AÇÃO ANTIINFLAMATÓRIA, IMUNOMODULADORA E CITOTÓXICA DO COMPOSTO RDV 8

MARCOS SCHUCH DE AZAMBUJA

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Orientador: Prof. Dr. Jarbas Rodrigues de Oliveira

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Dedico essa dissertação a meus pais,
Carlos Eugenio C. de Azambuja filho
e Liliane Mª Schuch de Azambuja,
meus irmãos Lucas S. de Azambuja
e Luiza S. de Azambuja, que sempre
me apoiaram e me deram todo suporte
necessário para eu atravessar mais
essa etapa de minha vida,
aprimorando meu crescimento profissional.

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RESUMO

A proposta deste estudo foi avaliar o efeito antiinflamatório, imunomodulador e citotóxico do composto derivado de 4-tioxopirimidinas denominado RDV 8.

Para investigar o efeito antiinflamatório usou-se o modelo de pleurisia induzida por carragenina em ratos, onde foram avaliados os parâmetros da fase aguda da inflamação. No modelo *in vitro* foi utilizado o método de cultura de células mononucleares de sangue periférico (PBMCs) humano, tanto para investigar a ação citotóxica quanto o efeito imunomodulador do RDV 8. No modelo *in vivo* foram utilizadas 30 ratas Wistar divididas em grupos controle e experimental. Meia hora (30min) após a injeção intraperitonial de RDV 8 (3,0 mg/kg) a carragenina (0.2mL) foi injetada na cavidade pleural para causar inflamação. Após 4 horas o volume de exudato, leucócitos totais e contagem diferencial, concentração de proteínas e óxido nítrico (NO) foram mensurados no liquido pleural aspirado. No modelo de cultura de células foram utilizadas células mononucleares de 6 indivíduos saudáveis, na faixa entre 17 e 40 anos. Estas células foram distribuídas em 11 grupos, 5 para avaliação citotóxica e 6 para investigação imunomoduladora. Após 96 horas (4 dias) as células foram contadas e retirados seus sobrenadantes, a fim de avaliar as concentrações de interleucinas-1 (IL-1) e (IL-6) e a proteína-1 quimioatraente de monócitos (MCP-1 ou CCL2).

Na pleurisia o RDV 8 reduziu significativamente ($P<0,05$) todas as variáveis inflamatórias exceto o numero de células polimorfonucleares (PMNs). Em nenhum dos testes de citotoxicidade ocorreu morte celularsignificativa nas concentrações utilizadas, mas nos testes de imunossupressão houve uma significante ($P<0,05$) imunossupressão (antilinfoproliferação) , diminuição de MCP-1 e aumento da IL-6 na concentração de 0,1µg/mL. Esses resultados indicam uma ação antiinflamatória e imunomoduladora do RDV 8, não apresentando ação citotóxica nas concentrações utilizadas nos experimentos.

Palavras-chaves: RDV 8; antiinflamatório; citotóxico; imunomodulação; *in vivo*; *in vitro*

ABSTRACT

The purpose of this study was to evaluate the anti-inflammatory, immunomodulatory and cytotoxic effect of the compound derivative of 4-tioxopirimidinas denominated RDV 8.

In order to investigate RDV 8 anti-inflammatory effect, the model used was pleurisy induced by carrageenan in rats, where the parameters of the acute phase of inflammation were evaluated. In the *in vitro* model, peripheral blood mononuclear cells (PBMCs from humans) culture was used to investigate both the cytotoxic action and the immunomodulatory effect of RDV 8. In the *in vivo* model, 30 female Wistar rats were used divided in control and experimental groups. Half hour (30min) after the intraperitoneal injection of RDV 8, 3.0 mg/Kg, carrageenan (0.2mL , 1%) was injected in the pleural cavity to cause inflammation.
After 4 hours the pleural liquid was aspirated and the volume of exudate, total and differential leukocytes were counted and protein concentration and nitric oxide (NO) were measured. In the cell culture model of mononuclear cells, we used blood samples from 6 healthy individuals, between 17 and 40 years olds. These cells were distributed in 11 groups, 5 for cytotoxic assessment and 6 for immunomodulatory research. After 96 hours (4 days) the cells were counted and their supernatants were withdrawn, in order to assess concentrations of interleukin (IL) 1 and 6 and the monocyte chemotactic protein 1 (MCP-1 or CCL2).

In the pleurisy model, RDV 8 significantly reduced ($P<0.05$) all variables except the number of polymorphonuclear cell (PMNs). The cytotoxic tests presented no significant change in the concentrations used, but there was a significant immunosuppressant effect ($P<0.05$) in immunomodulatory tests. There was a decrease in MCP-1 and a increase in IL-6 in the RDV 8 concentration of 0.1µg/ml. These results indicate a high immunomodulatory and anti-inflammatory action of RDV 8 without any cytotoxic action on the concentrations used in these experiments.

Keywords: RDV 8; anti-inflammatory; cytotoxic; immunomodulatory; *in vivo*; *in vitro*

LISTA DE ILUSTRAÇÕES

Artigo 1

Artigo 2

LISTA DE SIGLAS

FAP – Fatores Ativadores das Plaquetas

NO – Óxido Nítrico

IL 1 – Interleucina-1

IL 6 – Interleucina-6

TNF-α – Fator de Necrose Tumoral-alfa

MCP-1 – Proteína-1 Quimioatraente de Monócitos

COX – Ciclooxigenase

COX-1 – Ciclooxigenase-1

COX-2 – Ciclooxigenase-2

CAA – Células Apresentadoras de Antígenos

Th – Células T Auxiliares

DNA – Ácido Desoxirribonucléico

PHA – Fitohemaglutinina

RNA – Ácido Ribonucléico

TAP – transporte Associado de Proteínas

HIV – Infecção da Imunodeficiência Humana

HBV – Vírus da Hepatite B

HPV – Papiloma Vírus Humano

HSV – Herpes Vírus Simples

VEB – Virus Epstein Barr

HCMV – Citomegalovírus da Herpes

PBMCs – Células Mononucleares de Sangue Periférico

PMNs – Células Polimorfonucleares

NOS – Nitric Oxide Synthase

nNOS – neuronal Nitric Oxide Synthase

iNOS – inducible Nitric Oxide Synthase

eNOS – endothelial Nitric Oxide Synthase

cNOS – constitutive Nitric Oxide Synthase

PBS – Phosphate buffered saline

DMSO – Dimetil Sulfóxido

MTT – 3-[4-5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

NSAID – drogas antiinflamatórias não esteróides

SUMÁRIO

1. INTRODUÇÃO

A reação inflamatória é um mecanismo fisiopatológico de resposta à invasão por um agente infeccioso ou apenas reação a uma lesão de natureza variada (térmica, química ou mecânica), sendo representada por um conjunto de reações locais e gerais do organismo. Este mecanismo é composto por vários fenômenos complexos que se associam e se complementam entre si formando uma reação em cascata, que envolve uma complexa interação de células inflamatórias (neutrófilos, linfócitos, monócitos, macrófagos) e das células vasculares (endoteliais e células da musculatura lisa) (TEDGUI, MALLAT, 2001).

A resposta inflamatória visa destruir, diluir ou isolar o agente agressivo, sendo, portanto uma reação de defesa e reparação do dano tecidual (CHANDRASSOMA, TAYLOR, 1993).

A inflamação é caracterizada, em sua forma aguda, pelos sinais fisiológicos de dor, calor, rubor e edema, envolvendo uma série de eventos como aumento do fluxo sanguíneo, aumento da permeabilidade vascular, exsudação de fluidos, migração de leucócitos, liberação de agentes álgicos e dos efeitos induzidos pelos mediadores químicos no foco inflamatório (KUMAR et al., 2005; RANG et al, 2001; GUALILO et al 2000).

1.1.1 Inflamação aguda

A inflamação aguda tem duração relativamente curta, durando alguns minutos, horas ou dias e é independente da natureza do agressor, sendo a resposta muito similar aos diferentes estímulos (SIQUEIRA JÚNIOR e DANTAS, 2000). A resposta fisiológica que ocorre imediatamente após um estímulo agressivo é referida como uma fase precoce (0-1 hora) ao contrário do que ocorre de 5-6 horas após a lesão (fase tardia da inflamação aguda), onde as células inflamatórias se acumulam no local lesado (ALBERTINI et al., 2004).

Evidências demonstram que vários fatores desempenham importantes papéis na modulação da resposta inflamatória de cada uma das fases da inflamação aguda. Na fase precoce, mediadores como a histamina e bradicinina modulam a resposta inflamatória aumentando o

calibre e o fluxo vascular, responsáveis pelo calor e rubor presente no foco de inflamação (KUMAR et al., 2005; ALBERTINI et al., 2004).

Durante a fase tardia da inflamação aguda, há predominância de eventos celulares que se caracterizam pela marginação, adesão endotelial, diapedese e migração dos leucócitos para o foco da lesão, decorrentes dos estímulos quimiotáticos. Todos os granulócitos, monócitos e, em menor grau, os linfócitos respondem aos estímulos quimiotáticos com taxas variáveis de velocidade (KUMAR et al., 2005).

A inflamação é controlada (desencadeada, conduzida e extinta) pela presença de mediadores químicos, cada um com um papel específico atuando em estágios definidos da reação inflamatória (DE PAOLA, 1988). Os mediadores podem originar-se do plasma, das células ou do tecido agredido, sendo divididos nos seguintes grupos: aminas vasoativas (histamina e serotonina); proteases plasmáticas (sistema de cinina - bradicinina, sistema complemento, sistema de coagulação - fibrinolítico); metabólitos do ácido araquidônico (via ciclooxigenase e via lipoxigenase); proteases lisossômicas; radicais livres derivados do oxigênio; fatores ativadores das plaquetas (FAP); quimiocinas, citocinas e óxido nítrico (NO) (KUMAR et al., 2005; ALBERTINI et al., 2004).

Durante a evolução do processo de reparo, os eventos que se sucedem são a: infiltração de neutrófilos, infiltração de macrófagos, fibroplasia e deposição de matriz extracelular, angiogênese, cicatrização e reepitelização.

A transmigração dos neutrófilos para tecidos lesados é um fenômeno precoce do processo de reparo. Ocorre quase que de imediato após sinalização dos neutrófilos retidos no coágulo, macrófagos residentes e células estromais. Citocinas, principalmente a interleucina-1 (IL-1) e o fator de necrose tumoral-alfa (TNF-α), atuando sobre os receptores das células endoteliais, induzem a produção de NO, bem como a expressão de moléculas de adesão para neutrófilos. A expressão das proteínas de adesão é, neste momento, o elemento mais importante para a migração de neutrófilos (GERSZTEN et al., 1999).

A família das quimiocinas (citocinas com atividade atraente sobre leucócitos) é composta de aproximadamente 50 membros que se dividem em 4 famílias. Apesar da ação das quimiocinas ser mais evidente na quimiotaxia de macrófagos e linfócitos, alguns membros desta família de moléculas como a proteína-1 quimioatraente de monócitos (MCP-1) exerce esta função também sobre neutrófilos (CHRISTOPHERSON e HROMAS, 2001).

A interleucina (IL-6) é considerada como um mediador fundamental em diversas etapas da inflamação (GALLUCCI et al., 2000). Dentre os vários efeitos pró-inflamatórios que lhe são atribuídos, os intimamente relacionados ao processo de reparo são, na etapa mais tardia, a indução mitótica de queratinócitos e, na fase mais precoce, os seus efeitos quimioatrativos sobre neutrófilos (SATO et al., 1999).

A inflamação não infecciosa é tradicionalmente tratada com drogas antiinflamatórias esteróides e não-esteróides. Os glicocorticóides são potentes agentes antiinflamatórios esteróides largamente utilizados que apresentam capacidade de inibir a enzima fosfolipase A-2 e a ciclooxigenase-2 (COX-2). Esta inibição reduz os níveis de ativação do ácido araquidônico e a produção de prostaglandinas, respectivamente, proporcionando um alto poder antiinflamatório (LUNARDELLI et al., 2006).

1.1.2 Linfócitos

Devido a importantes características de memória, especificidade e reconhecimento, do sistema imunitário adaptativo os linfócitos são muito eficazes na defesa do organismo. Os tipos celulares que mediam esta reação são em particular os linfócitos B e T, e as células apresentadoras de antígenos (CAA), representadas por uma coleção de macrófagos e células dendríticas (ROITT et al., 2003).

Os linfócitos são as principais células responsáveis pela resposta imunológica adaptativa no ser humano. Nos indivíduos adultos, são continuamente produzidos à custa da proliferação controlada das células linfóides primitivas presentes na medula óssea, as quais dão origem a populações distintas de linfócitos.

Antes de se tornarem células maduras e capazes de exercer funções específicas, os linfócitos passam por processos sucessivos para aquisição de imunocompetência no timo ou na própria medula óssea, denominados órgãos linfóides primários. Os que requerem um período de diferenciação no timo são denominados linfócitos T. Outros que continuam a se diferenciar no próprio sítio produtor emergem como linfócitos B.

Como é no tecido linfóide periférico que ocorrem as maiores chances dos linfócitos T encontrarem antígenos, após a fase de amadurecimento no timo, essas células, agora imunocompetentes, ficam circulando entre a corrente sangüínea e o tecido linfóide periférico até encontrarem o seu antígeno específico, quando então são induzidas a proliferar e se diferenciar em células T efetoras (JANEWAY, 2002).

O encontro com um antígeno específico provoca a fase final do desenvolvimento e diferenciação dos linfócitos T, onde as células CD8 tornam-se células T citotóxicas para as células que expressam esses antígenos protéicos, enquanto as células CD4, sob influência de citocinas, tornam-se células T auxiliares subtipos 1 ou 2 (Th1 e Th2, respectivamente), sendo que o tipo celular que predominará dependerá do patógeno e do tipo de resposta imunológica requerida (PARHAM, 2001).

As células efetoras originadas da ativação de linfócitos T CD4 proliferam, dando origem a células auxiliares, responsáveis pela propagação da resposta imunológica por meio da produção e secreção de interleucinas. Os linfócitos T CD8, após contato com o antígeno, ativam-se e proliferam, originando também células capazes de produzir grandes quantidades de citocinas, que são secretadas com o objetivo de eliminar o agente agressor.

Em contraste com o processo de amadurecimento dos linfócitos T, os precursores dos linfócitos B são induzidos a diferenciar dentro da própria medula óssea, influenciados por várias citocinas produzidas pelas células estromais. No caso dos linfócitos B, após transformação, muitos proliferam, dando origem aos plasmócitos, células especializadas na síntese de imunoglobulinas que são, então, secretados como anticorpos (PARHAM, 2001).

A ativação de linfócitos decorre da interação entre um antígeno e o receptor presente na superfície celular com o qual ele pode interagir. Para que a proliferação celular ocorra, fatores de transcrição, que são ativados simultaneamente à interação ligante-receptor, representam intermediários essenciais, os quais traduzem e direcionam sinais extracelulares em respostas transcricionais específicas. A regulação combinada da transcrição envolve complexos multiprotéicos que se ligam de forma cooperativa a regiões específicas do DNA alvo. Esta interação permite a convergência de diferentes sinais para uma região definida do DNA, a qual, por sua vez, exerce controle regulatório rigoroso sobre a expressão dos genes alvos em resposta aos sinais recebidos (FESKE et al., 2000; TRAMA et al., 2000).

Certas substâncias possuem a habilidade de ativar e, subsequentemente, induzir a proliferação de linfócitos T, B ou de ambos *in vitro*, sendo genericamente denominados mitógenos (GERY et al., 1972; JANOSSY e GREAVES, 1972).

Enquanto a resposta dos linfócitos a antígenos *in vivo* é específica, gerando amplificação clonal, sua resposta a mitógenos *in vitro* é inespecífica e influencia, simultaneamente, um grande número de células, levando-as a sofrer transformação blástica e, posteriormente, proliferar. Diferente do que acontece *in vivo*, esse estímulo à proliferação não depende de células apresentadoras de antígenos, embora os mesmos mecanismos bioquímicos estejam aparentemente envolvidos (BURGERMEISTER et al., 2003). Esta propriedade favorece estudos experimentais e a Fitohemaglutinina (PHA), a Concanavalina A ou as lectinas extraídas e purificadas de plantas, que estimulam sub-populações de células T, têm sido usadas como mitógenos para estudos de proliferação dessas populações *in vitro* (MYERS, 1995).

São várias as metodologias *in vitro* utilizadas para quantificar a ativação e a proliferação lifocitaria. Entre estas estão os ensaios de imunomodulação que incluem a incorporação de nucleotídeos radioativos e a formação de sais tetrazólio pela mitocôndria (BRUNNER et al., 1986; GILLIS et al., 1978; MOSMANN, 1983).

1.1.3 Modelos animais

Modelos animais têm sido utilizados para avaliar o processo inflamatório através da indução de agentes químicos como a carragenina e/ou veneno de taturanas *Dirphia* sp. (GUERINO et al, 2000; DI ROSA 1972; LUNARDELLI et al., 2006). A principal fonte de carragenina é a alga *Chondrus Crispus*, também conhecida como *"Irish Nllos"*, que tem origem em *Carraghen* (Waterford – Irlanda), onde cresce abundantemente (DI ROSA, 1972).

A carragenina é um polissacarídeo freqüentemente usado em modelos animais experimentais para induzir reação inflamatória aguda. A carragenina induz a liberação de diferentes mediadores inflamatórios como a histamina, bradicinina e a prostaglandina. (MARTINS et al, 2005). Winter (1962) introduziu o uso da carragenina como um irritante para produzir edema na pata de rato, sendo o primeiro e mais popular método para avaliar as novas

terapias antiinflamatórias através da mensuração por hidropletismômetro do volume da pata inflamada.

O edema da pata induzido por carragenina é um modelo útil para avaliar a inflamação aguda, pois o pico do edema ocorre dentro de 3 a 5 horas (SALVEMINI et al., 1996). Entretanto esta técnica apresenta limitações na mensuração de células inflamatórias, proteínas e mediadores químicos onde não conseguimos extrair o exsudato inflamatório. Com o objetivo de avaliar quantitativamente e qualitativamente, Spector (1956), descreveu primeiramente o modelo de pleurisia em ratos que mais tarde foi adaptado para o porco e o camundongo (MARTINS et al., 2005; MIKAMI, MIYASAKA, 1983).

A pleurisia em ratos induzida por carragenina permite a quantificação do volume e da concentração protéica do exsudato formado, além da avaliação da migração de células inflamatórias para a cavidade pleural (SHIVKAR e KUMAR, 2004). Este tipo de pleurisia é utilizado na investigação da fisiopatologia da inflamação aguda e avaliação da eficácia de terapias antiinflamatórias (ARRUDA et al., 2003).

1.1.4 Derivados pirimidicos

Nas últimas duas décadas derivados pirimídicos da uracila e derivados de oxopirimidinas, vem sendo investigados extensivamente com relação às suas propriedades antivirais, antimicrobianas e antitumorais (COCCO et al., 2001).

As pirimidinas fazem parte do DNA e do RNA. Estão associadas aos ácidos nucléicos, a citosina, a timina e a uracila (derivados pirimídicos).

A enzima Deoxicitidiclato desaminase catalisa a conversão de 2'-deoxicitidina 5'-monofosfato (dCMP) para 2'-deoxiuridina 5'-monofosfato (dUMP), uma enzima importante no processo de síntese de timidina e novos nucleotídeos que são derivados pirimídicos e agentes anticancerígenos. Recentemente, foi descoberto que análogos de pirimidinas do DNA podem ser potentes agentes antivirais e antitumorais. Há ainda os interconversores enzimáticos da pirimidina do DNA, que fazem parte de um grupo de enzimas alostéricas que podem ser ativadas por 2’-deoxicitidina 5’-trifosfato, inibidas pelo transporte associado de proteínas (TAP)

(KUMAR, 2004). Foi demonstrado que essas enzimas podem catabolizar os monofosfatos de citarabina (MALEY e MALEY, 1972) e gemcitabina (JAMIESON et al., 1987), que são fármacos com propriedades antitumorais (HEINEMANN et al., 1992).

Nos últimos anos, esses análogos das pirimidinas estão sendo utilizados para o tratamento da infecção da imunodeficiência humana (HIV), pelo vírus da hepatite B (HBV) e para os cancros papiloma vírus humano (HPV) (NERSESYAN et al., 2006).

Os nucleosídeos de pirimidina têm tido um papel importante no tratamento das infecções por vírus. O estudo de fármacos com ação antiherpéticas foi desenvolvido a partir do advento de nucleosídeos de pirimidina, tais como o 5-carbamato, o 5-etil, o -5-(2-cloroetilo) ou o -5-(2-bromovinil) derivados de 2'-deoxiuridine. Esses são inibidores específicos do Herpes vírus simples (HSV), HSV-1, HSV-2. Contudo, o vírus Epstein Barr (VEB) e as estirpes são pouco sensíveis a estes agentes (KUMAR, 2004).

Tem sido investigado que o acréscimo de radicais funcionais na posição do C_1 da pirimidina pode determinar sua utilização como agente antiviral. Novos radicais acrescidos no C_1 e C_5 da cadeia lateral da pirimidina podem determinar o aumento do potencial de ampliação do espectro de antivirais, com ação para VEB, citomegalovírus humano (HCMV) e do Herpes vírus (KUMAR, 2004).

Resultados promissores das oxipirimidinas demonstraram que as tioxopirimidinas podem apresentar efeitos clínicos importantes. Dentre elas, a 4-tioxopirimidina (figura 1) que faz parte de uma família de compostos cuja estrutura essencial é formada pela união de um anel heterociclíco de pirimidina a um grupo fenol (C_6H_5) no C_2, um átomo de enxofre no C_4 (o que acrescenta o prefixo tioxo à molécula), a um aldeído no C_5, a um metil no C_6. Os locais na molécula onde podem ser adicionados diversos radicais são na posição do Nitrogênio (N_1) e no carbono (C_{14}) (CUNHA et al., 2007).

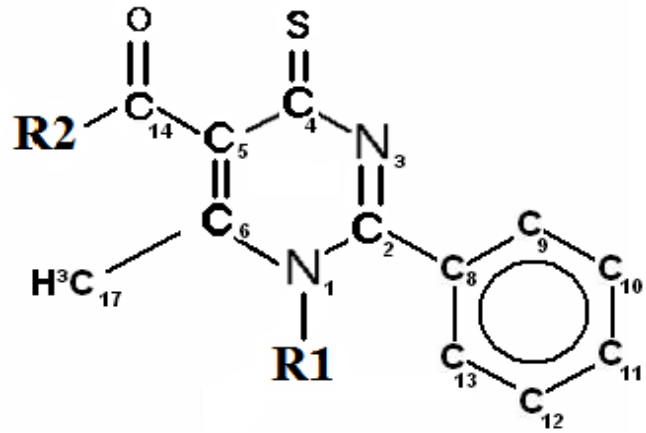

Figura 1: Estrutura utilizada como base para sintetizar os compostos de 4-tioxopirimidinas (Cunha S. et al 2007).

A partir da estrutura inicial, diversos radicais podem ser adicionados no N_1 e no C_{14}. Essas 4-tioxopirimidinas tiveram resultados satisfatórios quando testadas como antimicrobianas em *Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Streptococcus mutans, Salmonella* spp, *Escherichia coli, e Candida albicans*. Também foram avaliados os efeitos sobre o *Trypanosoma cruzi in vitro*. Todos os derivados de 4-tioxopirimidinas mostraram pouca atividade antitrypanocida. Este resultado sugere que a modificação estrutural das 4-tioxopirimidinas poderia melhorar as propriedades antimicrobianas como antitrypanocida (CUNHA et al., 2007).

1.1.5 RDV 8

O que diferencia as 4-tioxopirimidinas são os locais da molécula onde podem ser adicionados diversos radicais: posição do Nitrogênio (N_1) e no carbono (C_{14})

O RDV 8 é formado a partir da estrutura essencial utilizada como base (figura 1), e nela é adicionado um metil (CH_3) in N_1, mas sua principal diferença entre as outras 4-tioxopirimidinas é a adição de um carboxilato (C_2H_5O) no C_{14} (Figura 2).

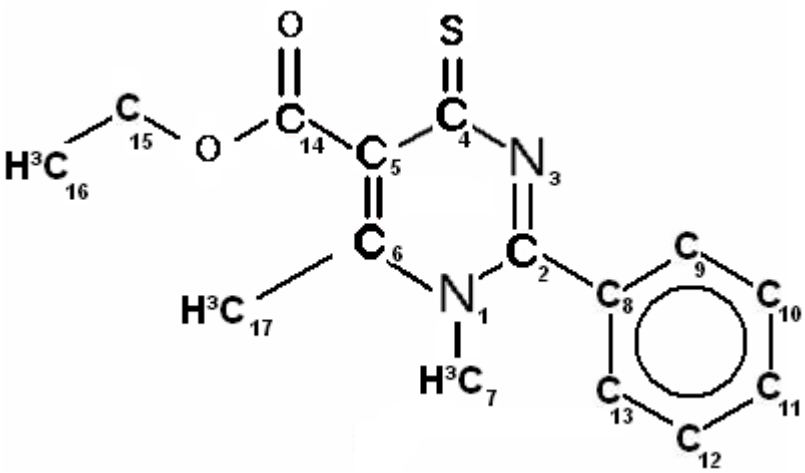

Figure 2. RDV 8 $C_{17}H_{16}N_2O_2S$ (1;6 methyl-2-phenyl-4-thioxo-5-aldehylde-12-carboxylate-pyrimidine) (Cunha et al., 2007).

1.2 Hipóteses

O composto de 4-tioxopirimidina denominado RDV 8 pode apresentar ação antiinflamatória *in vivo* em modelo experimental de pleurisia induzido por carragenina em ratos, e, *in vitro,* possíveis efeitos imunomoduladores em células mononucleares de sangue periférico (PBMCs) humano.

1.3 Objetivos

1.3.1 Objetivo Geral

- Verificar a ação antiinflamatória "*in vivo"* e avaliar a citotoxicidade e capacidade imunomoduladora "*in vitro* do composto de 4-tioxopirimidina denominado RDV 8.

1.3.2 Objetivos Específicos

- Avaliar a capacidade antiinflamatória do composto RDV 8, administrado via intraperitoneal, em modelo de pleurisia experimental induzida por carragenina ;

- Mensurar o volume do líquido, a quantidade de proteínas no exsudato inflamatório; verificar a migração de células inflamatórias para a cavidade pleural no processo inflamatório agudo; analisar a liberação de óxido nítrico (NO), e correlacionar as variáveis mensuradas com a migração de leucócitos polimorfonucleares para a cavidade pleural dos grupos estudados;

- Avaliar a capacidade imunomoduladora e a citotoxicidade do composto RDV 8 sobre PBMCs em cultura;

- Determinar as concentrações de IL-1, IL-6 e MCP-1 no sobrenadante de culturas de PBMCs.

2. ARTIGOS CIENTÍFICOS

Original Article

EVALUATION OF THE ANTI-INFLAMMATORY EFFECT OF RDV 8 IN A RAT PLEURISY MODEL

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ABSTRACT – OBJECTIVES: To assess the anti-inflammatory effect of RDV 8, (3,0mg/kg) a 4-thioxopyrimidine compound, using carrageenan-induced pleurisy in rats**. METHODS:** Injection of carrageenan into the pleural cavity of rats, eliciting an acute inflammation response, characterized by an accumulation of fluid in the pleural cavity which contained a large number of polymorphonuclear (PMNs) leukocytes. **RESULTS:** On our study, RDV 8 (3 mg/Kg) produced a reduction of 38% in the exudate volume, 37% in the leukocyte migration and 24% in the NO of pleural exudate, but the PMNs were not significantly affected by the treatment. **CONCLUSION:** This drug has anti-inflammatory actions suggesting that it may represent a novel strategy for the modulation of inflammatory response.

Keywords: RDV 8; Inflammation; pleurisy; carrageenan

INTRODUCTION

Inflammation is a pivotal component of a variety of diseases, such as atherosclerosis and tumor progression (1). It is an essential protective process for the preservation of the integrity of the organism against the physical, chemical and infectious damage (2). Inflammation is characterized by the classical signs such as pain, heat, redness and swelling, involving a complex series of events, including vasodilatation, increase permeability, fluid exudation and migration of leukocytes to the site of inflammation (3).

Carrageenan-induced pleurisy is a well-characterized experimental model of inflammation that permits the quantification of exudate formation and cellular migration. (4). The major characteristic of this model in rats is the biphasic profile of the inflammatory reaction, where early (4 hours) and late (8 hours) phases of both cell migration and exudation are clearly observed (5). Thus, this model constitutes a biologic system suitable for the investigation of possible correlations occurring between cell migration, fluid leakage, nitric oxide (NO), chemokine and cytokines.

One of the early cellular events in inflammation is the migration of leukocytes, primarily neutrophils. In addition, NO plays an important role in inflammation, such as plasma exudation and leukocytes infiltration. The NO synthase inhibitors can reverse several classic inflammatory symptoms (6).

Increased levels of arachidonic acid accompany inflammation and tissue damage. The cyclooxygenase (COX) enzyme, that converts arachidonic acid into prostaglandins, is present in two isoforms: cyclooxygenase 1 (COX-1), the constitutional isoform that is involved in normal homeostasis, which regulates physiological functions (7) and cyclooxygenase 2 (COX-2), wich is induced by inflammatory agents in case of inflammations, and is responsible for the increase in prostaglandins which

is characteristic of the inflammatory state (8), although it is not expressed under physiological conditions (9).

Drugs with analgesic and anti-inflammatory effects are commonly used in the treatment of chronic or acute inflammations, since they have the capacity to reduce the formation of prostaglandins, by inhibiting COX-1 and COX-2 (10).

The 4-thioxopyrimidine is part of a chemical family, whose essential structure is formed by a heterocyclic ring of pyrimidine (11). A compound, arbitrarily denominated as RDV 8, has a phenol group (C_6H_5) in the $C_{2,}$ was added to the ring, a sulphur atom (S) to the C_4 (and that adds the prefix thioxo to the molecule), an aldehyde (COH) was added to the C_5, and two methyl (CH_3) to N_1 and C_6. Different molecules added to C_{14} can form others compounds of 4-thioxopyrimidine. On the synthetic compound, carboxylate (C_2H_5O) was added to the C_{14} (Figure 1).

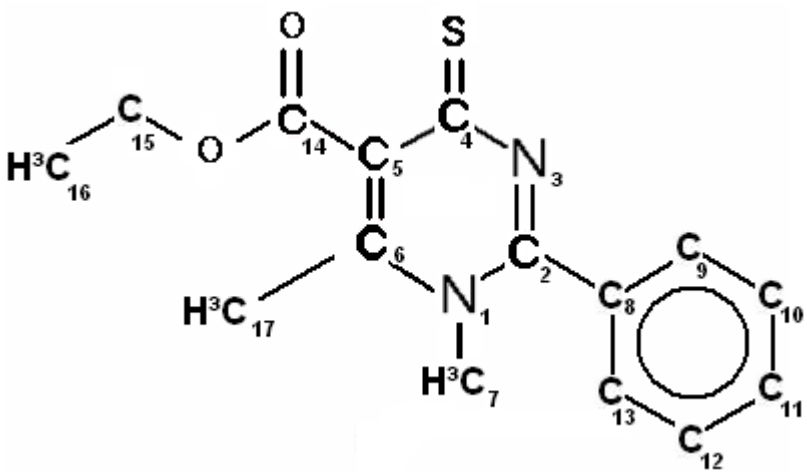

Figure 1. RDV 8 $C_{15}H_{16}N_2O_2S$ (1;6 methyl-2-phenyl-4-thioxo-5-aldehylde-12-carboxylate-pyrimidine) (11).

Promising results of the oxipyrimidines have demonstrated that the thioxopyrimidines present important clinical effects, (11) such as antimicrobial and antitumoral. The 4-tioxopyrimidine showed satisfactory results when tested as antimicrobial in *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Streptococcus mutans*, *Salmonella* spp, *Escherichia coli*, and *Candida albicans*. All the derivatives of 4-tioxopyrimidines showed little antitrypanocidal activity. This result suggests that the structural modification in the 4-tioxopyrimidine may improve the property of its action against the *Trypanosome cruzi* (11).

Recently, Anjos et al. demonstrated that one oxo-pirymidine (3,4-dihydro-2-phenyl-6-para-fluorophenyl-4-oxo-pirymidine-5-carbonitrile) has anti-edematogenic and analgesic activities through rat paw edema and number of abdominal contortions models, respectively (12).

The objective of the present study was to assess the anti-inflammatory potential of RDV 8, a 4-thioxopyrimidine compound, using a model of pleurisy in rats**.**

MATERIAL AND METHODS

This study was approved by the Ethics Committee for Use of Animals (CEUA) of PUCRS record 08/00021. Officious 054/08-CEUA.

2.1 Obtaining the compound of 4-Thioxopyrimidine *RDV 8*

The composite of 4-tioxopyrimidines was obtained through a partnership between the laboratory of Cellular Biophysics and Inflammation from the PUCRS, and the Institute of Chemistry from the Federal University of Bahia. The synthesized compound received the name of RDV 8.

2.2 Animals

Adult female Wistar rats (*Rattus norvegicus)* (around 3–4 months old, weighing 180 g–250 g) were used, all of the same ancestry and socialization with free access to food and water, kept in groups of ten rats. The animals were maintained in accordance with the "*Guiding Principles in the Care and Use of Animals"* approved by the Council of the American Physiological Society.

2.3 Carrageenan-induced pleurisy

Rats were anesthetized with isoforine. Saline solution 0.2 mL (control group) or saline solution containing 2% carrageenan (carrageenan group) 0.2 mL was injected in to the pleural cavity at the sixth level of the left intercostals space. In the carrageenan + RDV 8 group and carrageenan + dexamethasone group, the RDV 8 compound (3.0 mg/Kg) and dexamethasone (1.0 mg/Kg) were injected (intraperitoneally) 30 minutes before carrageenan-induced pleurisy. After 4h, the rats were killed with CO2. The pleural cavity was opened and the liquid that had accumulated was washed with 2.0 ml of sterile saline solution (NaCl 0.9 %) containing 1 % EDTA and then aspirated. Exudates contaminated with red blood cells were rejected (3). The RDV 8 was dissolved in Dimethyl sulfoxide (DMSO).

2.4 Exudate Analysis

The volume of the exudate was measured and the result expressed by subtracting the volume injected into the pleural cavity (2.0 ml of solution) from the total volume aspirated. Total leukocytes were diluted in Thomas solution (1:20) and counted in a Neubauer chamber using light microscopy. Cytological

slides stained with May-Grünwald/Giemsa were used for differential leukocyte counts in a light microscope (13).

The pleural liquid removed from the rats was centrifuged at 1200 × *g* for 10 min and the protein concentration measured by the Biuret technique. NO is a very unstable radical, rapidly metabolized from nitrate to nitrite in the presence of oxygen (14). Therefore, the amount of NO in the exudate was analyzed using the Griess reaction that measures nitrite, as previously described (15).

2.5 Statistical Analysis

The results were statistically evaluated by analysis of variance (one way ANOVA) with LSD *post hoc* test using SPSS (Statistical Package for the Social Sciences) 12.0 software and were expressed as the means ± standard deviation (S.D.). The level of statistical significance was defined as $P < 0.05$.

RESULTS

3.1 Exudate pleural

The amount of pleural exudate collected in control group (rats treated with saline solution) was 0.14 ± 0.12 mL, and 0.95 ± 0.40 mL in the carrageenan group, which was significantly different ($P < 0.05$) when compared with control group. In carrageenan + dexamethasone group, the exudates was 0.12 ± 0.09 mL and 0.59 ± 0.40 mL in carrageennan + RDV 8 group, which were significantly different ($P < 0.05$) when compared with carrageenan group (Figure 2).

3.2 Protein concentration

In control group, the plasma exudation rate estimated by protein concentration, reached 0.20 ± 0.21 g/dL. The rate estimated by protein concentration was 1.32 ± 0.38 g/dL in carrageenan group, which is significantly different ($P < 0.05$) when compared with control group. This rate was 0.50 ± 0.04 in carrageenan + dexamethasone group and 0.83 ± 0.61 in carrageenan + RDV 8 group. This decrease of protein concentration was significantly different ($P < 0.05$) when compared with carrageenan group (Figure 3)

3.3 Total leukocytes

In the control group the total count of leukocytes harvested from the lavage fluid from the pleural cavity was 13.39 ± 7×10^6 cells per cavity, the total leukocyte count was 55.08 ± $29\text{x}10^6$ cells per cavity in carrageenan group, wich was significantly different ($P < 0.05$) when compared with control group.

Leukocyte count was 18.24 ± 1.68 x 10^6 in carrageenan + dexamethasone group and 34.01 ± 4×10^6 cells per cavity in carrageennan + RDV 8 group, which were significantly different ($P < 0.05$) when compared with carrageenan group (Figure 4).

3.4 PMNs

The PMNs accounted for 8.22 ± 4.98% cells per cavity in control group, was 48.34 ± 23.00% cells per cavity in carrageenan group which was significantly different ($P < 0.05$) when compared with control group. PMNs count was 15.5 ± 3.10% cells per cavity in carrageenan + dexamethasone group and 33.51 ± 9.35% cells per cavity in carrageenan + RDV 8 group which were not significantly different with respect to the carrageenan group (Figure 5).

3.5 NO

In the control group, NO concentration was 21.60 ± 6.88 nmol / cavity, and 36.28 ± 7.90 nmol/cavity in carrageenan group which was significantly different ($P < 0.05$) when compared with control group. NO was 34.32 ± 10.09 nmol/cavity in carrageenan + dexamethasone group and 27.51 ± 5.59 nmol / cavity in carrageennan + RDV 8 group, being significantly different ($P < 0.05$) when compared with carrageenan group (Figure 6).

DISCUSSION

The synthesis of new pyrimidine compounds has been considered important in the last 30 years due to its biological relevance, once these molecules are intimately linked to the structure of the nucleic acids (12). Besides the antineoplastic activity previously described (18-22), many biological properties were attributed to this class of compounds, such as anti-hypertensive (23, 24), hypoglycemic (25, 26), anticonvulsive (27), anti-histaminic (28) and anti-inflammatory (29, 30). In recent researches, derivatives of pyrimidines and similar substances containing a carbonyl group in place of an amino group present in C_4 heterocyclic ring would incorporate pharmacological potential, besides presenting low toxicity (12).

The RDV 8 compound is a 4-thioxipyrimidines and it is formed by two main components, a pyrimidic ring and an atom of sulfur added to C_4, besides that, the RDV 8 receives an addition of a carboxylate in C_{12}. These characteristics may indicate why there are anti-inflammatory effects, since these three main components are present on some drugs with anti-inflammatory effects (12, 16, 17).

Immediately after an acute injury, the body initiates a series of biological responses. The inflammatory reaction consists of both vascular and cellular events. Injury responsive components such as

mast cells, bradykinins and prostaglandins are activated along with the vascular responses and cellular membrane reactions. All these combined processes and events are represented by the symptoms of edema, inflammation, pain and functional debility.

Carrageenan is a polysaccharide frequently used to induce acute inflammatory reaction in animal experimental models, since it induces the release of several inflammatory mediators, such as histamine and prostaglandins (7). The inflammatory response that occurs after carrageenan injection into the pleural cavity is characterized by a cellular infiltration, mainly composed by neutrophils (aprox. 90%) and to a lesser extent by monocytes/macrophages.

Our study showed that the RDV 8 compound has a significant anti-inflammatory activity because it decreased significantly the vascular permeability induced by carrageenan and reduced the inflammatory swelling. Parallel to this result, the compound reduced the leakage of protein, reinforcing its anti-inflammatory effect.

In this inflammatory framework, the migration of cells (leukocytes) to the inflamed site was reduced, showing that the studied compound must have intervened in the mechanism of cell migration. However, it was found that the compound has no significant effect on the PMN migration. This mechanism is unclear.

Glucocorticoids are powerful anti-inflammatory agents that are widely used in various diseases, such as rheumatoid arthritis, systemic lupus erythematosus, asthma and other chronic inflammatory and auto-immune disease. (7). Dexamethasone is a steroid anti-inflammatory with a powerful ability to inhibit phospholipase A_2 and COX-2. Our results show that the compound RDV 8, despite an anti-inflammatory action, is less potent that dexamethasone, however, was more efficient to reduce the NO production during the inflammatory process.

Sakaguchi et al. demonstrated that the non selective NO inhibitor shows an anti-inflammatory effect and the combination of an NOS inhibitor and COX inhibitor exerts a synergistic anti-inflammatory effect on acute inflammation, such as rat carrageenan-induced pleurisy. The combination of NOS and COX inhibitors showed greater decrease of the exudate volume (43%), leukocyte infiltration (31%) and exudate NO level (37%).

NO is produced by nitric oxide synthase (NOS), an enzyme existing in three isoforms, neuronal (nNOS or type I), inducible (iNOS or type II) and endothelial (eNOS or type III) (34). While nNOS and eNOS are constitutive (cNOS) (35), iNOS is calcium independent and it has been found in activated macrophages, neutrophils and endothelial cells challenged with endotoxin or cytokines (36, 37). The NO produced in large quantities by iNOS plays a key role in the host defense, in the pathogenesis of endotoxic shock and in autoimmune tissue destruction (38, 39, 40).

Nitric oxide (NO) plays an important role in inflammation (14). NO is considered a modulator of the interaction between leucocytes and vascular endothelium, although some conflicting results have been reported on its actual role in cell migration. Thus, NO has been shown to inhibit in vitro neutrophils adhesion to endothelial cells (31) as well as the adhesion of platelets and monocytes to microvascular endothelium (32, 33). Our study showed that the compound RDV 8 inhibited the release of NO in the pleural cavity and this effect may be related to inhibition of production of cytokines, such as TNF-α.

Our results confirm the anti-inflammatory effect of RDV 8, since caused a significant decrease of inflammatory parameters generated by carrageenan, such as expression of NO, release of proteins to the pleural space, producing swelling and migration of leukocytes to the site of inflammation, suggesting that it may be a clinical alternative to anti-inflammatory drugs. However, many questions regarding molecular and cellular mechanism remain answered.

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LIST OF FIGURES

Figure 2

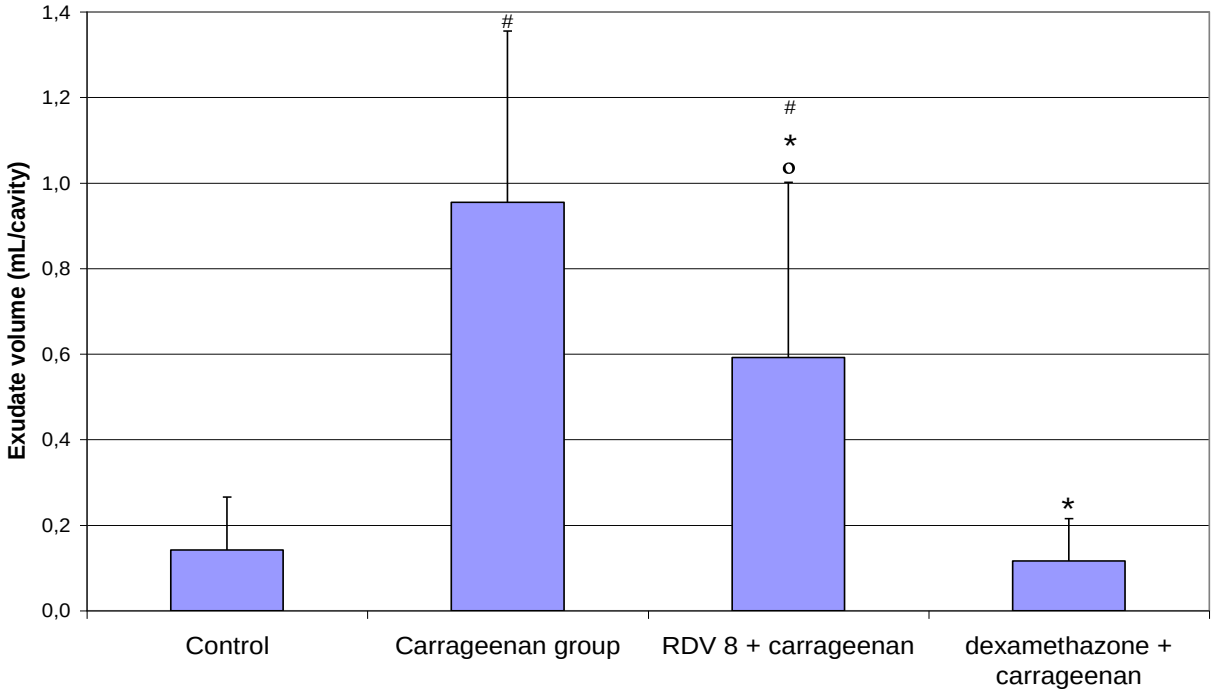


Figure 3

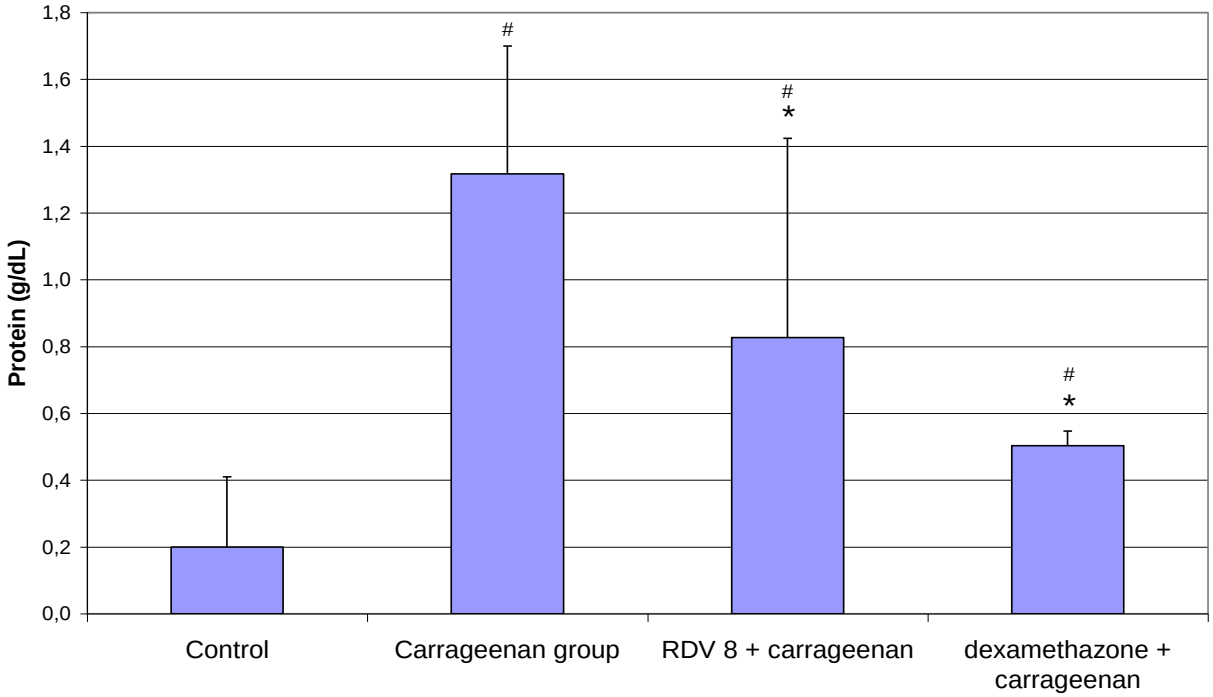


Figure 4

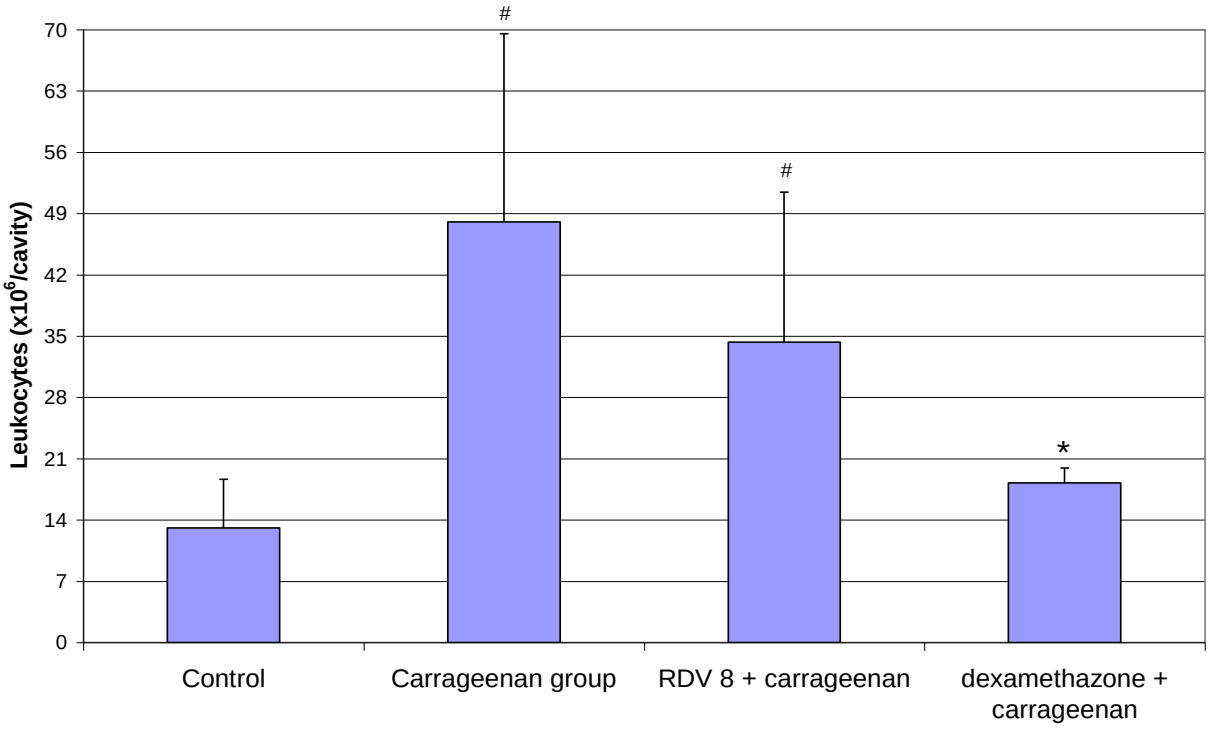


Figure 5

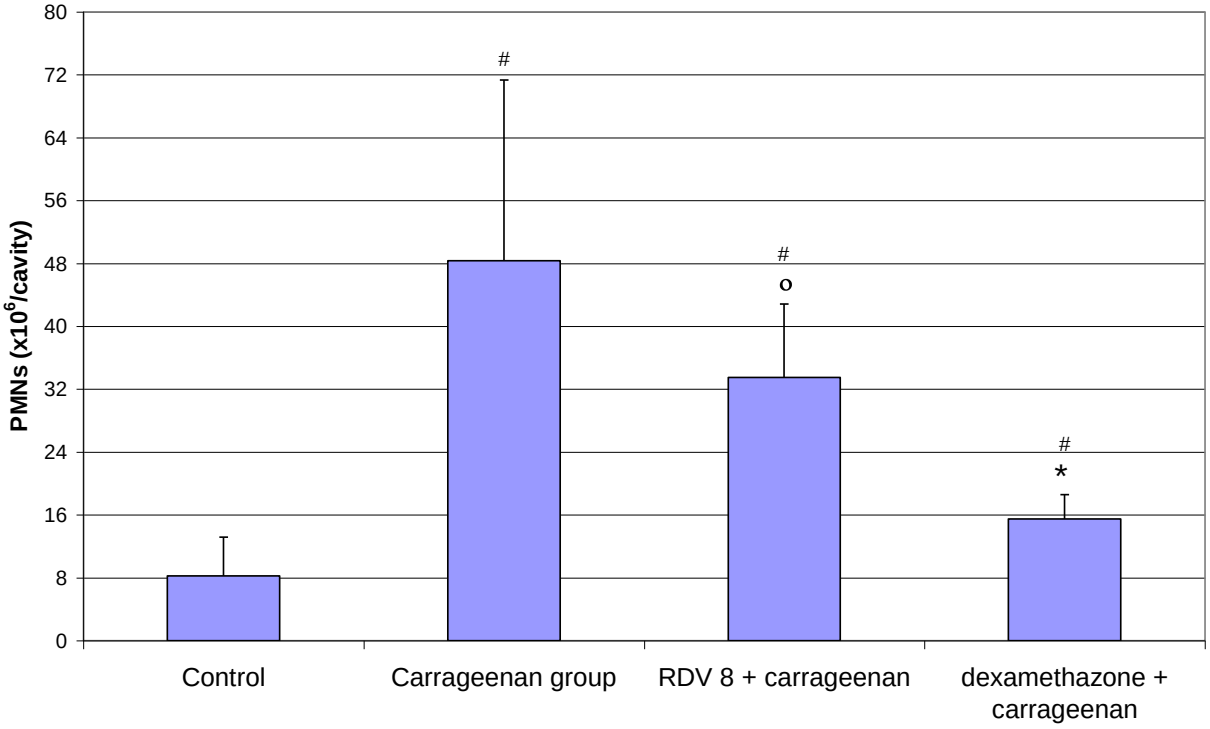
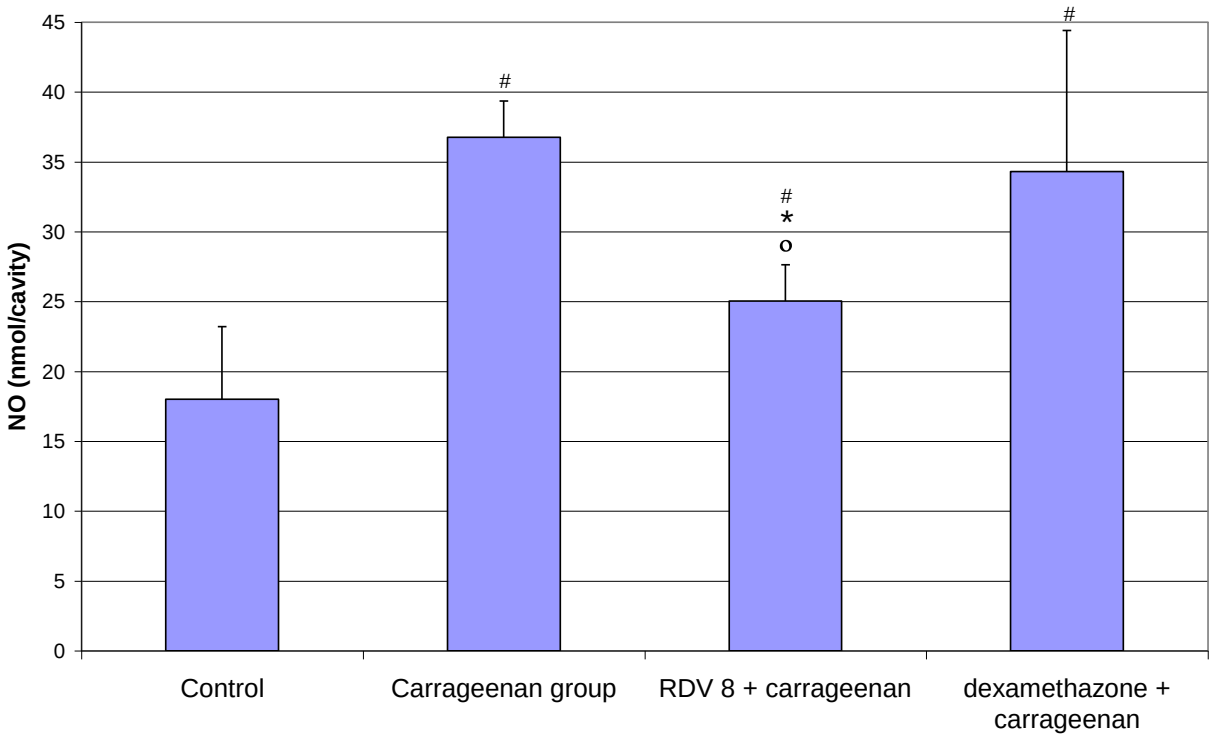


Figure 6


NO (nmol/cavity)
45
40
35
30
25
20
15
10
5
0
#
#
*
o
#
Control
Carrageenan group
RDV 8 + carrageenan
dexamethazone + carrageenan

LIST OF LEGENDS

Figure 2. Volume of exudate in the pleural cavity of control (0.2 ml of saline solution), carrageenan (0.2 ml–2 %), carrageenan + RDV 8 (3,0 mg/kg) and carrageenan + dexamethasone groups (1,0 mg/Kg). Results are expressed as means ± S.D. # $P < 0.05$ when compared with control group. * $P < 0.05$ when compared carrageenan group. o $P < 0.05$ when compared with carrageenan + dexamethasone group. n=10.

Figure 3. Protein concentration in the pleural cavity of control (0.2 ml of saline solution), carrageenan (0.2 ml–2 %) and carrageenan + RDV 8 (3,0 mg/kg) and carrageenan + dexamethasone groups (1,0 mg/Kg). Results are expressed as means ± S.D. # $P < 0.05$ when compared with control group. * $P < 0.05$ when compared carrageenan group. n=10.

Figure 4. Leukocytes totals in carrageenan-induced pleurisy in rats. Control (0.2 ml of saline solution), carrageenan (0.2 ml–2 %) and carrageenan + RDV 8 (3,0 mg/kg) and carrageenan + dexamethasone groups (1,0 mg/Kg). Results are expressed as means ± S.D. # $P < 0.05$ when compared with control group. * $P < 0.05$ when compared carrageenan group. n=10.

Figure 5. Concentration of PMNs in the pleural cavity of control (0.2 ml of saline solution), carrageenan (0.2 ml–2 %) and carrageenan + RDV 8 (3,0 mg/kg) and carrageenan + dexamethasone groups (1,0 mg/Kg). Results are expressed as means ± S.D. # $P < 0.05$ when compared with control group. o $P < 0.05$ when compared with carrageenan + dexamethasone group (1,0 mg/Kg). n=10.

Figure 6. Concentration of NO in the pleural cavity of control (0.2 ml of saline solution), carrageenan (0.2 ml–2%) and carrageenan + RDV 8 (3,0 mg/kg) and carrageenan + dexamethasone groups (1,0 mg/Kg). Results are expressed as means ± S.D. # $P < 0.05$ when compared with control group. * $P < 0.05$ when compared carrageenan group. o $P < 0.05$ when compared with carrageenan + dexamethasone group (1,0 mg/Kg). n=10.

3. ARTIGOS CIENTÍFICOS

Original Article

EVALUATION OF IMMUNOMODULATORY ACTIONS OF RDV 8 COMPOUND *IN VITRO* ON PROLIFERATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

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ABSTRACT – The objective of the present study was to investigate the potential effect of RDV 8 (1; 6-methyl-2-phenyl-4-thioxo-5-aldehyde-12-carboxylate-pyrimidine) on T-lymphocytes proliferation, since immunological alterations might contribute to the severity of inflammatory diseases, using a cell culture model of peripheral blood mononuclear cells (PBMCs). The PBMCs were isolated from the blood of healthy humans by gradient centrifugation. Phytohemagglutinin (PHA) was used for T-lymphocyte proliferation and PBMCs were plated directly with different concentrations of RDV 8 ranging from 0.0125μg/mL, 0.025μg/mL, 0.05μg/mL and 0.1μg/mL. The determination of cytokines IL-1, IL-6 and chemokines MCP-1 levels in cell culture supernates was performed (Kits ELISA). RDV 8 diminished cell proliferation, diminished the synthesis of MCP-1(Monocyte Chemotactic Protein 1**)** and increased IL-6 at concentration of 0.1μg/mL. However, the IL-1 levels and the cytotoxic effect were not significantly affected by RDV 8 treatment. This compound RDV 8 may have an immunomodulatory effect and mechanism action probably may involve cytokine modulations.

Keywords: RDV 8; cell culture; immunomodulatory; peripheral blood mononuclear cells (PBMCs); phytohemagglutinin (PHA).

INTRODUCTION

The development of an effective immune response involves lymphoid cells, inflammatory cells and hematopoietic cells. The complex interactions among these cells are mediated by a group of proteins collectively designated as cytokines to denote their role in cell-to-cell communication. Cytokines are low-molecular weight regulatory proteins or glycoproteins secreted by white blood cells and various other cells in the body in response to several stimuli. These proteins assist regulating the development of immune effector cells, and some cytokines and some are self-regulated (4).

Some cytokines are known by common names, including the interferons and tumor necrosis factors. Another subgroup of cytokines, the chemokines, has recently gained prominence. It is a group that affects chemotaxis and other aspects of leukocyte behavior. These molecules play an important role in inflammatory response (4).

The chemokines constitute a family of proteins of low molecular weight (8-14 kDa), they are important to both cellular organization of lymphoid organs under physiological conditions, and in regulating the recruitment of cells during inflammation (5). These chemokines are produced by different cell types (lymphocytes, macrophages, neutrophils, eosinophils and endothelial cells), and are present during inflammatory process. In addition, these proteins may also act in apoptosis, haematopoiesis,

angiogenesis, mitosis, tumor metastasis and secretion of inflammatory mediators, such as cytokines, free radicals and nitric oxide (6).

In the last two decades, pyrimidines derived from uracil and from oxopyrimidines have been extensively investigated for its antimicrobial and antitumor properties (7). The pyrimidines are part of the DNA and the RNA. They are associates to cytosine, timine and uracil nucleic acids.

Recent studies discovered that DNA pyrimidines analogues can be powerful antitumoural agents. These pyrimidines analogues are being used for the treatment of infection with the human immunodeficiency virus (HIV), the hepatitis B virus (HBV) and of human papiloma virus (HPV) (8, 9, 10, 11, 12).

The 4-thioxopyrimidine is part of a chemical family, whose essential structure is formed by a heterocyclic ring of pyrimidine (13). A compound, arbitrary denominated as RDV 8, a phenol group (C_6H_5) in the $C_{2,}$ was added to the ring, a sulphur atom (S) to the C_4 (and that adds the prefix thioxo to the molecule), an aldehyde (COH) was added to the C_5, and two methyl (CH_3) to N_1 and C_6. Different molecules added to C_{14} can form other compound of 4-thioxopyrimidine. On the synthetic compound, carboxylate (C_2H_5O) was added to the C_{14} (Figure 1).

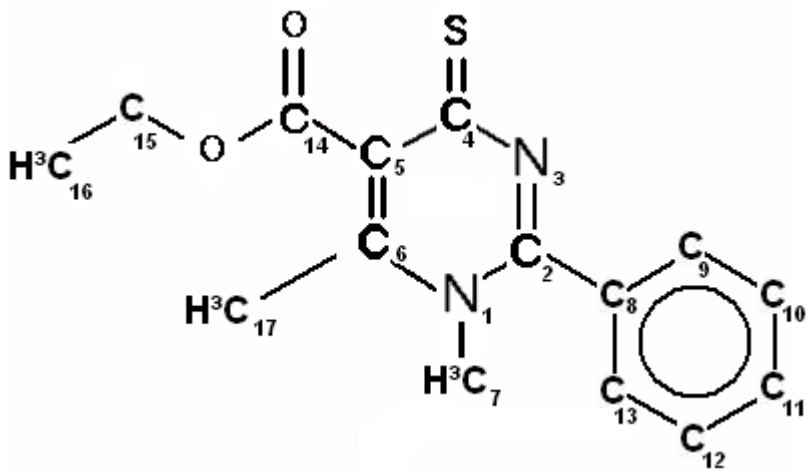

Figure 1. RDV 8: $C_{15}H_{16}N_2O_2S$ (1; 6 methyl-2-phenyl-4-thioxo-5-aldehyde-12-carboxylate-pyrimidine) (Cunha S. *et al* 2007).

Promising results of the oxipyrimidines have demonstrated that the thioxopyrimidines present important clinical effects, (14) such as antimicrobial and antitumoral. The 4-thioxopyrimidine showed satisfactory results when tested as antimicrobial in *Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Streptococcus mutans, Salmonella spp, Escherichia coli,* and *Candida albicans*. All the derivatives of 4-tioxopyrimidines showed little antitrypanocidal activity. This result suggests that the structural

modification in the 4-tioxopyrimidine may improve the property of its action against the *Trypanosome cruzi* (14).

The present study aims to investigate the potential immunomodulatory effect of RDV 8 on T-lymphocytes proliferation, using a model cell culture PBMCs.

MATERIAL AND METHODS

This study was approved by the Ethics Committee (CEUA) of PUCRS record 08/00021. Officious 054/08-CEUA.

2.1 Reagents

Medium RPMI 1640 and Phytohemagglutinin (PHA) were purchased from Invitrogen Corporation. Histopaque and Trypan blue 0.2% were obtained from Sigma. Phosphate buffered saline (PBS) was purchased from Hemgen Diagnostics. Isopropyl alcohol was obtained from Quimex. Dimethyl sulfoxide (DMSO) was purchased from Nuclear. Heparin was obtained from Cristalir. Garamicin sulfate 2.7mg/mL came from Schering-Plough (Brazil). MTT (3-[4-5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide) was obtained from Acros Organica.

2.2 Obtaining the compound of 4-Thioxopyrimidine *RDV 8*

The compound of 4-thioxopyrimidines obtained through a partnership between the laboratory of Cellular Biophysics and inflammation of the PUCRS, and the Institute of Chemistry of the Federal University of Bahia. The synthesized compound received the name of RDV 8.

2.3 Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from the blood of healthy humans (n=6) by gradient centrifugation. A total of 20mL of the heparinized blood was diluted 1:2 with RPMI 1640. This mixture was overlayed in 7mL partitions on to 3mL Histopaque and centrifuged at 800X*g* at room temperature for 20 minutes. The PBMCs, including T-lymphocytes, were harvested from the interface with a sterile transfer pipette and washed twice in the PBS. The cells were then resuspended in 3mL RPMI 1640 medium supplemented with garamicin sulfate 2,7mg/mL and 20% homologous serum at final cell density of l,6 x $l0^6$mL. Platelet contamination of these preparations was < 1%; after using trypan blue, the number of living cells should be greater than or equal to 95%.

2.4 Lymphoproliferation assay

Phytohemagglutinin (PHA) was used for T-lymphocyte proliferation. RDV 8 was dissolved in DMSO. PBMCs (1.6 x $l0^5$ cell/well) were plated directly with the concentrations of 0.0125μg/mL, 0.025μg/mL, 0.05μg/mL and 0.1μg/mL, which were cultured in the presence of mitogen (10μg/mL, PHA) in 96-wells microtiter plates (Corning Inc., Corning, NY) at 37°C in a 5% CO_2 humidified incubator for 96h.

Lymphocyte proliferation was determined by MTT assay as previously described (14). Briefly, MTT was dissolved in RPMI 1640 at 5mg/mL and added to all wells of an assay, then plates were incubated at 37°C in a 5% CO_2 humidified incubator for 4h. Isopropanol was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After 5 minutes, the plates were read on a Hyperion MicroReader reader, using a wavelength test of 540nm, at a wavelength reference of 650nm. The results are presented as optical density by means ± SD and all experiments were performed in triplicate.

2.5 Cytotoxic assay

RDV 8 was dissolved in DMSO and this solution was dissolved in RPMI 1640 and added directly to PBMCs (1,6 x $l0^5$ cell/well), which were incubated in 24-well microtiter plates (Corning Inc., Corning, NY) at 37°C in a 5% CO_2-humidified incubator. The cellular viability was performed by trypan blue dye exclusion after 96h of the incubation. The results are presented as percentage by mean ± SD and all experiments were performed in triplicate.

2.6 Dosage of cytokines and chemokines in cell culture supernates assay

The cytokines interleukin 1 (IL-1) and interleukin 6 (IL-6) and chemokines monocyte chemotactic protein 1 (MCP-1), were evaluated in the cell culture supernatantes by Biosourse Reagents (Kits ELISA).

2.7 Statistical analysis

The results were evaluated statistically by analysis of variance (one way ANOVA) with LSD *post hoc* test using SPSS (Statistical Package for the Social Sciences) 12.0 software and were expressed by means ± standard deviation (S.D.). The level of statistical significance was defined as $P < 0.05$.

RESULTS

3.1 Immunomodulatory effect of RDV 8 on PBMCs stimulated with PHA *in vitro*

We evaluated the immunomodulatory effect of compounds 4-thioxopyrimidine, RDV 8 on proliferation of PBMCs, *in vitro,* in the presence of PHA (10μg/mL). The results presented (Figure 2) show the absorbance, in control group (0.1580 ± 0.0447) in PHA (0.4020 ± 0.0773) (255%) with significant increase ($P < 0.05$) when compared with control group, in PHA + RDV 8 (0.1 μg/mL) 0.3400 ± 0.1429 (115%) showed significant increase ($P < 0.05$) when compared with control group and significant decrease (15.4%) in the cellular proliferation when compared with PHA group.

3.2 Cytotoxic effect of composite of 4-Thioxopyrimidines, RDV 8, on PBMCs

To determine the cellular viability of RDV 8 in PBMCs, we used control groups, on RDV 8 (0.1 μg/mL), on RDV 8 (0,05μL/mL), on RDV 8 (0,025μL/mL) and on RDV 8 (0,0125μL/mL). (Figure 3).

3.3 Concentration of Monocyte chemotactic protein 1 in supernates on the PBMCs

We evaluated the liberation of the MCP-1/CCL2 on supernatant on the PBMCs, *in vitro*. The results shown 4963.33 ± 1091.37 on control group, 5360.00 ± 1492.92 on PHA group, and 2582.50 ± 1122.15 on RDV 8 (0.1μg/mL) the decreased (47.97%) significantly when compared with control group and also diminished (51.82%) when compared with PHA group (Figure 4).

3.4 Concentration of interleukin 1 on supernatant on the PBMCs

We evaluated the liberation of the IL-1 on supernatants on the PBMCs, *in vitro*. The results showed no variation on control group, on PHA group, on PHA + RDV 8 (0.1μg/mL) and on RDV 8 (0.1μg/mL). (Figure 5).

3.5 Concentration of interleukin 6 on supernatant on the PBMCs

We evaluated the liberation of the IL-6 on supernatants in PBMCs, *in vitro*. The results showed 1088.67 ± 41.14 pg/mL on control group, 1259.33 ± 140.54 pg/mL on PHA group, 1230.00 ± 57.26 pg/mL on PHA + RDV 8 (0.1μg/mL) and 1295.00 ± 139.90 pg/mL on RDV 8 (0.1μg/mL). All treatment significantly increased (15.68%, 12.98% and 18.95% respectively) the IL-6 levels when compared with control group (Figure 6).

DISCUSSION

The objective of this work was to investigate the potential effect of RDV 8 on T-lymphocytes proliferation using a cell culture model PBMCs, since immunological alterations might contribute to the severity of inflammatory diseases. Lymphocytes are the main causes of immune response of the adaptive cells. The first step towards their activation lies in the interaction of receivers present in cell surface with a simulative agent. The cellular events that occur after this activation are called collectively blast process (15).

The RDV 8 is a compound of 4-thioxipyrimidines, composed by two main components: a pyrimidic ring and an atom of sulfur in C_4. On RDV 8 there is an added a carboxylate in C_{12}. In relation to pyrimidine, the synthesis of other similar compounds has received a larger importance in the last 30 years due to its biological relevance, once these molecules are intimately linked to the structure of the nucleic acids (16). Besides the antineoplastic activity previously described (17-21), many biological properties were attributed to this class of compound such as antiviral (22, 23), anti-hypertensive (24, 25), hypoglycemic (26, 27, 28), anti-histaminic (29) and anti-inflammatory (30, 31). In recent researches, derivatives of pyrimidines and similar substances containing a carbonyl group in place of amino group present in C_4 heterocyclic ring would incorporate pharmacological potential (16).

This study demonstrates that the RDV 8 compound has an immunomodulatory effect at concentration of 0.1 μg/L (figure 2). To determine whether the inhibitory effect on lymphoproliferation was due to cellular death, the cellular viability of RDV 8 in PBMCs was investigated. As shown on figure 3, the compound demonstrated no toxicity in concentrations used.

To further investigate the mechanism responsible by the inhibitory effect on T-lymphocyte, the effect of RDV 8 on IL-1, IL-6 and MCP-1 production was investigated.

Evidence suggests massive inflammatory reactions resulting from systemic cytokine release and this is a common pathway underlying inflammation. Tumor necrosis factor-alfa (TNF-α), IL-1 and IL-6 are the three first cytokine involved in its pathogenesis (32). The maintenance of leukocyte recruitment during inflammation requires intercellular communication between infiltrated leucocytes and the endothelium. These events are mediated by the generation of early response cytokines, e.g., IL-1, IL-6, the expression of cell-surface adhesion molecules and the production of chemotactic molecules, such as chemokines.

The compound RDV 8 did not inhibit the increase caused by PHA in IL-6 test, moreover caused an increase when in contact with the cells, when compared to the control group. The RDV 8 compound has an immunomodulatory effect and, possibly, antinflammatory effect. However, the increasing of IL-6, is not clear, being necessary that more studies to elucidate this event.

In our study, the production of IL-1 by PBMCs was not affected by PHA or the RDV 8 compound action. This result shows that the immunomodulating action of RDV 8 is not related to the release of IL-1.

The chemokines are responsible by the addition of chemotaxis, mainly to macrophages and lymphocytes. In this family only the MCP-1 is responsible for that, it has also effects on neutrophils (33), NK cells, basophils, eosinophils, and hepatic stellate cells (34-36). On the analysis of the concentration of MCP-1 on supernatants (Figure 4), we observed that both, PHA and PHA+RDV 8, did not increase the production of this chemokine, but the RDV 8 compound decreased its MCP-1 production, when only the cells were incubated. These results showed that the RDV 8 compound is an immunomodulatory agent and its mechanisms of action involve modulation of MCP-1. This result has a therapeutic interest. Intratracheal instillation of MCP-1 in lungs of mice was recently shown to cause increased alveolar monocyte accumulation, in absence of lung inflammation. Besides, it was found that after endotoxin challenge in baboons, there is an increase in TNF-α at 2 h postchallenge, which is followed at 4 h with a peak in MCP-1 levels. Administration of exogenous MCP-1 protects mice from a lethal challenge of bacteria or endotoxin. MCP-1 seems to shift the balance in favor of anti-inflammatory cytokines, with an increase in IL-10 and decrease in IL-12 (37).

In conclusion, the results reported here suggest that the immunomodulatory effect of RDV 8 and these results can be correlated with the protection against inflammatory disease. However, many questions regarding molecular and cellular mechanisms remain unanswered.

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LIST OF FIGURE

Figure 2

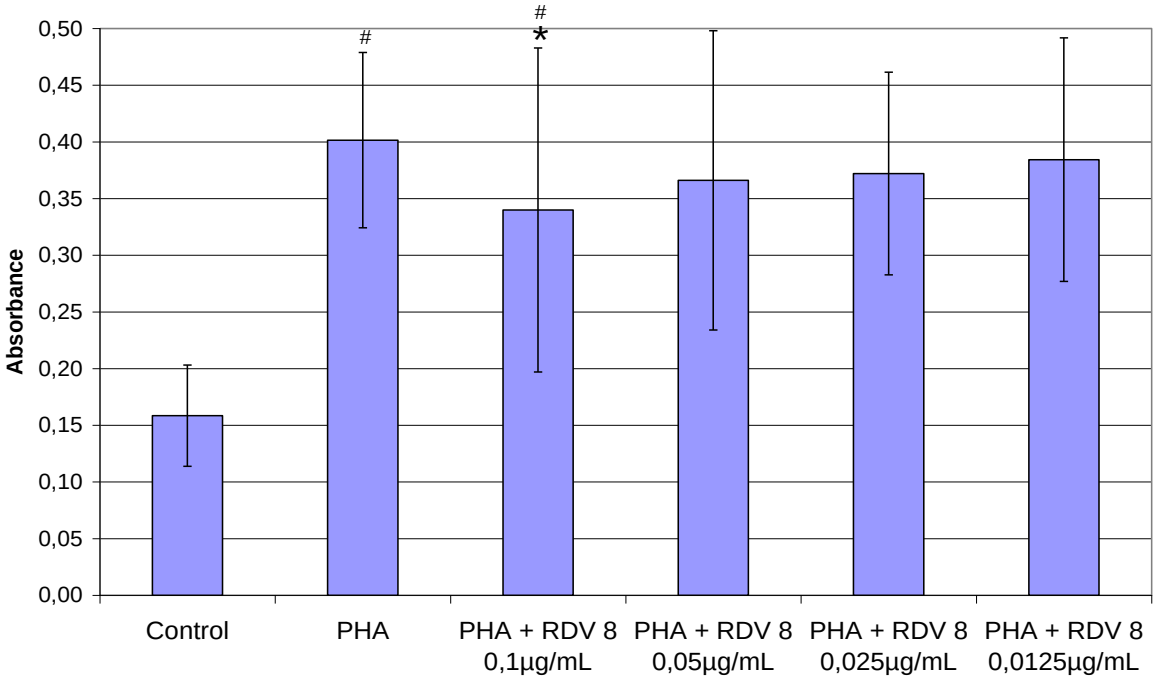
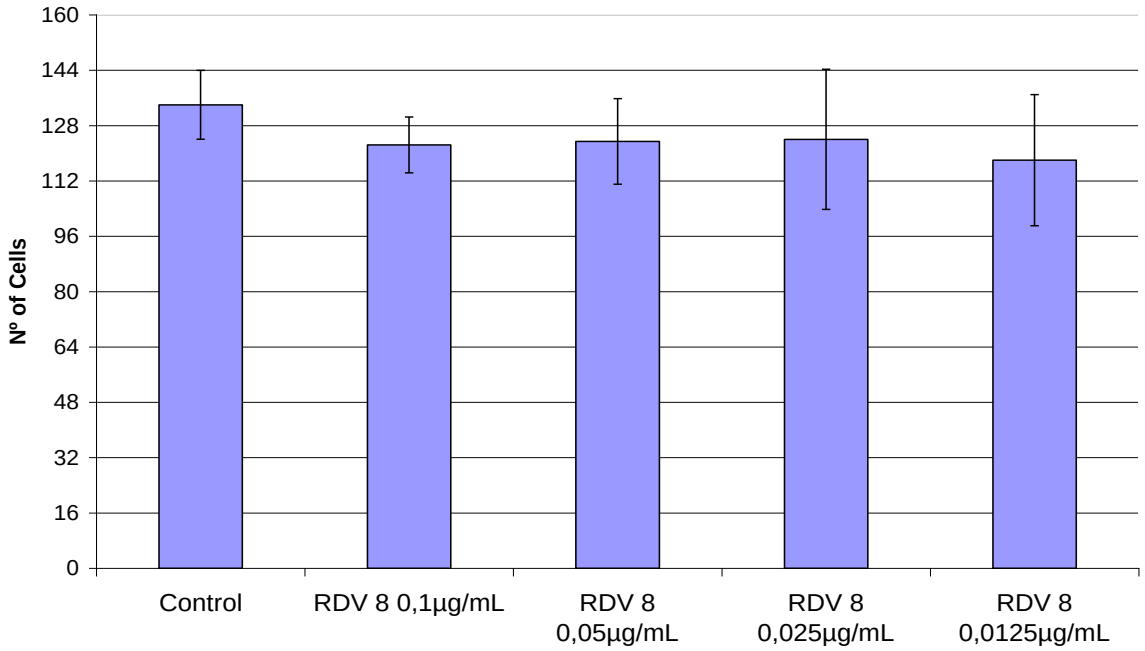


Figure 3



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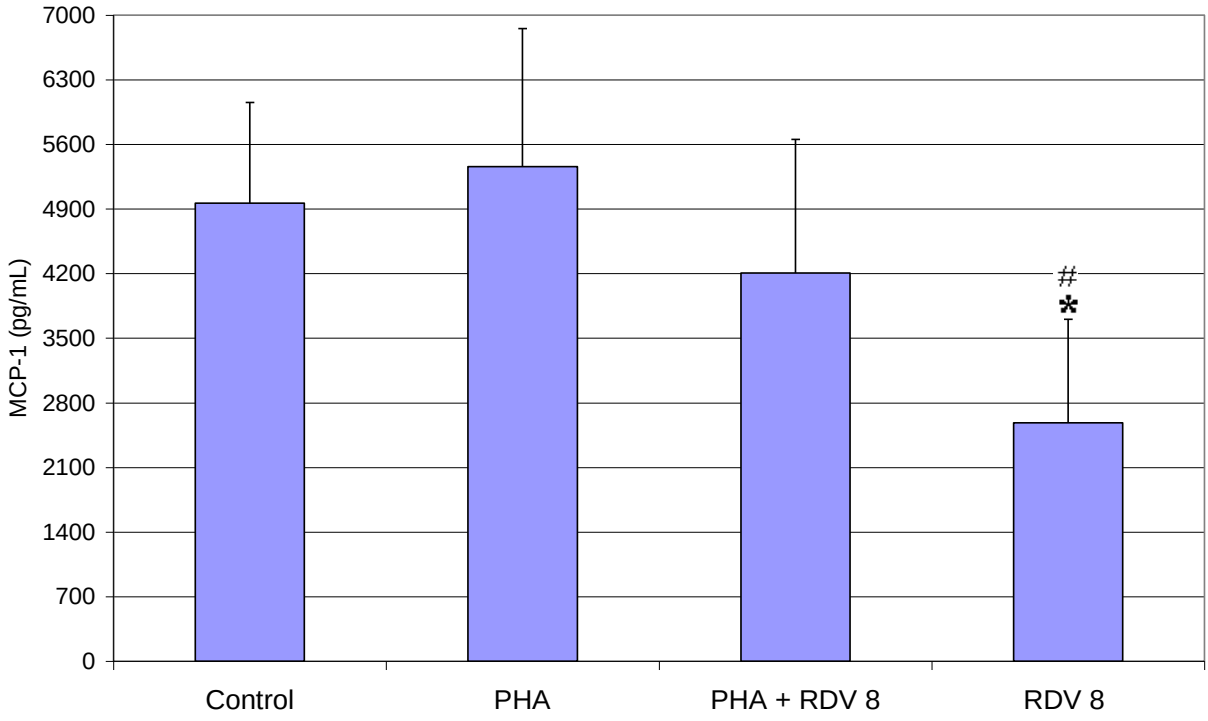

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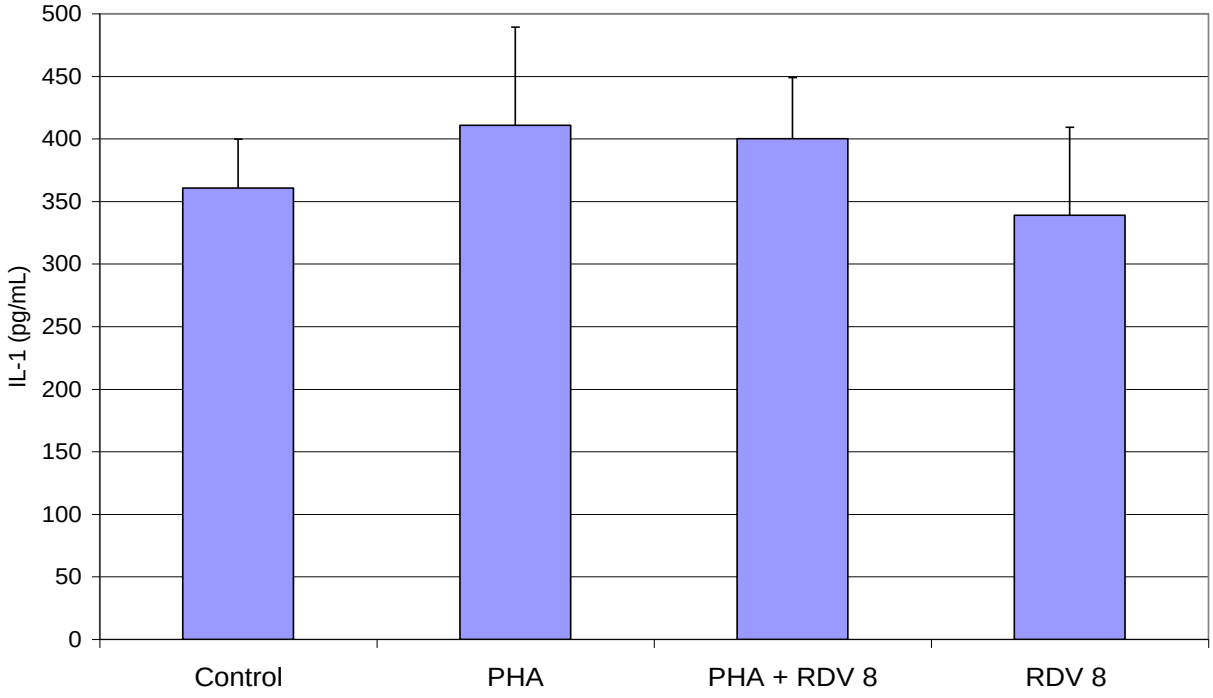
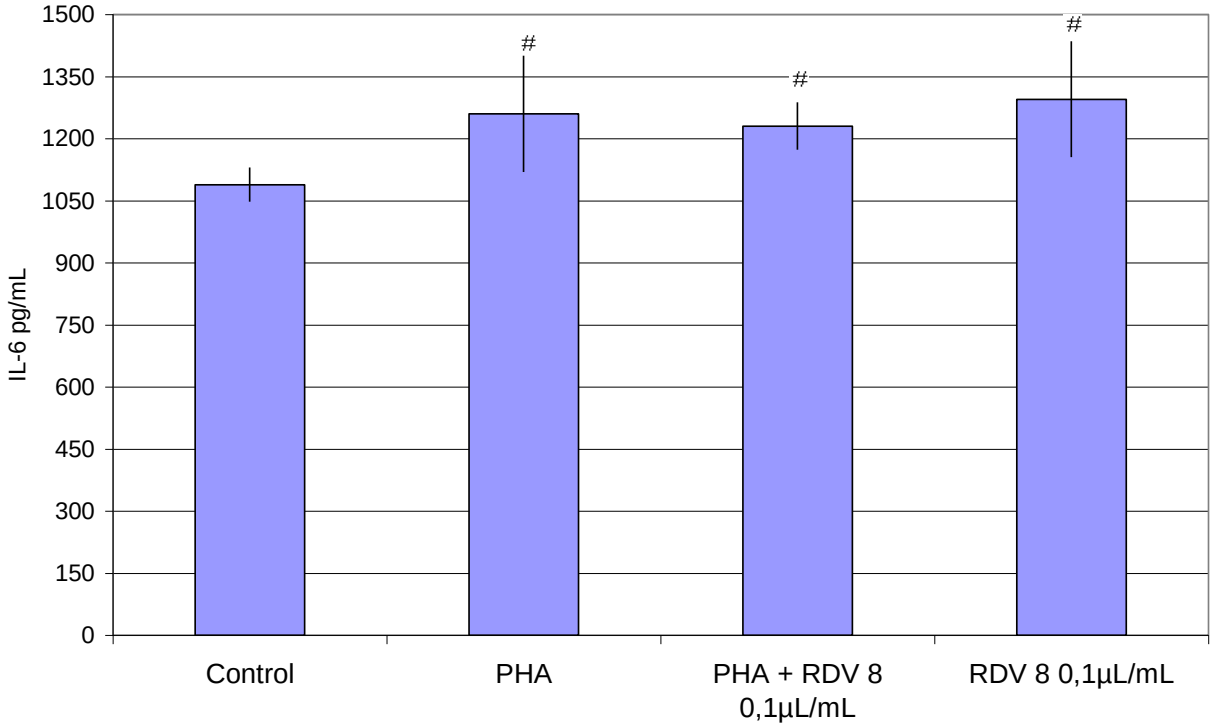


Figure 6


1500
1350
1200
1050
900
750
600
450
300
150
0
IL-6 pg/mL
#
#
#
Control
PHA
PHA + RDV 8 0,1µL/mL
RDV 8 0,1µL/mL

LIST OF LEGEND

Figure 2. Immunomodulatory effect, in control (100μL cells + 100μL RPMI 1640), PHA (100μL cells + 50μL PHA +50μL RMI 1640) and PHA + RDV 8 (100μL cells + 50μL PHA + 50μL RDV 8 {in 4 different concentrations, 0,4μL/mL; 0,2μL/mL; 0,1μL/mL; 0,05μL/mL}). Results are expressed as means ± S.D. # $P < 0.05$ when compared with control group. * $P < 0.05$ when compared PHA group.

Figure 3. Curve of cytotoxic, in control (100μL cells + 100μL RPMI 1640) and RDV 8 (100μL cells + 100μL RDV 8 {in 4 different concentrations, 0,2μL/ml; 0,1μL/mL; 0,05μL/mL; 0,025μL/mL}). Results are expressed as means ± S.D. $P < 0.05$.

Figure 4. Concentration of MCP-1 in supernatants from PBMC, in control (100μL cells + 100μL RPMI 1640), PHA (100μL cells + 50μL PHA +50μL RMI 1640), PHA + RDV 8 (100μL cells + 50μL PHA + 50μL RDV 8 {0,4μL/mL}) and RDV 8 (100μL cells + 100μL RDV 8 {0,2 μL/mL}). Results are expressed as means ± S.D. # $P < 0.05$ when compared with control group. * $P < 0.05$ when compared PHA group.

Figure 5. Concentration of IL-1 1 in supernatants from PBMC, in control (100μL cells + 100μL RPMI 1640), PHA (100μL cells + 50μL PHA +50μL RMI 1640), PHA + RDV 8 (100μL cells + 50μL PHA + 50μL RDV 8 {0,4μL/ml}) and RDV 8 (100μL cells + 100μL RDV 8 {0,2 μL/ml}). Results are expressed as means ± S.D. $P < 0.05$.

Figure 6. Concentration of IL-6 1 in supernatants from PBMC, in control (100μL cells + 100μL RPMI 1640), PHA (100μL cells + 50μL PHA +50μL RMI 1640), PHA + RDV 8 (100μL cells + 50μL PHA + 50μL RDV 8 {0,4μL/ml}) and RDV 8 (100μL cells + 100μL RDV 8 {0,2 μL/ml}). Results are expressed as means ± S.D. $P < 0.05$.

4. CONSIDERAÇÕES FINAIS

As publicações científicas acerca dos análogos de pirimidinas indicam sua relevância farmacológica. A maioria desses estudos revela as propriedades biológicas e farmacológicas desses análogos, porém alguns desses compostos como os da família das 4-tioxopirimidinas (entre eles o RDV 8) possuem uma escassa literatura, o que dificulta e justifica maiores estudos na área.

A inflamação é uma resposta inespecífica do corpo as diferentes formas de agressão tecidual, sendo um mecanismo protetor essencial para o inicio do processo de reparo.

Intervenções farmacológicas, incluindo drogas antiinflamatórias não esteróides (NSAID) e injeções de esteróides, são terapias comumente usadas para aliviar a dor e bloquear a inflamação em um curto espaço de tempo. O pequeno tempo de ação destas drogas, os mecanismos de ação biológica e as ótimas doses são freqüentemente investigados em estudos *in vivo* de modelos animais e *in vitro* cultura de células.

O modelo de pleurisia é um dos mais utilizados para estes propósitos pela facilidade de coletar o exsudato inflamatório e assim avaliar os mediadores e os mecanismos biológicos de ação de terapias antiinflamatórias. Já o modelo de culturas celulares, principalmente humanas, é amplamente utilizado por ser um meio não invasivo, mais econômico e apresenta resultados extremamente importantes para avaliação da reação celular humana em contato com o composto ou fármaco utilizado na pesquisa. Combinando os dois modelos obtém-se um ótimo mecanismo para avaliar as ações de novas drogas com ação antiinflamatória e imunomoduladora.

No estudo *in vivo,* onde se usou o modelo de pleurisia induzida por carragenina para avaliar a ação antiinflamatória, observou-se que o composto RDV 8 provocou diminuição do liquido pleural (exsudado), da concentração de proteínas, da contagem de leucócitos totais e da concentração de óxido nítrico. Todos esses parâmetros apresentaram diferenças significativas em relação ao grupo carragenina (controle positivo). Esses resultados confirmam a ação antiinflamatória do RDV 8, pois o óxido nítrico desempenha um papel importante na resposta inflamatória, pois é um potente vasodilatador e regula o recrutamento de leucócitos (KUMAR et al., 2005). A diminuição da produção de NO promove a diminuição da vasodilatação provocada pela carragenina, com conseqüente diminuição do edema. Também reduz a migração leucocitária

para o sítio de inflamação. Esses parâmetros evidenciam que o RDV 8 pode ser utilizado como antiinflamatório.

Ao compararmos com a ação do composto em estudo com a dexametazona, verificamos que o esteróide sintético apresentou uma ação antiinflamatória mais potente, entretanto, o composto RDV 8 foi mais eficiente em reduzir a liberação de NO, sugerindo que a inibição da síntese deste radical livre de nitrogênio pode estar envolvido no mecanismo de ação da droga em estudo. Novos estudos envolvendo a expressão da enzima óxido nítrico sintase induzível devem ser feitos para elucidar este provável mecanismo.

Já no estudo *in vitro* utilizou-se o modelo de cultura de células mononucleares de sangue periférico (PBMCs) humano para avaliar a capacidade imunomoduladora do RDV 8. Observou-se uma inibição significativa na proliferação linfocitária, ativada pela fitohemaglutinina (PHA), quando o RDV 8 foi usado na concentração de 0.1μL/mL. Nesta concentração o composto não apresentou nenhuma toxicidade. Tendo em vista que os linfócitos são as principais células responsáveis pela resposta imune através de síntese e secreção de citocinas e quimioquinas, procurou-se avaliar o seu efeito sobre a liberação da IL-1, Il-6 e MCP-1. Nosso estudo mostra que o composto possivelmente tenha seu mecanismo imunomodulador e antinflamatório relacionado com a inibição da MCP-1, já que houve uma diminuição significativa desta citoquina no sobrenadante da cultura de linfócitos. Este resultado apresenta grande interesse terapêutico, já que estudos recentes mostram que quando se instila MCP-1 em pulmão de camundongos, temos um grande acúmulo de monócitos no alvéolo, sem haver sinais de inflamação. Também foi verificado que após a injeção de LPS (lipopolissacarídeo) intratraqueal em camundongos há um aumento de TNF α em aproximadamente 2 horas e em 4 horas um aumento de MCP-1. Neste mesmo estudo foi verificado que a admnistração exógena de MCP-1 protegeu os camundongos da morte provocada pela endoxina, levando a crer que possa ter uma importante ação antiinflamatória Neste contexto, esta citoquina parece ter um papel fundamental no processo inflamatório.

Nosso estudo verificou que a síntese de IL-6 foi aumentada quando o composto RDV 8 foi colocada em contato com as células, sem a presença de PHA, mostrando que possui ação sobre a síntese de interleucinas. Acreditamos que este efeito também pode estar envolvido no mecanismo de ação, porém mais estudos serão necessários para poder investigar o real mecanismo de ação do RDV 8.

A combinação dos estudos *in vivo* e *in vitro* nos permitem concluir que o composto pirimidínico RDV 8 é um potencial agente imunossupressor e antiinflamatório.

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ANEXO 1

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Há um aumento crescente do número de casos de sepse em diversos países. Este aumento é acompanhado por maiores taxas de mortalidade, aumento do tempo de internação e conseqüentemente aumento dos custos associados ao manejo destes pacientes. No nosso estudo propõe-se avaliar a ação das 4-tioxopirimidinas na sepse de origem bacteriana, alem de possuir propriedades antivirais, antimicrobianas e antitumorais. A busca de novos medicamentos para o tratamento de sepse torna-se de extrema importância, visto que o índice de mortalidade e morbidade dessa patologia continua aumentando, mesmo com os avanços de medicamentos nessa área. Verificaremos o efeito dos compostos de 4-tioxopirimidinas sobre a citotoxidade proliferação celular *in vitro.* Este estudo intitulado "**Determinação da Citotoxicidade e da Capacidade Linfoproliferativa dos compostos de 4-tioxopirimidnas sobre Células Mononucleares de Sangue Periférico Humano**", tem por objetivo observar o efeito dos compostos de 4-tioxopirimidinas sobre células de defesa (linfócitos) do organismo humano. Para isso, será coletado 20 mL de sangue por punção venosa dos doadores. Os resultados desta pesquisa trarão avanços acerca do conhecimento da ação citotóxica das 4-tioxopirimidinas, e contribuirá para um melhora auxilio às vítimas.

Eu.....,RG: ....., fui informado dos objetivos desta pesquisa. Recebi informações acerca dos procedimentos a serem realizados e minhas dúvidas foram esclarecidas. Sei que meu nome será mantido em sigilo. Poderei obter resultados da pesquisa se julgar necessário, bem como solicitar a exclusão dos meus dados no momento que desejar. Estou ciente de que na punção venosa pode ocorrer desconforto e, eventualmente, um pouco de dor no local. Fui informado de que caso existirem danos a minha saúde, causados diretamente pela pesquisa, terei direito ao tratamento médico, conforme estabelece a lei, sem nenhum tipo de ônus.

Caso houver perguntas sobre o estudo, posso entrar em contato com o pós-graduando Marcos Schuch de Azambuja no fone (51) 99690329. Para qualquer pergunta sobre os meus direitos como participante deste estudo ou se penso que fui prejudicado pela minha participação, posso entrar em contato com o orientador do projeto Prof. Dr. Jarbas R. de Oliveira, pelo telefone (51) 33203500 ramal 4147.

Declaro que recebi cópia do presente TERMO DE CONSENTIMENTO.

_____ _____ _____

Assinatura do paciente Nome Data

_____ _____

Assinatura do pesquisador Data

Este formulário foi lido para _____

_____, em ____/____/____ por _____, enquanto eu estava presente.

_____ _____ _____

Assinatura do testemunha *Nome* *Data*

ANEXO 2

Aprovação do comitê de ética

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA PARA O USO DE ANIMAIS

Ofício 054/08-CEUA

Porto Alegre, 14 de agosto de 2008.

Senhor Pesquisador:

O Comitê de Ética para o Uso de Animais apreciou e aprovou seu protocolo de pesquisa, registro CEUA 08/00021, intitulado: **"Avaliação da ação citotóxica, imunomoduladora e antimicrobiana dos compostos de 4-tioxopirimidinas".**

Sua investigação está autorizada a partir da presente data.

Relatórios do andamento do projeto devem ser entregues a este Comitê.

Atenciosamente,

Profª. Dr. Anamaria Feijó
Coordenadora do CEUA - PUCRS

Ilmo. Sr.
Prof. Dr. Jarbas Rodrigues de Oliveira
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ANEXO 3

Confirmação da submissão do artigo.

EVALUATION EFFECT ANTI-INFLAMMATORY OF RDV 8 IN A RAT PLEURISY MODEL

Editorial Manager(tm) for Journal of Pharmacy and Pharmacology
Manuscript Draft
Manuscript Number:
Title: EVALUATION EFFECT ANTI-INFLAMMATORY OF RDV 8 IN A RAT PLEURISY MODEL
Article Type: Research Paper
Keywords: RDV 8; Inflammation; pleurisy; carrageenan
Corresponding Author: Marcos Schuch Azambuja, Masters student
Corresponding Author's Institution: Pontifícia Universidade Católoca do Rio Grande do Sul
First Author: Marcos Schuch Azambuja, Masters student
Order of Authors: Jarbas R de Oliveira, PhD; Robson H Amaral, Student; Denizar Alberto S Melo, PhD

ANEXO 4

Confirmação da submissão do artigo.

EVALUATION OF IMMUNOMODULATORY ACTIONS OF RDV 8 COMPOUND *IN VITRO*, ON PROLIFERATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

Elsevier Editorial System(tm) for Cellular Immunology

Manuscript Draft

Manuscript Number:

Title:

EVALUATION OF IMMUNOMODULATORY ACTIONS OF OF RDV 8 COMPOUND IN VITRO, ON PROLIFERATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

Article Type: Regular Article

Keywords: RDV 8; cell culture; immunomodulatory; peripheral blood mononuclear cells (PBMCs); phytohemagglutinin (PHA).

Corresponding Author: Azambuja M. S. Marcos Schuch Azambuja, Master student

Corresponding Author's Institution: PUCRS - Pontifícia Universidade Católica do Rio Grande do Sul

First Author: Marcos Schuch Azambuja, Master student

Order of Authors: Vinicius L Costa, Student; Eduardo Caberlon, Master Student; Denizar Alberto S Melo, Doctor and Teacher; Jarbas R Oliveira, Pos Doctor and Teacher.