (annexin V binding) and cell membrane integrity (PI staining) in S-180 cells undergoing apoptosis. The cells were treated with *cis*-$[RuCl_2(NH_3)_4]Cl$ (19 and 95 µM) for 24

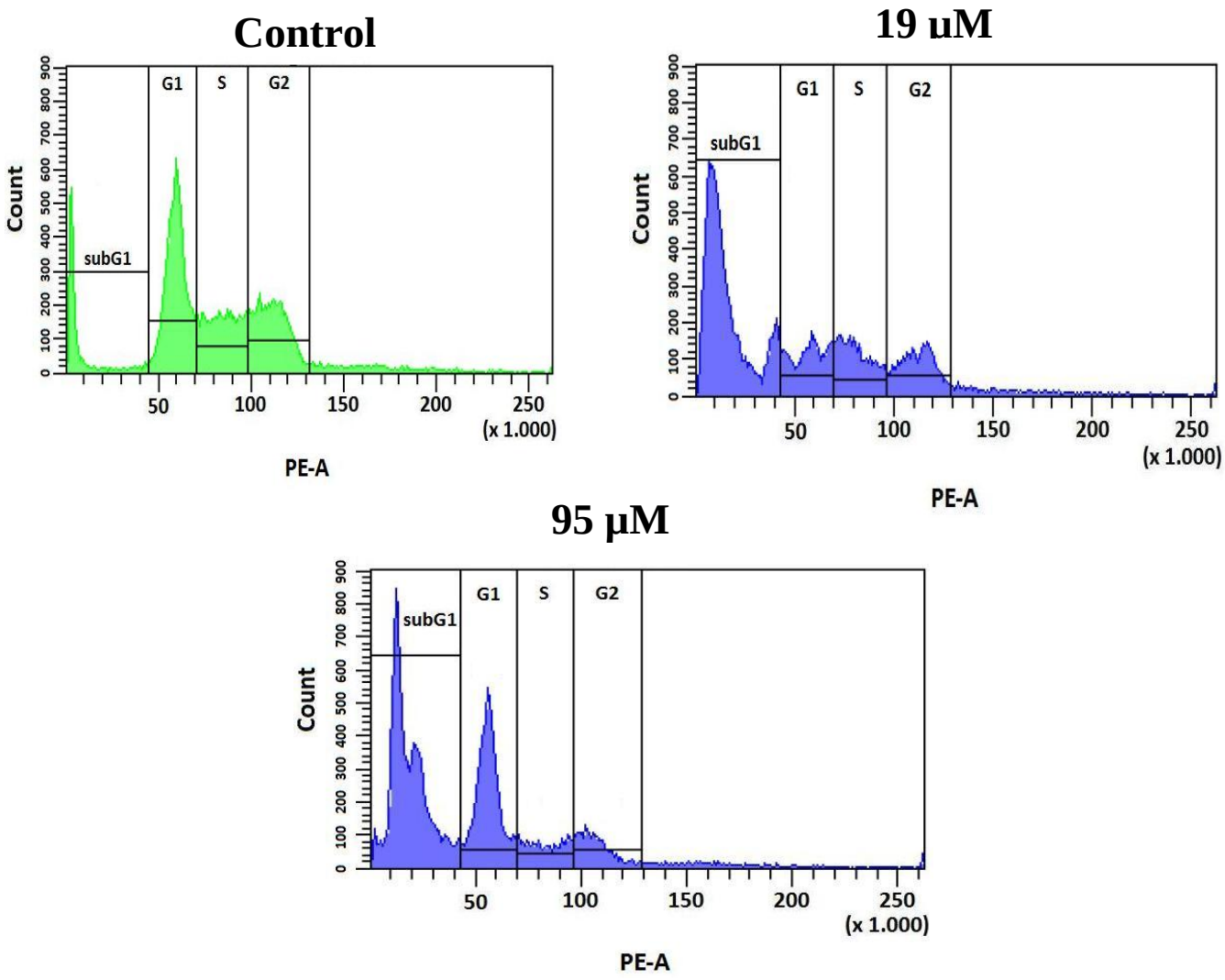
(A) and 48 h (B). The dual parametric dot plots combining annexin V-FITC and PI fluorescence show the viable cell population in the lower left quadrant Annexin V(-)/PI(-), the early apoptotic cells in the lower right quadrant Annexin V(+)/PI(-), and the late apoptotic cells in the upper right quadrant Annexin V(+)/PI(+).

A


Control
19 uM
95 µM
G1
S
G2
subG1
Count
PE-A
(x 1.000)


Percentage of cells (%)
Control
19 µM
95 µM
Sub G1
G1
S
G2

B


Control
19 uM
95 µM
G1
S
G2
subG1
Count
PE-A
(x 1.000)

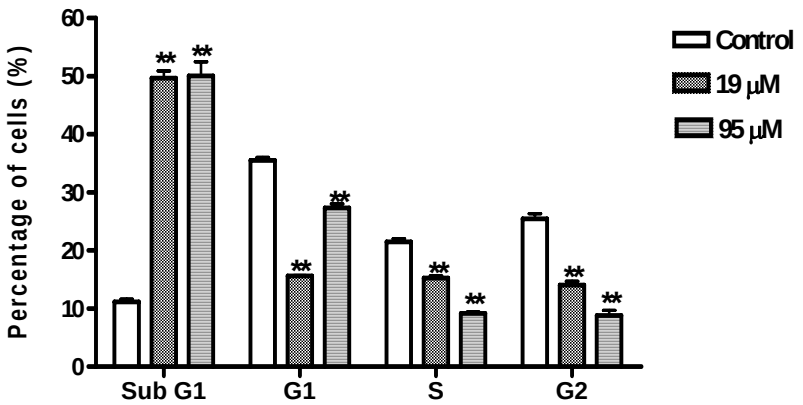
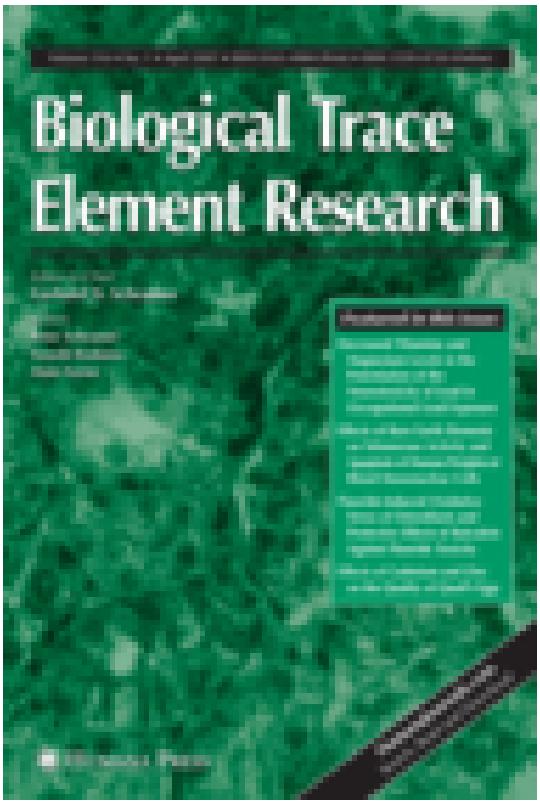

Percentage of cells (%)
Control
19 µM
95 µM
Sub G1
G1
S
G2

Figure 4. Effect of *cis*-[$RuCl_2(NH_3)_4$]Cl on the cell cycle distribution of S-180 cells. Cells were treated with different concentrations of *cis*-[$RuCl_2(NH_3)_4$]Cl (19 and 95 μM) for 24 h (A) and 48 h (B). The percentages of cell cycle distribution were then evaluated by flow cytometric analysis. Data represent the mean ± SD of two experiments. Significant differences compared to the untreated control are indicated by *$p < 0.05$, **$p < 0.01$.

ARTIGO 3



Artigo Submetido ao periódico **Biological Trace Element Research**

<http://www.editorialmanager.com/bter/>

Ciências Biológicas I: B3

Human lung carcinoma cells (A549) show resistance to the ruthenium complex *cis*-(dichloro)tetramineruthenium(III) chloride

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Abstract

Lung cancer is one of the leading causes of death in the world, and non-small cell lung carcinoma (NSCLC) accounts for approximately 75-85% of all lung cancers. In the present work, we studied the antitumor activity of the compound *cis*-(dichloro)tetramineruthenium(III) chloride {*cis*-$[RuCl_2(NH_3)_4]Cl$} against human lung carcinoma tumor cell line A549. The results of MTT and trypan blue assays showed that *cis*-$[RuCl_2(NH_3)_4]Cl$ causes reduction in the viability of A549 cells when treating with 95 and 383 μM of the compound for 48 and 72 h. Lower concentrations of the compound (19, 3.8 and 0.38 μM), however, only slightly affected cell viability. The IC_{50} value for the compound was about 383 μM. Survival analysis of the A549 cells after treatment with ruthenium (III) compound using long term clonogenic assay showed that it reduced colony formation ability at concentrations of 0.38 and 3.8 μM, and at concentrations of 95 and 383 μM, no colonies were observed. Furthermore, we used DNA fragmentation assays to demonstrate that ruthenium(III) compound did not cause any visible ladders at the concentrations studied. Our findings indicate that A549 tumor cells are particularly resistant to treatment with ruthenium(III) complex, and further studies evaluating the mechanisms of this resistance should be pursued.

Keywords: A549; Antitumor activity; *cis*-(dichloro)tetramineruthenium(III) chloride; Cytotoxicity; Lung cancer

1.0 Introduction

Lung cancer is one of the leading causes of death in the world, and non-small cell lung carcinoma (NSCLC) accounts for approximately 75-85% of all lung cancers. At the time of diagnosis, 40% of NSCLC cases are often at an advanced stage [1]. Currently, chemotherapy is the main method of treating NSCLC [2].

The chemotherapeutic drug cisplatin is one of the most frequently used agents in the treatment of lung cancer. Still, the therapeutic effect of cisplatin is weakened by its severe toxicity and the chemoresistance of most NSCLC [3-5]. It is thought that clinical multidrug resistance to chemotherapeutic agents is a key obstacle to curative treatment for advanced NSCLC [6]. Thus, the development of chemotherapeutic agents to maximize antitumor activity and minimize toxicity is the prime target for NSCLC management.

Recently, much attention has been focused on ruthenium (III) complexes as potential antitumor agents [7]. Despite exhibiting lower cytotoxicity to tumor cells compared to cisplatin and requiring a higher therapeutic dose, ruthenium complexes are better tolerated *in vivo* [8, 9].

In comparison to the platinum(II) antitumor complexes currently used clinically, ruthenium compounds offer potentially reduced toxicity, a novel mechanism of action, the prospect of non-cross-resistance and a different spectrum of activity. The reduced toxicity is in part due to the ability of ruthenium complexes to mimic the binding of iron to biologically significant molecules, exploiting the mechanisms that the body has evolved for non-toxic transport of iron. This reduced toxicity, which allows larger doses of the compounds to be given,

together with non-cross-resistance in cisplatin-resistant cancer cells, makes these complexes particularly attractive [7,8,10,11].

Studies have shown ruthenium complexes to be active against certain tumors both *in vivo* and *in vitro,* with fewer side effects than cisplatin [12,13]. In addition, two ruthenium-based drugs, ImH[*trans*-RuCl4(DMSO)Im] (NAMI-A) and indazolium *trans*-[tetrachlorobis (1*H*-indazole)ruthenate(III)] (KP1019), are the first ruthenium-based anticancer drugs transferred into to enter clinical trials [14,15]. Many other compounds that include ruthenium centers are being developed and tested [7]. Among these, the ruthenium(III) complex *cis*-(dichloro)tetramineruthenium(III) chloride (*cis*-$[RuCl_2(NH_3)_4]Cl$) is particularly promising. It is being characterized by very low toxicity and has potential as an antitumor drug. Recently, the antitumor activity of *cis*-$[RuCl_2(NH_3)_4]Cl$ was evaluated *in vivo* using Sarcoma 180 (S180) murine ascitic tumor cells and a number of murine(S180, A-20) and human (Jurkat, SKBR-3) tumor cell lines. In all cases *cis*-$[RuCl_2(NH_3)_4]Cl$ demonstrated a very encouraging activity profile [9,16]. Based on these findings, we studied the antitumor activity of *cis*-$[RuCl_2(NH_3)_4]Cl$ against the human lung carcinoma tumor cell line A549.

2. Materials and Methods

2.1. Synthesis of *cis*-$[RuCl_2(NH_3)_4]Cl$

The *cis*-$[RuCl_2(NH_3)_4]Cl$ complex (Figure 1) was prepared as follows. Air-free concentrated ammonium hydroxide (25 mL) was added to $[RuCl(NH_3)_5]Cl_2$ (1.0 g, 3.4×10^{-3} mol), which was then refluxed under argon until a burgundy-colored

solution was produced. Sodium dithionate (0.7 g) was added to the hot solution, which was subsequently cooled in an ice bath. An off-yellow/cream solid, $RuOH(NH_3)_5]S_2O_6$, precipitated and was collected by filtration and air dried. A second crop of crystals was obtained by the addition of anhydrous ethanol (50 mL). This material was collected by filtration, washed with ethanol and air dried. The $[RuOH(NH_3)_5]S_2O_6$ was dissolved in saturated oxalic acid (11 mL) and then refluxed for approximately 10 minutes under argon, forming a yellow solid. The mixture was then cooled to complete the precipitation. The crystals of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2S_2O_6$ were isolated by filtration, washed with ethanol and air dried. The tetraammineoxalatoruthenium(III) dithionate crystals were dissolved in 5M HCl (12 mL), and the solution was refluxed for 10 minutes. The hot solution was filtered, and absolute ethanol (30 mL) was added to the yellow filtrate, precipitating a yellow solid, *cis*-$[RuCl_2(NH_3)_4]Cl$. After the mixture had cooled, the *cis*-product was collected by filtration, washed with ethanol and air dried. The yield was 55%. The compound was characterized by electronic spectra at room temperature using quartz cells in an HP 8453 spectrophotometer with a diode arrangement that interfaced with a compatible PC, HP Vectra XM. The electronic spectrum of the *cis*-$[RuCl_2(NH_3)_4]Cl$ complex presented bands at 260, 308 and 350. Briefly, the absorption spectra in aqueous solution were dominated by the ligand-to-metal charge transfer bands, with λ_{max} at 350 nm (ε = l.5×10^3 M^{-1} cm^{-1}), 308 nm (ε = l.3×10^3 M^{-1} cm^{-1}) and 260 nm (sh) (ε = 500^3 M^{-1} cm^{-1}). Carbon, hydrogen and chloride microanalyses were performed by the staff of the Analytical Center of the Chemical Institute of Universidade de São Paulo. The calculated

proportions were as follows: N, 20.3; H, 4.4; Cl, 38.6. The observed proportions were as follows: N, 20.2; H, 4.5; Cl, 38.5.

2.2. Cell culture

The human lung carcinoma cell line A549 (ATCC number CCL-185^{TM}) was obtained from the Rio de Janeiro Cell Bank (RJ, Brazil). The cells were cultured in DMEM medium (pH 7.2-7.4) supplemented with 100 U mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine, 1.5 g. L^{-1} sodium bicarbonate, 10 mM HEPES and 10% fetal calf serum 1% (w/v) (all reagents were obtained from Gibco, Grand Island, NY) at 37°C under a 5% CO_2 humidified atmosphere. A549 tumor cells were routinely sub-cultured using a 2.5 g L^{-1} trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO).

2.3 MTT assay

The effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ on A549 cell viability were studied using an MTT assay as described previously [17]. Briefly, 1×10^5 viable A549 cells were plated in 96-well tissue culture plates and treated with different concentrations of *cis*-$[RuCl_2(NH_3)_4]Cl$ (0,01, 0.1, 0,2, 0,5 and 1 $mg.mL^{-1}$) that correspond to (0.38 to 383 μM) for 48 h. After treatment, 10 μL of MTT (5 $mg.mL^{-1}$) was added to each well, and the plates were incubated at 37°C for another 3 h. The purple formazan crystals were dissolved in 50 μL SDS (10%), and the absorbance was determined at 545 nm using a Stat Fax 2100 microplate reader (Awareness Technology, Palm

City, FL, USA). Cell viability was expressed as a percentage relative to control (100% viability) and was calculated as follows: viability (%) = (Absorbance of the treated wells)/(Absorbance of the control wells)×100. Each concentration was tested in three different experiments run in triplicate. The IC_{50} value (i.e., the concentration (μM) that produced a 50% reduction in cellular viability) was obtained from the dose-response curves using GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA, USA).

2.4 Trypan blue assay

The viability of A549 cells was also evaluated by the trypan blue exclusion method. Cells at a concentration of 1×10^5 cells/well were treated with different concentrations of *cis*-$[RuCl_2(NH_3)_4]Cl$ (0.38 to 383 μM) and incubated for 48 or 72 h at 37 °C in a humidified air atmosphere with 5% CO_2. After incubation, the medium was removed by aspiration, and the cells were washed with a phosphate-buffered saline solution. The cells were then harvested with a 2.5 g L^{-1} trypsin-EDTA solution, suspended in complete media and centrifuged at 1,500 rpm for 10 min. The cells were then resuspended, and 10 μL of the cell suspension was incubated with 10 μL of 0.4% trypan blue solution (Sigma). The number of cells was then estimated using a hemocytometer. Thereafter, an Olympus BX41 microscope was used to identify nonviable cells, which were dark blue, and viable cells, which excluded the dye.Cell viability (%) was determined as described in item 2.3. The IC_{50} was calculated and defined as the drug concentration (μM) causing a 50% reduction in cellular viability.

2.5 Clonogenic assays

Cell sensitivity to *cis*-$[RuCl_2(NH_3)_4]Cl$ was assessed using a colony-forming assay. Briefly, 300 viable A549 cells were plated in culture bottles (25-cm^2) containing 5 mL of growth medium and allowed to adhere for 24 h. After the 24-hour incubation, the medium was removed, and the cells were exposed to *cis*-(dichloro)tetraminеruthenium(III) chloride (0.38 to 383 μM) or cisplatin (25 μM) for 48 or 72 h. After incubation, the drug-containing medium was removed, and the cells were washed with a phosphate-buffered saline solution and supplemented medium. The culture bottles were then kept in an incubator at 37 ºC in a humidified atmosphere of 5% CO_2 and air mixed for 10 days, allowing viable cells to grow into macroscopic colonies. The medium was then removed, and the cells were fixed [methanol/acetic acid/water (1:1:8, v/v)] and stained using 5% Giemsa. The software Clono-Counter was used to evaluate the clonogenic growth by densitometric analysis of scanned grey-scale photos (200 dpi jpeg files) of the culture flasks. Plating efficiency was calculated as the number of colonies divided by the number of cells plated. Survival was calculated by dividing the plating efficiency of the drug-treated cells by the plating efficiency of untreated control cells.

2.6 DNA laddering assay

Briefly, 1×10^6 cells (A-549) were treated with different concentrations of *cis*-$[RuCl_2(NH_3)_4]Cl$ (3.8 to 383 μM) for 48 h at 37 °C with 5% CO^2. Cells were harvested and centrifuged at 300 × *g* for 15 min at 10°C and washed with PBS.

The cells were then resuspended in extraction buffer (10 mmol L^{-1} Tris-HCl, 0.1mol L^{-1} EDTA, 5 g mL^{-1} SDS) and treated with 20 mg L^{-1} RNase A at 37°C for 60 min, followed by incubation with proteinase K (10mg.mL^{-1}) at 37°C for 60 min. An equal volume of saline solution (6M NaCl) was added to the cells, which were then centrifuged at 13,000 × *g* for 10 min. The supernatant was collected, and two volumes of ethanol (-20°C) were added. The samples were centrifuged at 13,000 × *g* for 30 min at 4°C. The supernatant was then discarded, and the pellets were dissolved in TE buffer (1X). The concentration of DNA was detected using a UV spectrophotometer (Beckman DU-640, USA). The DNA was transferred to a 1.5% horizontal agarose gel, and electrophoresis was performed at 100V cm^{-1} for 90 min. The DNA in the gels was visualized by ultraviolet transillumination after staining with ethidium bromide (5 µg mL^{-1}) using an Omega® molecular imaging system (UltraLum Inc., Claremont, CA, USA).

2.7 Statistical analysis

Statistical analysis of the results was performed using one-way ANOVA and Tukey's or Dunnett's post test for multiple comparisons with a control. All statistical analyses were performed using the statistical software GraphPad Prism, version 4. A probability of 0.05 or less was deemed statistically significant. The following notation was used throughout: *p <0.05, **p <0.01, relative to control.

2.0 Results

3.1. The *cis*-$[RuCl_2(NH_3)_4]Cl$ reduces the viability of A-549 cells

The effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ on the viability of the A549 cell line were analyzed by treating the cells with different concentrations using trypan blue and MTT assays. As shown in Figs. 2 and 3, A549 cell viability was inhibited after treatment with 95 and 383 μM *cis*-$[RuCl_2(NH_3)_4]Cl$. At lower concentrations (0.38, 3.8 and 19 μM), however, cell viability was only slightly affected. It was not possible to calculate the IC_{50} values precisely from the concentrations tested (IC_{50} > 383 μM); this would have required testing even higher concentrations.

3.2. *cis*-$[RuCl_2(NH_3)_4]Cl$ reduced the colony formation ability of the A549 tumor cell lines

To investigate possible differences in the long term effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ in A549 cells, we used a clonogenic assay, which showed that *cis*-$[RuCl_2(NH_3)_4]Cl$ reduced the number of colonies at concentrations of 0.38 and 3.8 μM. In addition, no colonies were observed when A549 cells were treated with 95 or 383 μM *cis*-$[RuCl_2(NH_3)_4]Cl$ or 25 μM Cisplatin for 48 or 72 h (Fig. 4 A and B).

3.3 DNA ladder analysis

To investigate whether the inhibition of cell viability by *cis*-$[RuCl_2(NH_3)_4]Cl$ involves apoptotic cell death, we examined DNA fragmentation using agarose gel

electrophoresis. As shown in Fig. 5, no visible ladders were observed at the concentrations studied.

4.0 Discussion

Chemotherapy is one of the primary treatments for lung cancer; however, primary or secondary drug-resistance is a significant reason why chemotherapy is often ineffective against non-small cell lung cancer (NSCLC) [3,5,6]. This presents a major obstacle to successfully treatment of this disease. It is therefore crucial to develop better therapeutic strategies for the management of lung carcinomas.

Here we presented the antitumor effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ on the human lung carcinoma cell line A549.

Using the trypan blue exclusion test, which evaluates the structural integrity of the A549 cell membrane, and the reduction of 3-(4,5-dimethylthi-azole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which assesses mitochondrial function via the activity of succinate dehydrogenase [17,18], we demonstrated that *cis*-$[RuCl_2(NH_3)_4]Cl$ produces a mild antitumor effect on A549 cells. The results obtained from the initial viability studies showed that the IC_{50} values for both viability assays were above the highest concentration studied ($IC_{50} > 383$ μM).

Based on the IC_{50} value demonstrated for *cis*-$[RuCl_2(NH_3)_4]Cl$ on A549 cells, it appears that A549 cells show resistance to treatment with this compound compared to other ruthenium (II) compounds [19]. Despite the finding that *cis*-$[RuCl_2(NH_3)_4]Cl$ showed low antitumor potency in this study, requiring higher doses

to exert its antitumor effect, ruthenium complexes are better tolerated *in vivo* compared to cisplatin [8,12]. The results from previous experiments in which mice transplanted with sarcoma 180 cells were treated with *cis*-$[RuCl_2(NH_3)_4]Cl$ at different time intervals and doses revealed that *cis*-$[RuCl_2(NH_3)_4]Cl$ is remarkably well tolerated at therapeutic doses, with little or no toxicity to the host [9].

Although A549 tumor cells did not show great sensitivity to treatment with *cis*-$[RuCl_2(NH_3)_4]Cl$, previous observations have shown that *cis*-$[RuCl_2(NH_3)_4]Cl$ is cytotoxic to other tumor cells such as human Jurkat, SK-BR-3, and S-180 cells, in addition to murine A-20 tumor cells. Furthermore, it is important to note that this compound is less toxic to normal cells when compared to cisplatin [9,16]. Table 1 shows the IC_{50} values for *cis*-$[RuCl_2(NH_3)_4]Cl$ on tumor and normal cells found in previous studies.

Previous studies have indicated that the A549 cell line is particularly resistant to a variety of different antitumor agents [20,21]. Recently, the effects of cisplatin were investigated in a variety of lung cancer cell lines (A549, H522, H23, H520 and H460), and it was found that A549 cells were more resistant to cisplatin than the other lung cancer cell lines [22].

The development of resistance to cisplatin is a major obstacle for the treatment of lung cancer. Mechanisms of resistance to cisplatin are of two types: the first type involves the formation of DNA adducts and includes decreased drug accumulation and increased drug inactivation by cellular proteins, while the second type involves the prevention of cell death after DNA adduct formation and includes increased DNA adduct repair and increased DNA damage tolerance [3,23].

The mechanisms by which A549 cells show resistance to ruthenium complexes are still unknown. Ru(III) complexes might interact with DNA to cause cross-linking (similar to cisplatin) or induce strand breaks [24,25]. This suggests the possibility that *cis*-$[RuCl_2(NH_3)_4]Cl$ resistant A549 cells are able to tolerate the induced DNA lesions and continue to perform their cellular functions (e.g., replication). These hypotheses are corroborated by the results of agarose gel electrophoresis in which *cis*-$[RuCl_2(NH_3)_4]Cl$ was unable to induce DNA fragmentation in A549 cells.

A clonogenic assay was performed to assess the long term survival of A549 cells after treatment with *cis*-$[RuCl_2(NH_3)_4]Cl$. Figure 4 shows that *cis*-$[RuCl_2(NH_3)_4]Cl$ at concentrations of 0.38 and 3.8 μM inhibited the colony-formation ability of A549 cells. Furthermore, at concentrations of 95 and 383 μM, no colonies were generated, suggesting that the treatment induced subtle cell damage.

Interestingly, the results of the MTT and Trypan blue short term assays in A549 cells showed approximately 60% cell viability in samples treated with 383 μM of the compound, in contrast to the low survival rates in the clonogenic assay. This suggests that cellular damage undetected by the MTT and trypan blue assays can be reflected in the long term survival of the treated cells. This indicates the importance of long term studies for the evaluation of *cis*-$[RuCl_2(NH_3)_4]Cl$ cytotoxicity.

Additionally, an interesting response was observed in the A549 cells after treatment with *cis*-$[RuCl_2(NH_3)_4]Cl$. During the process of trypsinization of the A549 cells treated with *cis*-$[RuCl_2(NH_3)_4]Cl$, it was observed that these cells were highly resistance to detachment from the plate when compared to the untreated cells. These observations seem to indicate that this compound acts on adhesion

molecules, which could be an interesting result for studies of anti-metastatic activity.

The hypothesis that *cis*-[$RuCl_2(NH_3)_4$]Cl acts on adhesion molecules is supported by previous studies with the ruthenium (III) complex NAMI-A, which was demonstrated to inhibit the activity of MMP-2 and MMP-9 (metalloproteinases capable of degrading the extracellular matrix) and to selectively bind collagen of the lung extracellular matrix (a substrate of metalloproteinases). This led to a pronounced increase in extracellular matrix components, which would likely hinder metastasis formation [26]. We postulated that this direct inhibition of metalloproteinases and binding to substrate, however, could lie beneath the effects of *cis*-[$RuCl_2(NH_3)_4$]Cl on A549 cells. This would suggest that *cis*-[$RuCl_2(NH_3)_4$]Cl may alter the levels adhesion molecules or bind to them, reducing the activity of trypsin.

In summary, our results demonstrate that *cis*-[$RuCl_2(NH_3)_4$]Cl has a mild effect antitumor on A549 cells, with an IC_{50} value about 383 μM, suggesting great resistance to this compound. Further studies of the mechanisms of resistance and anti-metastatic activity, however, must be pursued.

Acknowledgments

The authors gratefully acknowledge the financial support of Research and Projects Financing (FINEP) (Grant No.01.06.0941.00/CT-Saúde to Elisângela de Paula Silveira-Lacerda). Coordination for the Advancement of Higher Education Staff (CAPES) was provided through a fellowship to Aliny Pereira de Lima and Cesar Augusto Sam Tiago Vilanova-Costa, and the Brazilian National Counsel of

Technological and Scientific Development (CNPq) through a fellowship to Flávia de Castro Pereira (Grant No. 131960/2008-3).

Funding sources

There are no financial or personal interests that might be viewed as inappropriate influences on the work presented herein. This manuscript was completely financed by governmental and nonprofit institutions, the Brazilian National Counsel of Technological and Scientific Development (CNPq), Research and Projects Financing (FINEP) and Coordination for the Advancement of Higher Education Staff (CAPES).

Ethical Approval

No studies involving humans or experimental animals were conducted in this work. The human lung carcinoma A549 cells were purchased from the Rio de Janeiro Cell Bank (RJ, Brazil) and cultured *in vitro*.

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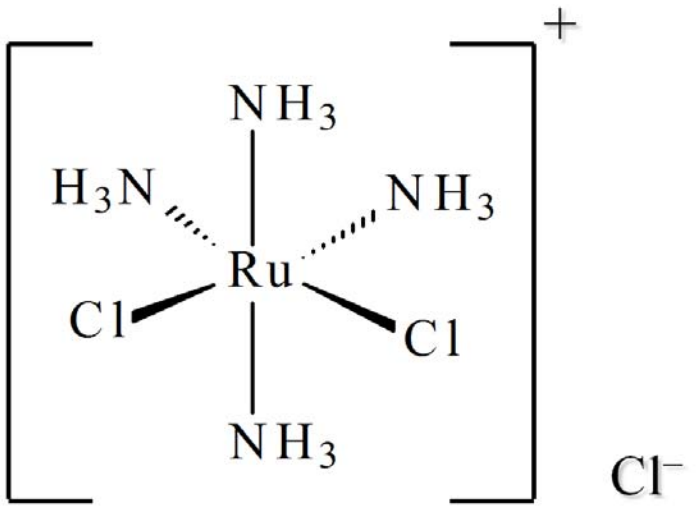

Figure 1. Chemical structure of *cis*-$[RuCl_2(NH_3)_4]Cl$

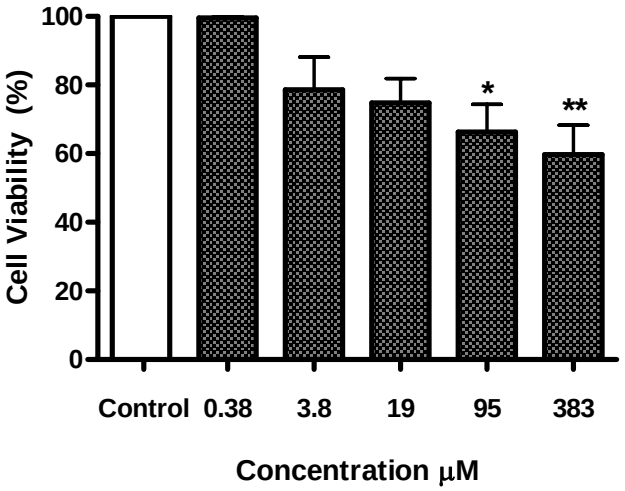

Figure 2. Effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ on the viability of A549 tumor cells assessed by the MTT assay. Cells were treated for 48 h in the presence of *cis*-$[RuCl_2(NH_3)_4]Cl$. Cell viability was then determined by an MTT assay. Data show the mean ± SD of two separate experiments. IC_{50} > 383μM. Significant differences from the untreated control are indicated by *$p < 0.05$; **$p < 0.01$

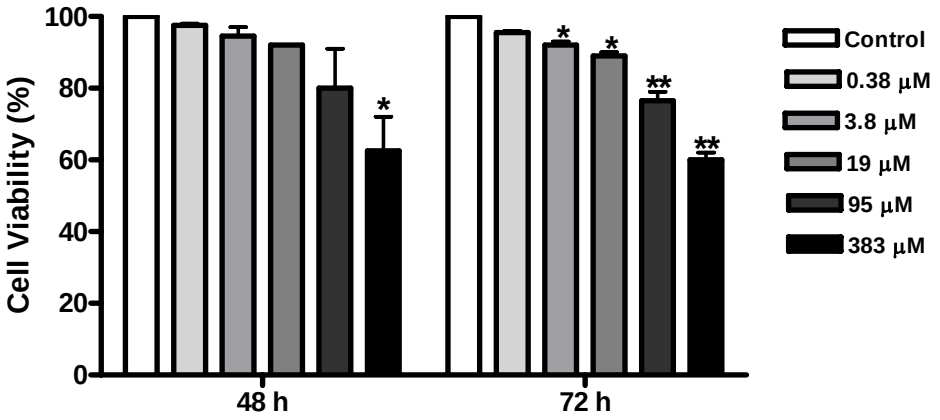

Figure 3. Effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ on the viability of A549 tumor cells assessed by trypan blue assay. Cells were treated for 48 or 72 h in the presence of compound. Cell viability was then determined using a trypan blue assay. Data show the mean ± SD of three separate experiments. IC_{50} > 383 μM. Significant differences from the untreated control are indicated by *$p < 0.05$; **$p < 0.01$

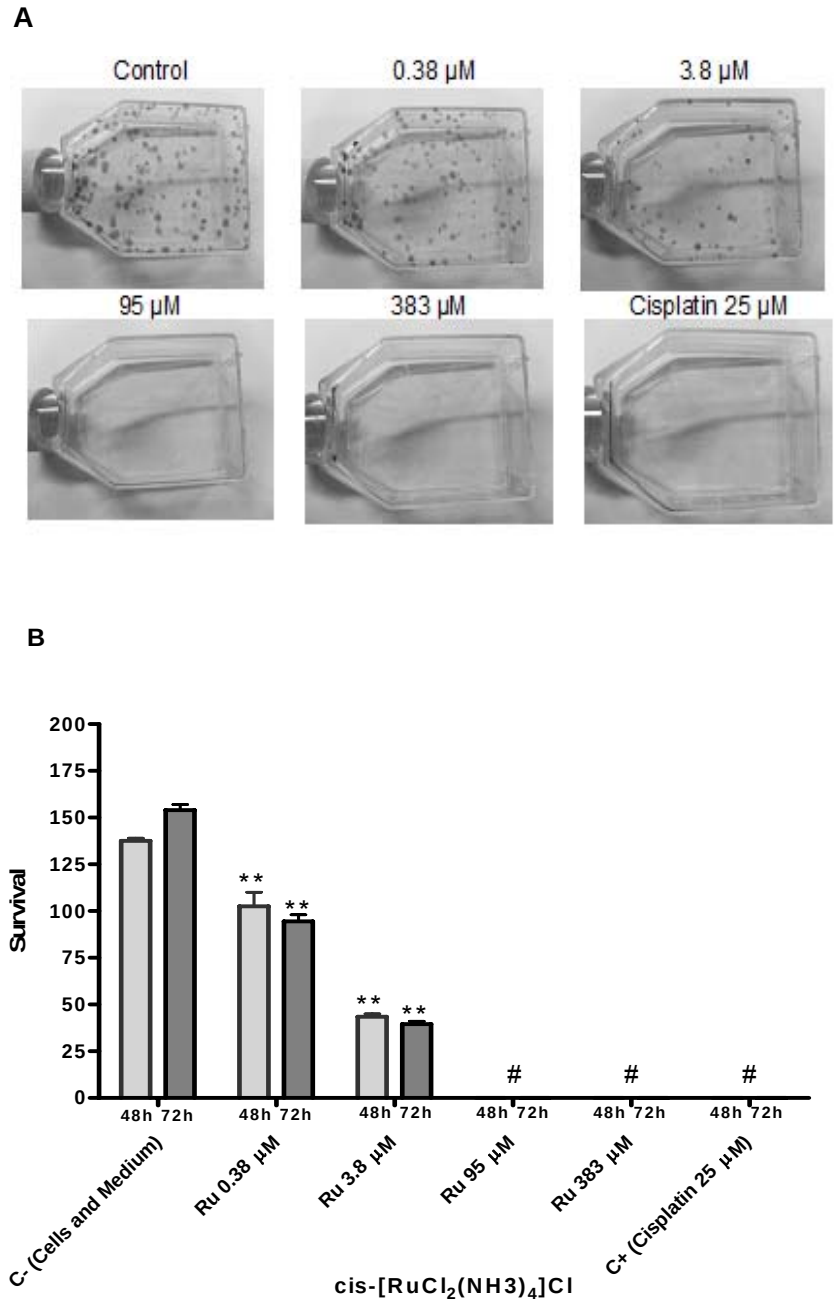

Figure 4. Effect of *cis*-$[RuCl_2(NH_3)_4]Cl$ on the survival of A549 cells. A549 cells were treated with different concentrations of *cis*-$[RuCl_2(NH_3)_4]Cl$ (0.38-383 µM) for 48 or 72 h, and the percentage of surviving cells was determined. Significant differences from untreated controls are indicated by **p < 0.01 (A). Images show bottles with A549 cells [control, *cis*-$[RuCl_2(NH_3)_4]Cl$ (0.38-383 µM) and cisplatin (25 µM)] (B).

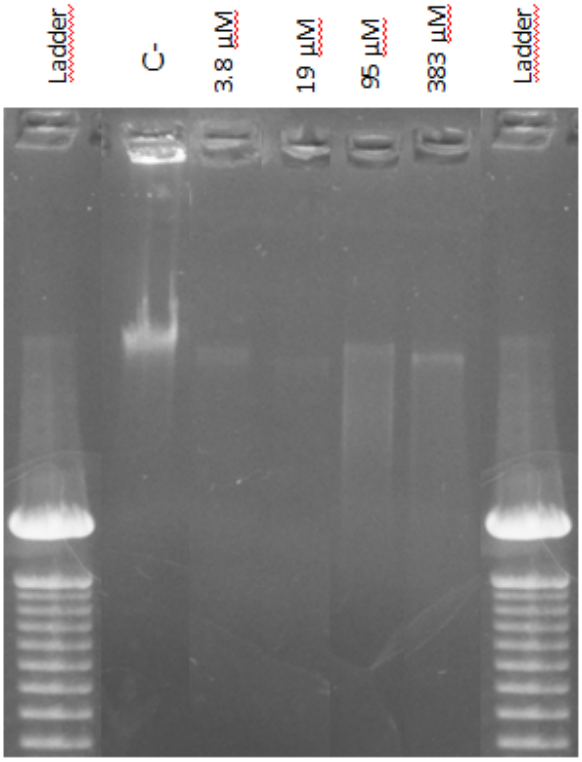

Figure 5. DNA agarose gel electrophoresis profile of A549 cells cultured in the presence of *cis*-[$RuCl_2(NH_3)_4$]Cl. A549 cells were cultured with different concentrations of *cis*-[$RuCl_2(NH_3)_4$]Cl (3.8 - 383 µM) for 48 h. The results shown are representative of at least three similar experiments.

Table 1

Previously reported IC_{50} values for *cis*-$[RuCl_2(NH_3)_4]Cl$ on tumor cells and normal cells

Cell Line	Type Cell	IC_{50}	Authors
A-20	Murine B-cell lymphoma	13 μM	Silveira-Lacerda et al., 2009
SK-BR3	Human Breast Adenocarcinoma	13.53 μM	Silveira-Lacerda et al., 2009
S180	Murine Ascitic Sarcoma 180	63 μM	Menezes et al., 2007
Jurkat	Human T-cell Leukemia	1982 μM	Silveira-Lacerda et al., 2009
K-562	Chronic Myeloid Leukemia	10.74 μM	Unpublished data
PBMC	Human Peripheral Blood Mononuclear Cells	8157 μM	Silveira-Lacerda et al., 2009
MRC-5	Human Lung Fibroblast	>383 μM	Unpublished data

6. CONCLUSÕES

A partir dos resultados obtidos neste trabalho, foi possível concluir que:

- O composto *cis*-$[RuCl_2(NH_3)_4]Cl$ apresentou atividade antitumoral *in vitro* sobre a linhagens tumorais K-562 (IC_{50} 10,74 e 73,45 μM) e A549 (IC_{50} > 383 μM) e baixa atividade citotóxica sobre células normais MRC-5 (> 383 μM);

- O composto *cis*-$[RuCl_2(NH_3)_4]Cl$ em baixas concentrações (0,38 e 3,8 μM) diminui a capacidade de células A549 de formarem colônias, e em altas concentrações (19 e 383 μM) nenhuma houve a formação de nenhuma colônia ;

- Por meio do ensaio cometa pôde-se verificar que o composto *cis*-$[RuCl_2(NH_3)_4]Cl$ induziu um alto índice de dano no DNA de células S180;

- O composto *cis*-$[RuCl_2(NH_3)_4]Cl$ alterou a distribuição do ciclo celular de células S180 e K562, diminuindo a percentual de células nas fases G1, S e G2, e consequentemente aumentando o percentual de células em sub-G1;

- O composto *cis*-$[RuCl_2(NH_3)_4]Cl$ induziu morte celular via apoptose em células S180 e K562;

- A apoptose induzida por *cis*-$[RuCl_2(NH_3)_4]Cl$ em células K-562 envolveu o aumento da ativação de caspase 3, 8 e 9.

7. CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Estudos experimentais envolvendo a interação *in vitro* fármaco-célula são de grande importância na pesquisa inicial sobre novas drogas. Sendo assim, neste trabalho um dos objetivos foi avaliar os possíveis efeitos antitumorais *in vitro* de um novo organometálico e os possíveis mecanismos envolvidos.

Como demonstrado neste trabalho e em estudos anteriores, o composto *cis*- tetraaminodiclororutênio(III) mostrou-se bastante promissor sobre células tumorais, e através dos ensaios realizados pôde-se observar que os mecanismos envolvidos deste composto sobre células tumorais estão relacionados a alterações das fases do ciclo celular, danos à molécula de DNA e indução de apoptose. No entanto, o mecanismo pelos quais complexos de rutênio exercem seus efeitos antitumorais, a forma como se ligam ao DNA e como induzem processo de apoptose são ainda mecanismos muito questionados pelos pesquisadores. Diante disso, diversos mecanismos têm sido propostos como: capacidade de fazer cross-link e induzir quebras no DNA e, além disto, como complexos de rutênio pertencem ao mesmo grupo de ferro e possuem a capacidade de ligar à transferrina, sugere-se que complexos de rutênio (III) possam substituir o Fe (III) gerando, assim, radicais livres, que por sua vez atuarão sobre o DNA levando a morte celular.

Sendo assim, estudos adicionais envolvendo a formação de radicais livres, tipos de ligação ao DNA e avaliação de alguns fatores determinantes no mecanismo de morte celular podem assim contribuir para o estudo do mecanismo de ação deste composto. Atualmente diante da grande resistência apresentada

pelas drogas antitumorais convencionais estudo tem focado sobre a expressão de genes relacionados a apoptose tais como genes anti-apoptóticos (Bcl-2 e Bcl-X) assim como genes envolvidos a Multi Drogas Resistentes (MDR). Contudo, estudos adicionais abordando a modulação destes genes poderiam assim contribuir para o estudo do mecanismo de resistência apresentado pelas células tumorais A549 neste trabalho, assim como os genes envolvidos no processo de morte celular apoptótica.

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9.0 ANEXOS

9.1. Anexo 1

Elsevier Editorial System(tm) for Toxicology in Vitro
Manuscript Draft

Manuscript Number:

Title: The organometallic compound cis-(dichloro)tetramminеruthenium(III) chloride induces caspase-mediated apoptosis in K562 cells

Article Type: Research Paper

Section/Category: Mechanisms

Keywords: Apoptosis; cis-(dichloro)tetramminеruthenium(III) chloride; Cytotoxicity; K-562; MRC-5.

Corresponding Author: Professor Elisângela de Paula Silveira-Lacerda, Ph.D.

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Abstract: Ruthenium(III) complexes are increasingly attracting the interest of researchers due to their promising pharmacological properties. In the present study, we investigated the ability of cis-(dichloro)tetramminеruthenium(III) chloride to produce lethal effects in human chronic myelogenous leukemia K-562 cells. The MTT tetrazolium reduction test and the trypan blue exclusion assay revealed that the IC50 for the compound after 48 h of incubation with K562 cells was approximately 10.74 and 73.45 μM, respectively. Interestingly, it was observed that this compound exhibits mild cytotoxicity towards MRC-5 human fibroblast cells (IC50 > 383 μM). Flow cytometric analysis revealed that cis-(dichloro)tetramminеruthenium(III) chloride was capable of change cell cycle distribution since the percentage of cells in the G1, S and G2 phases decreased. In addition, treatment with this compound induced apoptotic cell death in K562 cells, demonstrated by increased DNA content in the sub-G1 peak and a significant increase in caspase-3 activity. In summary, cis-(dichloro)tetramminеruthenium(III) chloride displayed a significant anti-tumor effect through cell cycle arrest and apoptotic induction in K562 cells, which suggests that cis-(dichloro)tetramminеruthenium(III) chloride might have therapeutic potential against leukemia.

Federal University of Goiás
Biological Sciences Institute
Cytogenetics and Molecular Genetics Laboratory

LGMC - UFG

Goiânia, 15 January, 2010.

Editorial Board
Toxicology in Vitro

Dear Editors,

We would like to submit the manuscript **"The organometallic compound *cis*-(dichloro)tetramminеruthenium(III) chloride induces caspase-mediated apoptosis in K562 cells"** to be considered for publication in Toxicology in Vitro .

In this work, we studied the antitumor activity of the compound *cis*-(dichloro)tetramminеruthenium(III) chloride {*cis*-$[RuCl_2(NH_3)_4]Cl$} against the chronic myelogenous leukemia tumor cell line K-562. Leukemia is one of the most common malignancies worldwide. Approximately 44,270 new cases of leukemia were diagnosed in 2008 just in the United States of America. Since chemotherapy for leukemia frequently causes resistance and side effects in patients, the development of effective therapeutic agents for this pathology is both important and urgent.

Ruthenium(III) complexes are increasingly attracting the interest of researchers due to their promising pharmacological properties. We investigated the ability of *cis*-$[RuCl_2(NH_3)_4]Cl$ to produce lethal effects in human chronic myelogenous leukemia K-562 cells. Using the MTT tetrazolium reduction test and the trypan blue exclusion assay, the IC_{50} for the ruthenium(III) compound after 48 h incubation with K-562 cells were approximately 10.74 and 73.45 μM, respectively. Interestingly, it was observed that this compounds exhibited low cytotoxicity towards normal MRC-5 human fibroblast cells (IC_{50} >383 μM). Flow cytometry revealed that *cis*-$[RuCl_2(NH_3)_4]Cl$ caused the percentage of cells in G1, S and G2 phases to decrease, indicating that this compound is capable of

preventing cell cycle progression. In addition, treatment with this compound induced apoptotic cell death in K-562 cells, as demonstrated by increased DNA content in the sub-G1 peak and a significant increase in caspase-3 activity. In summary, *cis*-$[RuCl_2(NH_3)_4]Cl$ displayed significant anti-tumor effects via cell cycle arrest and apoptotic induction in K562 cells, which suggests that *cis*-$[RuCl_2(NH_3)_4]Cl$ might have therapeutic potential against leukemia.

The development of new antitumor medications demands adequate knowledge in order to avoid inefficient and/or toxic treatments. Thus, a mechanistic understanding of how metal complexes achieve their effects is crucial to their clinical success and to the rational design of new compounds with improved potency.

We affirm that this paper has not been submitted to any other journal and that all the authors have approved the submission of the manuscript.

Best regards,

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Manuscript title: Organometallic compound cis-(dichloro)tetramminеruthenium(III) chloride induce caspase-mediated apoptosis in K562 cells

Authors: Lima AP & Silveira-Lacerda EP

Key: E10A-D511-A171-7A35-2EC8

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9.1. Anexo 2

Elsevier Editorial System(tm) for Chemico-Biological Interactions
Manuscript Draft

Manuscript Number:

Title: The ruthenium complex cis-(Dichloro)tetramminеruthenium(III) Chloride induce apoptosis and damage DNA in murine ascitic sarcoma 180 (S-180) cells

Article Type: Research Paper

Keywords: Apoptosis; cis-(dichloro)tetramminеruthenium(III) chloride, cycle cell, DNA damage, S180.

Corresponding Author: Professor Elisângela de Paula Silveira-Lacerda, Ph.D.

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Abstract: Ruthenium(III) complexes are increasingly attracting the interest of researchers due to their promising pharmacological properties. Recently, we have reported that cis-(dichloro)tetramminеruthenium(III) chloride compound presented cytotoxic effects on murine sarcoma 180 cell lines (S180). In an effort to understand the mechanism behind their cytotoxicity, this study investigated the genotoxicity, cell cycle distribution and the induction of apoptosis caused by cis-(dichloro)tetramminеruthenium(III) chloride in S180 tumor cells. cis-(dichloro)tetramminеruthenium(III) chloride treatment induced significant DNA damage in S180 cells, as detected by alkaline comet assay. In cell cycle analysis, cis-(dichloro)tetramminеruthenium(III) chloride caused an increase in the number of cells in G1 phase accompanied by a decrease in the S and G2 phase after 24 h treatment. On the other hand, cell cycle distribution of S180 cells treated with cis-(dichloro)tetramminеruthenium(III) chloride for 48 h showed a dose-dependent increase in the sub-G1 phase (indicating of apoptosis), with a corresponding decrease in cells in G1, S and G2 phase. In addition cis-(dichloro)tetramminеruthenium(III) chloride treatment induced apoptosis in a time-dependent manner, as observed by a increase numbers of Annexin V-positive cells. Taken together, these findings strongly demonstrate that DNA damage, process apoptotic and change cell cycle may correlate with their antitumor effects presented for cis-(dichloro)tetramminеruthenium(III) chloride on S180 cells.

Federal University of Goiás
Biological Sciences Institute
Cytogenetics and Molecular Genetics Laboratory

LGMC - UFG

Goiânia, 18 January, 2010.

Editorial Board

Chemico-Biological Interactions

Dear Editors,

We would like to submit the manuscript "**The ruthenium complex *cis*-(dichloro)tetramminеruthenium(III) chloride induce apoptosis and damage DNA in murine ascitic sarcoma 180 (S-180) cells** " to be considered for publication in Chemico-Biological Interactions.

In this work, we investigated the genotoxicity, cell cycle distribution and the induction of apoptosis caused by *cis*-(dichloro)tetramminеruthenium(III) chloride in murine sarcoma 180 cell lines .

Ruthenium(III) complexes are increasingly attracting the interest of researchers due to their promising pharmacological properties. Recently, we have reported that *cis*-(dichloro)tetramminеruthenium(III) chloride compound presented cytotoxic effects on murine sarcoma 180 cell lines (S180). In an effort to understand the mechanism behind their cytotoxicity, this study investigated the genotoxicity, cell cycle distribution and the induction of apoptosis caused by *cis*-(dichloro)tetramminеruthenium(III) chloride in S180 tumor cells. *cis*-(dichloro)tetramminеruthenium(III) chloride treatment induced significant DNA damage in S180 cells, as detected by alkaline comet assay. In cell cycle analysis, *cis*-(dichloro)tetramminеruthenium(III) chloride caused an increase in the number of cells in G1 phase accompanied by a decrease in the S and G2 phase after 24 h treatment. On the other hand, cell cycle distribution of S180 cells treated with *cis*-(dichloro)tetramminеruthenium(III) chloride for 48 h showed a dose-dependent increase in the sub-G1 phase (indicating of apoptosis), with a corresponding decrease in cells in G1, S and G2 phase. In

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Best regards,

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9.1. Anexo 3

Elsevier Editorial System(tm) for Chemico-Biological Interactions
Manuscript Draft

Manuscript Number:

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Article Type: Research Paper

Keywords: Apoptosis; cis-(dichloro)tetramminеruthenium(III) chloride, cycle cell, DNA damage, S180.

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We affirm that this paper has not been submitted to any other journal and that all the authors have approved the submission of the manuscript.

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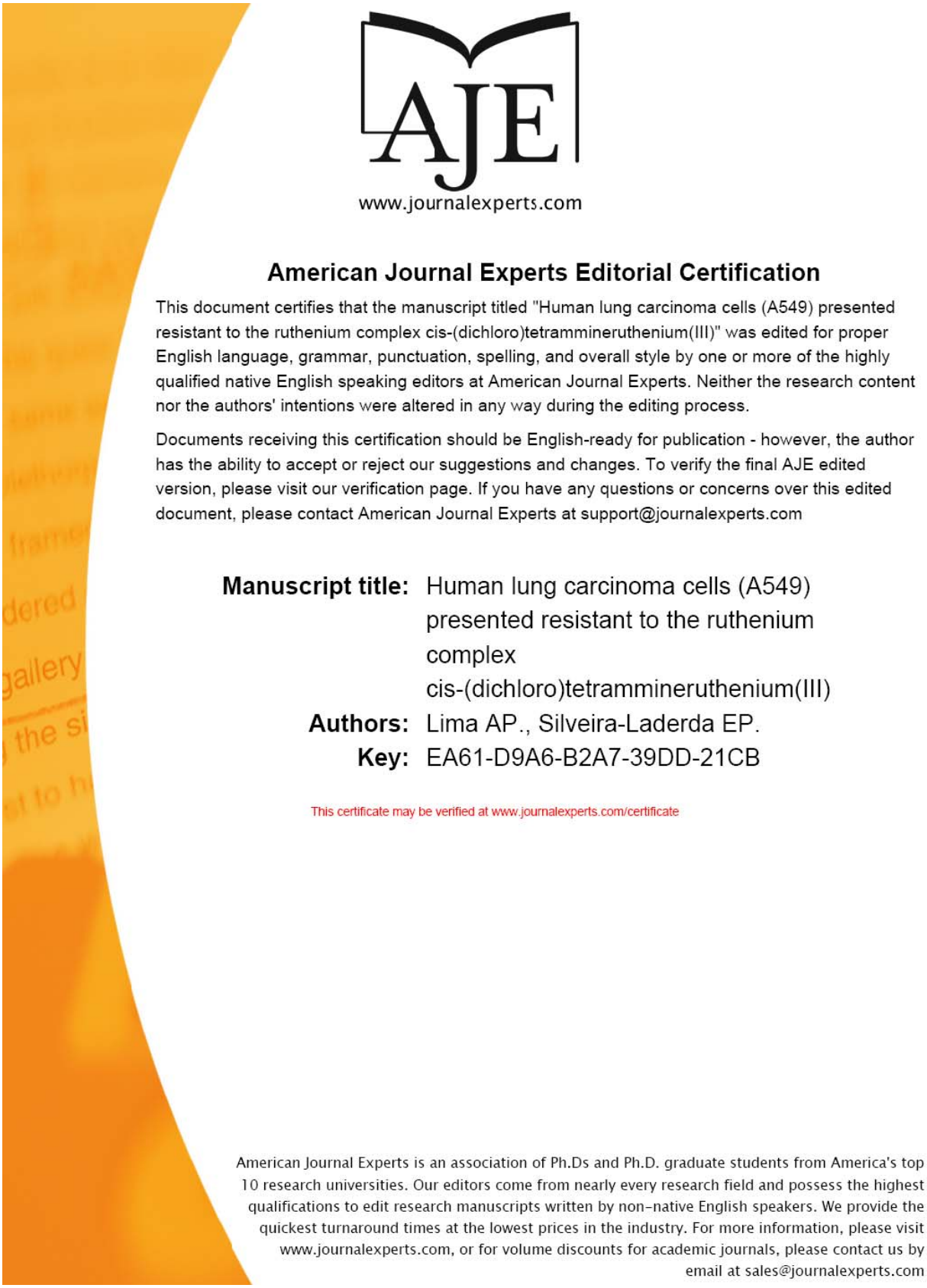

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