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Dissertação apresentada ao Programa de Pós-Graduação em Microbiologia da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Mestre.

Orientador: Prof. Carlos Nozawa

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Londrina, 24 de fevereiro de 2006.

DEDICATÓRIA

Para meus pais, Sinésio e Maria, pelos ensinamentos da vida e pelo amor que sentem por mim.

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“Deus nos fez perfeitos e não escolhe os capacitados, capacita os escolhidos. Fazer ou não fazer algo só depende de nossa vontade e perseverança” (Albert Einsten)

SUMÁRIO

1 INTRODUÇÃO

As doenças virais acompanham o homem, provavelmente, desde a formação dos primeiros assentamentos. Provavelmente, o primeiro caso conhecido de infecção viral foi o da varíola, relatado em 2000 a.C., na China e leste da Ásia, sendo responsável pela morte de milhões de pessoas antes de ser erradicada, contemporaneamente, no final da década de 70 (HENDERSON, 1987).

Doenças virais como a varíola e a poliomielite estão erradicadas ou controladas pelo homem, no entanto, para várias outras infecções virais, as vacinas ainda são indisponíveis. Esta situação propicia a ocorrência de viroses na população humana, de grande importância na saúde pública. Cerca de 40 milhões de pessoas estão infectadas pelo vírus da imunodeficiência humana (HIV), 170 milhões estão infectadas pelo vírus da hepatite C (HCV), 300 milhões são portadoras do vírus da hepatite B (HBV), 25-35% da população sexualmente ativa do ocidente estão infectadas com herpes simplex genital (HSV-2) (UNAIDS/WHO 2003; JONES, 1998; LAUER & WALKER, 2001).

WHITLEY & ALFORD, 1978 descreveram: “a quimioterapia antiviral permanece sendo o maior desafio da ciência médica”. Passado quase 28 anos, houve um considerado avanço na pesquisa de antivirais, principalmente com a modernização das técnicas de cultivo de vírus *in vitro.* Apesar deste avanço, as pesquisas ainda esbarram em uma característica fundamental dos vírus, são parasitas intracelulares obrigatórios, assim dificultam o desenvolvimento de compostos que atuem de forma específica na replicação viral, sem serem nocivos à célula.

Nos últimos 50 anos, portanto, tem havido um grande empenho na pesquisa e desenvolvimento de compostos antivirais, inicialmente a grande maioria sintéticos, porém hoje, os compostos isolados de plantas também têm sido explorados.

O primeiro composto antiviral usado foi a tiosemicarbazona, um antimicrobiano usado para tratamento da tuberculose, com atividade contra o vírus vacinia (HAMRE *et al*., 1950; BROWNLEE & HAMRE, 1951; THOMPSON *et al.,* 1951). A partir de 1960, a metisazona, derivado da tiosemicarbazona, passou a ser usada na profilaxia e tratamento da varíola em humanos (JONES, 1998). Desde então, as pesquisas se concentraram no desenvolvimento de medicamentos efetivos contra as doenças virais, porém, inócuos ou de baixa toxicidade para o hospedeiro (GALASSO, 1998).

A idoxiuridina, um análogo de nucleotídeo, foi testada contra o HSV por HERMANN (1961) com excelentes resultados *in vitro*, tendo sido demonstrado razoável seletividade. KAUFMAN *et al.* (1962) demonstraram que a idoxiuridina foi eficiente no tratamento da ceratite herpética em humanos, mas devido a sua toxicidade, só foi liberada para uso tópico (WEBER & CINATL, 1996). O primeiro análogo nucleosídico liberado para uso sistêmico foi a vidarabina, que atua na infecção do HSV e varicela-zoster (SCHABEL, 1968; BUCHANAN & HESS, 1985).

A partir da década de 80, segundo WIGG (2002), devido ao elevado custo das pesquisas com agentes antivirais, os pesquisadores concentraram seus esforços nas viroses de maior importância epidemiológica, tais como, viroses respiratórias, doenças causadas por herpesvírus e a síndrome da imunodeficiência adquirida (AIDS).

Atualmente, existem mais de 40 compostos liberados para uso clínico e muitas drogas foram aprovadas nos últimos cinco anos. Pelo menos metade destas drogas é usada para tratamento de infecção do HIV (zalcitabina, amprinavir, zidovudina, didanosina, neviparina, ritonavir, indinavir, estavudina, abacavir, lamivudina, emtricitabina, neviparina, disoproxil tenofovir, delavirdina, efavirenz, saquinavir, nelfinavir, atazanavir, lopinavir e eufuvirtide). As outras, são usadas no tratamento de infecções do HBV (dipivoxil adenofir, emtricitabina e lamivudina), do HSV (aciclovir, valaciclovir, penciclovir, famciclovir, trifluridina, brivudina, e idoxuridina) do citomegalovírus (CMV) (ganciclovir, valganciclovir, foscarnet, formivirsen e cidofovir), do influenza (amantadina, rimantadina, zanamivir, oseltamivir e ribavirina) (DE CLERQ, 2004).

Os mecanismos de ação destes antivirais referem-se à inibição dos processos iniciais da replicação viral (ex., amantadina), inibição de proteases virais (ex., indinavir) e inibição da enzima transcriptase reversa (ex., zidovudina) (DE CLERQ, 2004).

Apesar do grande número de compostos antivirais disponíveis, os efeitos tóxicos ainda continuam sendo uma barreira em muitos tratamentos. O aciclovir e ganciclovir podem apresentar neurotoxicidade e pacientes tratados com ganciclovir devem ter sua função renal monitorada (EMST & FRANEY, 1998; WAUGH *et al.,* 2002). Além disso, o uso contínuo de alguns compostos tem favorecido a seleção de mutantes resistentes, como por exemplo, as drogas usadas no tratamento de infecções por herpesvírus e pelo HBV. (KIMBERLIN & WHITLEY, 1995; WEBER & CINATL, 1996; ZOULIM, 2001).

Com o intuito de encontrar novas classes de antivirais que apresentem uma menor toxicidade e que atuem de maneira alternativa aos antivirais disponíveis, diversos extratos de plantas têm sido testados em todo o mundo.

De acordo com VLIETINCK & VANDER-BERGHE (1991) as espécies de plantas a serem testadas podem ser selecionadas de acordo com três metodologias: (1) seleção baseada em dados etnofarmacológicos, (2) baseadas em dados da literatura ou (3) dados quimotaxonômicos e por final, aleatoriamente. Comparações destes métodos têm mostrado que o método baseado em dados etnofarmacológicos é 25% mais eficiente. Na maioria dos casos as plantas são selecionadas com base na combinação destas informações.

O primeiro trabalho com extratos de plantas foi realizado na Inglaterra, no qual avaliou-se a eficácia de 228 extratos contra o vírus influenza A, sendo que destas 12 foram ativas (CHANTRILL *et al.,* 1952). Na década de 70, pesquisadores canadenses relataram que algumas frutas e seus respectivos sucos apresentavam atividade contra o HSV, poliovírus 1, coxsackievírus B5 (Cox) e echovírus 7 (KONOWALCHUK & SPEIR, 1976a, 1976b, 1978a, 1978b).

YIP *et al.* (1991) estudaram o extrato etanólico de 31 espécies de planta medicinais utilizadas na província de Yanan, na China, dos quais 16 foram ativas contra CMV murino e vírus Sindbis.

McCUTCHEON *et al.* (1995) avaliaram a atividade de 100 plantas tradicionais da Columbia Britânica (Canadá) dentro as quais se destacaram *Amelanchier alnifolia*, *Rosa mutkana* e *Sambucus reacemosa*, as duas primeiras apresentaram atividade contra o coronavírus e a última contra o vírus respiratório sincicial (RSV).

TAYLOR *et al.* (1996) estudaram plantas medicinais do Nepal, na proteção contra as infecções pelos HSV, Sindbis e poliovírus, sendo que as espécies *Hypericum*, *Lygodium* e *Maesa* apresentaram expressiva atividade.

MEYER *et al.* (1997) demonstram a atividade antiviral de um composto isolado de brotos de *Helichrysum aureonitens*, contra o HSV-1, Cox B, adenovirus (Ad) e reovirus (Reo), tendo sido ativo contra o HSV-1 e Cox, mas ineficaz contra os demais.

Plantas historicamente utilizadas pela população aborígine australiana foram testadas contra CMV humano, vírus Ross River e poliovírus-1. Duas espécies apresentaram atividade contra poliovírus, duas contra o vírus Ross River e duas contra o CMV humano (SEMPLE *et al.,* 1998*)*.

Estudando extratos de *Geranium sanguineum*, planta medicinal da Bulgária, SERKEDJIEVA & IVANCHEVA (1999) demonstraram que as partes aéreas e raízes eram eficazes contra o HSV-1. YOOSOOK, *et al.* (1999) estudaram plantas nativas da Tailândia e atestaram a sua atividade contra o HSV-2.

BARRIO & PARRA (2000) avaliaram a atividade do extrato aquoso de *Phyllanthus orbicularis* contra HSV-2, herpes vírus bovino tipo 1 (BHV-1), Ad tipo 5 e mengovirus, sendo que o extrato apresentou atividade contra o HSV-2 e BHV-1. Os autores sugeriram a atuação do extrato diretamente na partícula viral ou na fase de penetração.

SCHMITT *et al.* (2001) estudaram algumas espécies do gênero *Hypericum* e verificaram atividade contra o vírus da imunodeficiência felina, pela inibição do efeito citopático e diminuição da quantidade de ácido nucléico, detectado por RT-PCR, no sobrenadante celular.

ESQUENAZI *et al.* (2002) estudaram o extrato de *Cocos nucifera* L. (Palmae), uma espécie da região nordeste do Brasil, normalmente utilizada na medicina popular contra diarréia e artrite, e demonstraram atividade contra o HSV-1 resistente ao aciclovir.

Dois compostos da *Stylogne cauliflora* foram testados contra o HCV e apresentaram efeito inibidor sobre a protease NS3 do mesmo (HEGDE *et al.,* 2003).

OOI *et al.* (2004) avaliaram a atividade dos extratos etanólico e aquoso da *Youngia japonica* (L.) contra o RSV e influenza A, sendo que para o RSV não houve atividade, mas, para o influenza A, ambos os extratos foram capazes de reduzir o título viral em mais de 50%.

GEKKER *et al.* (2005) demonstraram que a própolis apresentou atividade contra o HIV, inibindo 85% e 98% da expressão viral, em células CD_4^+ e culturas de células microgliais, respectivamente.

POLIOVÍRUS

Os poliovírus são membros do gênero *Enterovirus* da família *Piconarviridae*, cujo vírion apresenta simetria icosaédrica (26 a 30 nm de diâmetro), sem envelope e o seu genoma é pequeno (do latim, pico significa pequeno), constituído de RNA de fita simples de polaridade positiva. Os poliovírus são considerados modelos da família *Piconarviridae*, a qual também pertence os gêneros: *Rhinovirus, Hepatovirus, Cardiovirus* e *Apthovirus* (MUELLER *et al.,* 2005).

É estimado que um bilhão de pessoas no mundo são acometidos pelos membros do gênero *Enterovirus*, que, além do poliovírus, também apresenta

importantes patógenos, como Cox e Echovirus (OBERSTE *et al.,* 2000; PALLANSCH & ROSS, 2001).

A partícula icosaédrica é constituída por 60 cópias de proteínas que formam o capsídeo constituído de VP1, VP2, VP3 e VP4. Existem três sorotipos de poliovírus, (tipo 1, 2 e 3) e não há imunidade cruzada entre eles. Os humanos são considerados os únicos hospedeiros naturais, embora, os primatas não humanos possam ser infectados experimentalmente (MINOR, 1996).

A transmissão do vírus obedece a via fecal-oral e a multiplicação inicial ocorre no intestino delgado e na garganta. Após a replicação inicial, o vírus pode ser encontrado no sangue, por um breve período, e pode espalhar-se para sítios distantes, incluindo o sistema nervoso central. Muitas infecções são inteiramente assintomáticas ou apresentam sintomas clínicos moderados (MINOR, 1996).

A patogenia clássica do poliovírus é a poliomielite, caracterizada por um quadro de paralisia flácida, acometendo em geral membros inferiores, de forma assimétrica, tendo como principais características flacidez muscular, com sensibilidade conservada e arreflexia no segmento atingido (PALLANSCH & ROSS, 2001). Estes sintomas acometem menos do que 1% dos indivíduos infectados por poliovírus (NATHANSON & MARTIN, 1979), sendo que a poliomielite é considerada um “acidente” nesta infecção entérica. De uma maneira geral, a poliomielite pode apresentar-se de forma assintomática, ou com sintomas leves, tais como, febre, dor de cabeça e de garganta (MUELLER *et al.,* 2005).

A Organização Mundial da Saúde adotou um plano em 1988 para erradicar os poliovírus selvagens no mundo, até o ano 2000. A campanha de vacinação através do imunógeno constituído de vírus atenuado (Sabin), tem

proporcionado resultados eficazes, reduzindo a incidência de poliomielite de 350.000 casos em 1988 para 784 em 2003 (MUELLER *et al.,* 2005). Atualmente, no entanto, surtos endêmicos da doença ainda ocorrem em vários países do continente Africano e Asiático (ROBERTS, 2005).

ROTAVÍRUS

O termo rotavírus é derivado da palavra latina *rota,* que significa roda, e deve-se ao aspecto morfológico da partícula viral quando observada em microscópio eletrônico (FLEWETT *et al.,* 1974). A partícula viral íntegra tem aproximadamente 75 nm de diâmetro, não possui envelope e o capsídeo, de simetria icosaédrica, é constituído por três camadas (ESTES, 2001). O gênero *Rotavirus* é um dos nove gêneros da família *Reoviridae* que apresentam propriedades morfológicas e bioquímicas em comum (ICTV, 2004). São caracterizados por apresentarem genoma composto por 11 segmentos de RNA fita dupla. Cada segmento codifica uma proteína (monocistrônico) com exceção do segmento 11 que codifica duas proteínas (policistrônico) (LUNDGREN & SVENSSON, 2001; JAYARAM *et al.,* 2004).

O capsídeo externo é constituído por duas camadas de proteínas estruturais, a VP4 e VP7, e o capsídeo interno pela VP6. O cerne viral é delimitado por uma estrutura que consiste de 60 dímeros de VP2, com função ligante de RNA, pela VP1 com função transcritase/replicase e VP3 com função guanililtransferase, além dos 11 segmentos genômicos (ESTES, 2001).

A transmissão do vírus é feita por via fecal-oral (KAPIKIAN, 2001), no entanto, há especulações que a transmissão possa ocorrer por via respiratória, principalmente em países desenvolvidos. A detecção do antígeno viral na secreção traqueal de crianças reforça essa hipótese (SANTOS, 2002). Os rotavírus infectam as células epiteliais das vilosidades do intestino delgado (STARKEY *et al.,* 1986), onde realizam a sua replicação no citoplasma destas células, levando-as a morte (ESTES, 2001).

A infecção produz um espectro de respostas que varia de diarréia suave à severa, com intensa desidratação. As manifestações clínicas mais freqüentes são diarréia, vômitos, febre, desidratação e dor abdominal (KAPIKIAN, 2001), sendo a tríade clássica da infecção febre, vômitos e diarréia (OFFIT, 1998).

Os rotavírus são os principais agentes etiológicos das gastroenterites agudas em crianças por todo mundo e também importante patógenos em muitas outras espécies de mamíferos (CLARE *et al.,* 2000; ARIAS *et al.,* 2002). É estimado que eles causem cerca de 870.000 mortes de crianças abaixo de dois anos de idade, por ano, nos países em desenvolvimento (ARIAS *et al.,* 2002).

Devido aos altos índices de morbidade e mortalidade há um considerável interesse no desenvolvimento de vacina efetiva e estratégias terapêuticas contra os rotavírus (ARIAS *et al, 2002*).

Existem sete grupos de rotavírus (A-G), sendo que os vírus do grupo A são subdivididos em 14 sorotipos (G1 a G14) baseados na VP7 e 12 sorotipos baseados na VP4 (P1 a P8). O grupo A, sorotipo G1, ocorre com maior freqüência mundial e somente os grupos A, B e C foram diagnosticados em humanos (TANIGUCHI & URASAWA, 1995).

Atualmente, diversas vacinas vêm sendo desenvolvidas, principalmente, nos Estados Unidos, com o intuito de atenuar o quadro grave da doença diarréica, e que apresentem padrão de segurança (SANTOS & HOSHINO, 2005). Uma das vacinas desenvolvidas, em 1998, foi a denominada Rotashield, porém, devido a problemas de intussuscepção, foi retirada de uso em 1999. Outras estão em fase avançada de desenvolvimento, como a Rotarix, Rotateq e UK-recombinante, sendo que, algumas já estão liberadas para o uso, como o Rotarix, com previsão de ser implementada no Brasil, no começo de 2006.

HERPESVÍRUS BOVINO

O Herpesvírus bovino tipo 1 (BHV-1), antigamente denominado Vírus da Rinotraqueíte Infecciosa Bovina ou Vulvovaginite Pustular Infecciosa é o agente etiológico de uma série de enfermidades, incluindo a rinotraqueíte bovina (IBR), a vulvovaginite pustular (IPV), conjuntivites, balanopostites, abortos e encefalites (GIBBS & RWEYEMAMU, 1977; KAHRS, 1977).

O BHV pertence à família *Herpesviridae,* subfamília *Alphaherpesvirinae,* gênero *Varicellovirus*. Apresenta simetria icosaédrica, nucleocapsídeo de 95-110nm de diâmetro (partícula íntegra de 150-200nm), envelope lipídico e seu genoma é constituído de DNA fita dupla. A sua replicação ocorre no núcleo da célula infectada (ARMSTRONG *et al.,* 1961, TIKOO *et al.,* 1995).

O BHV-1 é capaz de estabelecer latência nos neurônios sensoriais e motores, sendo este o principal obstáculo às campanhas de erradicação da doença

através da vacinação. No caso de uso do vírus vacinal atenuado há a possibilidade deste estabelecer infecções latentes, podendo, durante a reativação, sofrer mudança para a forma patogênica (TIKOO *et al.,* 1995).

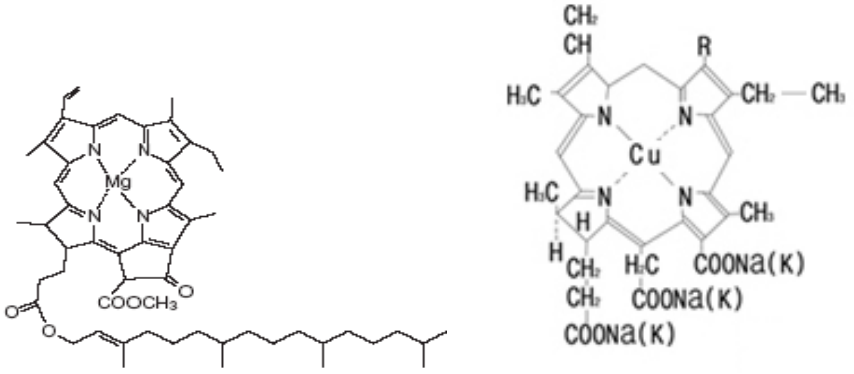
O BHV-1 tem ampla distribuição mundial, estando presente em quase todos os países de bovinocultura expressiva (GIBBS & RWEYEMAMU, 1977; KAHRS, 1977), impondo grandes prejuízos. Nos Estados unidos, por exemplo, o BHV-1 custa para a indústria de gado cerca de 500 milhões de dólares por ano (BOWLAND & SHEWEN, 2000). No Brasil, o vírus foi isolado inicialmente por ALICE (1978) e desde então, anticorpos anti-BHV-1 têm sido amplamente encontrados nos rebanhos (CASTRO, 1988; LOVATO *et al.,* 1995; VIDOR *et al.,* 1995; MELO *et al*., 1997; MELO *et al*., 1999,).

CLOROFILINA

A clorofila, molécula porfirínica planar, possui um anel, em cujo centro é encontrado um átomo de magnésio. Contém uma cadeia de ácido propiônico em ligação éster com o álcool diterpênico fitol, tornando o pigmento solúvel em gorduras (ALLINGER *et al*., 1978). As clorofilas a e b e seus derivados são encontrados em muitos vegetais verdes e vêm sendo estudadas pela sua atividade protetora aos danos à molécula do DNA causados por agentes químicos e físicos (SARKAR *et al*., 1994; NEGISHI *et al*., 1997).

Devido à sua instabilidade e insolubilidade em água a clorofila vem sendo substituída pela clorofilina (CHLN), um derivado sintético da clorofila, no qual o átomo central de magnésio é substituído por cobre, ferro ou cobalto. Os grupos

éster fitil e metil são substituídos por sódio ou potássio, o que a torna solúvel em água (Fig. 1) (KEPHART, 1955; ARIMOTO *et al*., 1993; SARKAR *et al*., 1994). Além disso, o átomo de metal substituto no núcleo da CHLN confere uma maior estabilidade quando comparado à da clorofila (SARKAR *et al.*, 1994; NEGISHI *et al*., 1997).



a- Clorofila b- Clorofilina

Fig. 1- Estrutura química da Clorofila (a) e a Clorofilina (b). O átomo central da clorofila, Mg, é substituído pelo cobre e os grupamentos éster fitil e metil são substituídos por sódio ou potássio (FAHEY *et al.,* 2005, com modificações).

A CHLN tem sido alvo de amplos estudos uma vez que apresenta propriedades de grande importância clínica, com ampla aplicação em seres humanos.

NEGISHI *et al*. (1990) testaram a atividade antimutagênica da clorofila (extraída de *Chlorella vulgaris*) e CHLN usando cepas de *Drosophila melanogaster* e obtiveram redução na freqüência de indução de mutação provocada pelo 3-amino-1 metil-5*H*-pirido[4,3-*b*] indol (Trp-p-2).

GENTILE & GENTILE (1991) constataram o potencial antimutagênico da clorofila extraída de *Gossypium hirsutum* e da CHLN ao mutágeno 4-nitro-o-fenilenodiamina (NOP) em *Salmonella typhymurium* cepa TA98.

ARIMOTO *et al.* (1995) demonstraram o efeito inibitório da CHLN na mutagenicidade do composto benzo[a]pireno e seus metabólicos, relacionando este efeito à capacidade da CHLN em formar um complexo com o composto e acelerar a sua degradação.

PARK & SURH. (1996) demonstraram atividade quimioprotetora da CHLN à indução de tumores de pele, em camundongos, por benzo[a]pireno, relatando uma significativa redução da incidência e multiplicidade de tumores.

NEGISHI *et al.* (1997) apontaram intensa atividade antigenotóxica das clorofilas naturais extraídas (extrato bruto e purificado) de espinafre e da alga verde *Chlorella vulgaris*, além da clorofilina cúprica ao óxido de nitroquinolina (4NQO), em larvas de *Drosophila melanogaster.*

DASHWOOD *et al.* (1998) realizaram experimentos em trutas e caracterizaram as propriedades inibitórias da CHLN na mutagenicidade e hepatocarcinogenicidade da aflotoxina B_1. Efeitos similares foram descritos por EGNER *et al*. (2003) em humanos submetidos à dieta com aflatoxina.

PIMENTEL *et al.* (1999) descreveram o efeito protetor da CHLN sobre os danos provocados pela irradiação de raios gama em células somáticas de *Drosophila melanogaster*. HAYATSU *et al.* (1999) mostraram que a CHLN prevenia o efeito carcinogênico de compostos amino heterocíclicos, compostos que são encontrados principalmente em carnes.

BOLOOR *et al.* (2000) observaram que as membranas mitocondriais das células de fígado de rato pré-tratadas com CHLN, não sofriam danos ou apresentavam danos reduzidos quando expostas à radiação gama, demonstrando o efeito radioprotetor da CHLN. KAMAT *et al.* (2000) verificaram o efeito antioxidativo da CHLN nestas membranas.

BEZ *et al.* (2001) estudaram a antigenotoxicidade das clorofilas a e b e CHLN aos danos induzidos ao DNA por metil metanosulfonado (MMS) em fibroblasto de pulmão de hamster chinês (V79). A hipótese foi confirmada pela redução do número de células com micronúcleo em relação ao controle positivo.

SUGIYAMA *et al.* (2002) estudaram o efeito protetor da CHLN em camundongos submetidos a um composto hepatocarcinogênico, 2-amino-1-metil-5H-pirido[4,3-b]indol (Trp-2). Constataram a formação do complexo CHLN-Trp-2 e conseqüentemente a diminuição da absorção do Trp-2 pelo organismo dos camundongos, funcionando desta forma como um agente anticarcinogênico.

NEGRAES *et al.* (2004) pré-trataram células de mamíferos com CHLN e as submeteram ao composto clastogênico, etil-metano-sulfonato. Concluíram que a droga apresentou potencial anticlastogênico com redução significativa do efeito.

NEGISHI *et al.* (1997); DASHWOOD *et al.* (1998); OLVERA, *et al.* (2000); TORRES-BEZAURI *et al.* (2002) sugeriram os possíveis mecanismos de ação da CHLN, dentre os quais, inibição da ativação do mutágeno, degradação do carcinógeno ou formação de complexo molecular com o pró-mutágeno.

BOTELHO *et al.* (2004) explorando a interação da CHLN com a infecção viral *in vitro* demonstraram que a droga protegeu células HEp-2 da fragmentação nuclear induzida pelo poliovírus.

Embora os potenciais efeitos antimutagênico e anticarcinogênico da CHLN a vários agentes genotóxicos tenham sido demonstrados, poucos trabalhos têm sido relacionados aos efeitos causados pelos vírus e/ou os efeitos do composto sobre a replicação viral (DUNHAM, 1954; MEKLER *et al*., 1969; DRZENIEK, *et al*., 1971, BOTELHO, *et al.,* 2004). Assim sendo, este trabalho visa estudar a interação da CHLN na replicação do poliovírus, rotavírus e herpesvírus bovino.

2 MATERIAIS E MÉTODOS

CÉLULAS

As culturas de células MA-104 (rim fetal de macaco Rhesus-ATCC-CRL-2378.1) e HEp-2 (carcinoma de laringe humana-ATCC-CCl-23) foram cultivadas em Meio Mínimo Essencial modificado por Dulbecco (DMEM- Gibco-BRL-USA), acrescido de 10% de soro fetal bovino (Gibco-BRL, EUA), 100µg/ml de estreptomicina (Sigma, Chem. Co., EUA), 100 UI/ml de penicilina (Sigma, Chem. Co.) e 2,5µg/ml de fungizona (Bristol Myers-Squibb, Brasil).

DROGA

A CHLN (Sigma Chem. Co., USA) foi preparada na forma de solução estoque a 250 µg/ml em tampão fosfato-salina (PBS) pH 7.4, alicotada e mantida a -20ºC protegida da luz.

VÍRUS

O rotavírus símio (cepa SA-11) é da coleção do Laboratório de Virologia, do Departamento de Microbiologia da UEL. O BHV-1 foi cedido pelo Prof. Amauri Alfieri, do Departamento de Medicina Veterinária Preventiva-UEL e o poliovírus foi obtido da American Type and Culture Collection (ATCC, VR-58).

O estoque de rotavírus foi preparado em cultura de células MA-104, enquanto que os estoques de poliovírus e BHV-1 foram preparados em HEp-2, posteriormente congelados a -80ºC até o momento de uso.

TESTE DE CITOTOXICIDADE

Células MA-104 e HEp-2 cultivadas em microplacas de 96 escavações foram submetidas ao tratamento com concentrações variadas de CHLN (450µg/ml, 380µg/ml, 300µg/ml, 200µg/ml, 100µg/ml, 50µg/ml e 20µg/ml) em meio DMEM, (doze escavações por concentração da droga) a 37ºC, em ambiente com tensão de 5% de CO_2, sendo a viabilidade celular avaliada pelo método do dimetilthiazol difenil brometo de tetrazólio (MTT-Sigma Chem. Co., USA). Após 72h, o reagente MTT foi adicionado e as células incubadas novamente a 37ºC por 2 horas, após o qual, a leitura da absorbância foi realizada em 490 e 630nm.

ÍNDICE DE SELETIVIDADE

O índice de seletividade (IS) foi calculado através da concentração considerada 50% tóxica (CC_{50}) para as células MA-104 e HEp-2, dividida pela concentração considerada capaz de inibir 50% da atividade viral (IC_{50})

TRATAMENTO COM A DROGA

Três protocolos foram utilizados para avaliar a interação da CHLN na replicação viral: No primeiro, as células foram tratadas com a CHLN por uma e duas horas, a 37ºC, antes da infecção (atividade profilática - uma hora e duas horas). No segundo, as cepas virais foram tratadas com a CHLN por uma hora a 37Cº antes da infecção (atividade virucida), e no terceiro, a CHLN foi adicionada no meio de cultura celular, no momento da infecção (terapêutico 0 hora), após uma hora e duas horas de infecção (terapêutico uma hora e duas horas, respectivamente).

ATIVIDADE ANTIVIRAL POR ENSAIO DE PLAQUE

As células foram cultivadas em placas de fundo chato com 24 escavações, e após confluência de 90%, foram tratadas com variadas concentrações da droga (tratamentos profiláticos e terapêuticos). Posteriormente, inoculadas com multiplicidade de infecção (MOI) igual a um, seguido de incubação a 37ºC por 1h. Após lavagens das células, foi adicionado meio DMEM 2 vezes concentrado, livre de SFB, adicionado de antibióticos e agarose 1% (previamente fundida e mantida em banho maria a 46 ºC). Após a solidificação da agarose nutriente, as placas foram incubadas invertidas a 37ºC em ambiente com 5 % de CO_2. Após 48 horas, a agarose nutriente foi retirada, as células fixadas com formalina a 10% e coradas com solução alcoólica de cristal violeta a 0,5%. Paralelamente, foram feitos os controles de célula, de vírus e da substância. A atividade antiviral, definida pela porcentagem de inibição de plaque (I), foi calculada pela fórmula:

I = [1 – (Nº de plaques no teste/Nº de plaques no controle de vírus) x 100].

Para o tratamento virucida, 10^8 e 10^7 unidades formadoras de plaques (UFP), respectivamente, de poliovírus e BHV-1 foram previamente tratadas com as respectivas concentrações da droga por uma hora, a 37 ºC, e posteriormente avaliadas pelo ensaio de plaque, similarmente ao processo descrito anteriormente.

IMUNOFLUORESCÊNCIA INDIRETA (IFI)

Culturas de células HEp-2 e MA-104 foram cultivadas em tubos de Leighton com lamínulas, infectadas e tratadas conforme protocolos citados anteriormente. Após, aproximadamente, 24 horas, as culturas foram coletadas,

lavadas com PBS e fixadas com acetona gelada (-20ºC), durante 20 minutos. Posteriormente, foram tratadas com soro de camundongo anti-rotavírus (DMVP/UEL), soro de coelho anti-herpesvirus bovino tipo 1 (DMVP/UEL) ou soro de coelho anti-poliovírus tipo 1 (cedidos pelo Dr. E.C.Leal, INCQS/FIOCRUZ, RJ) por 30 minutos em câmara úmida a 37ºC, seguido de 3 lavagens com PBS. Posteriormente, foram tratadas com soro de carneiro anti-imunoglobulina de camundongo (rotavírus) ou soro de cabra anti-imunoglobulina de coelho (poliovírus e herpesvirus) conjugados com isotiocianato de fluoresceína (Sigma Chem. Co., USA), por 30 minutos em câmara úmida e escura, a 37ºC. As células foram, novamente, lavadas por três vezes com PBS, montadas em lâminas de vidro, com 50% glicerina-tamponada (pH 7.3), e observadas ao microscópio de luz ultravioleta.

QUANTIFICAÇÃO DO RNA VIRAL DE ROTAVÍRUS

Culturas de células MA-104 cultivadas em tubos 13X100mm foram inoculadas com rotavírus símio SA-11, a uma MOI de aproximadamente um. Em seguida, foi acrescentado meio com CHLN em variadas concentrações.

Paralelamente, foram mantidos controles de células infectadas com vírus sem tratamento com a droga e controle de células não infectadas, na ausência e presença da CHLN. Após, aproximadamente, 24 horas, as respectivas culturas de células foram congeladas e descongeladas por 3 ciclos e submetidas à extração do RNA viral (HERRING *et al*., 1982). Os extratos previamente tratados com tampão dissociante (0,065M Tris/HCL, pH 6.8; 5M uréia; 5% 2-mercaptoethanol; 3% SDS; 0,01% azul de bromofenol e 10% de glicerol), a 60ºC por 30 minutos, foram submetidos à eletroforese em gel de poliacrilamida (EGPA) (HERRING *et al*., 1982). O DNA do fago Lambda digerido com *Hind* III (Gibco- BRL, EUA), em concentração

definida, foi utilizado como padrão. Após a coloração com nitrato de prata, o gel foi analisado pelo sistema de vídeo documentação (Pharmacia) e as bandas de RNA quantificadas pelo software Pharmacia Image Master 1D Prime.

INIBIÇÃO DAS CEPAS VIRAIS COM INTERFERON E ARA-C

Poliovírus, BHV-1 and rotavírus foram submetidos ao ensaio antiviral com 100.000U/ml, 10.000 U/ml e 1000U/ml de interferon humano alfa B-2 (Meizler-Com. Intern. SA, Brazil). Adicionalmente, o BHV-1 foi também testado com arabinosideo citosina (Ara-C) (Pharmacia NV/SA-Bélgica) na concentração de 50μg/ml. Ambos composto virais foram monitorados por plaque, exceto rotavírus que foi monitorado por IFI.

ESTATÍSTICA

Todos os experimentos foram avaliados em quadruplicata. Os dados foram analisados por ANOVA, seguido do teste de Dunnett. Os valores com $P \leq 0,05$ foram considerados significativos. O CC_{50} e o IC_{50} foram calculados por regressão linear.

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CHLOROPHYLLIN INHIBITS THE REPLICATION OF POLIOVIRUS 1, ROTAVIRUS (SA-11), AND BOVINE HERPESVIRUS 1, *IN VITRO*

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Abstract

Chlorophyllin (CHLN) was assayed in poliovirus (PV), bovine herpesvirus (BHV-1) and rotavirus (R) in HEp-2 and MA-104 cell cultures. Three protocols were used: I) Cells were treated with CHLN for 1h and 2h, before infection (prophylactic activity, -1h and -2h). II) Virus strain was treated with the drug for 1h, before infection (virucide activity). III) CHLN was added to the culture at the moment of infection (therapeutic, zero h), and 1h and 2h after infection, therapeutic 1h and 2h, respectively. Effects were monitored by plaque assay (PFU) and inhibition of fluorescent cell (IFA) for PV and BHV-1, and viral nucleic acid quantification (RNA) and IFA for R. Virucide activity demonstrated: a) Inhibition of R replication in 100% and 70% by RNA and IFA, respectively, representing selectivity indexes (SI) of 33.6 and 22.5, respectively. b) Inhibition of PV and BHV-1 in 62% (SI=22.4) and 66% (SI=22.0) by PFU, respectively. By IFA, inhibition were 57.7% (SI=32.1) and 66% (SI=33.1) for PV and BHV-1, respectively. The time-of-addition study demonstrated: for PV, the highest inhibition, 70% (SI=57.0) was observed under therapeutic protocol 1h (IFA). For BHV-1, the maximum percent of inhibition was found in prophylactic activity -2h (IFA), 77% (SI=18.7). Under therapeutic protocol 0h (PFU), high rate was also found, 66,5% (SI=33,1). For R, the highest percentage was 60% (SI=11.8) in therapeutic protocol 0h (IFA). CHLN activity is possibly on virus particles and/or on virus-receptor sites. We suggest that drug complexation with virion and/or receptors seem to be one of the mechanisms.

Keywords: chlorophyllin, poliovirus, bovine herpesvirus, rotavirus, antiviral

1 INTRODUCTION

Although search for drugs against viral diseases has increased significantly, viruses still pose a challenge in development and the use of these compounds clinically, partially, due to their toxicity (Emst and Franey, 1998; De Clercq, 2004) as well as for the occurrence of drug resistant strains (Gilbert et al., 2002; Zoulim, 2001). These are the underlying aspects that have stimulated the research for new substances either synthetic or natural with antiviral activity (Field, 2001).

Chlorophyllin (CHLN) is a synthetic derivative of chlorophyll (CHL) in which the phytyl and methyl groups are substituted by sodium or potassium, making it water soluble and, therefore, more useful than CHL which is only soluble in organic solvents (Arimoto et al., 1993). CHLN has been used as a coloring agent and shows a potent antimutagenic agent against a variety of mutagens in vitro and in vivo (Ong et al*.,* 1986; Waters et al*.,* 1996; Chernomorsky et al., 1997; Egner et al., 2001). It was also shown to exhibit anticarcinogenic activity in animal models (Young and Bergei, 1980; Guo et al., 1995; Hasegawa et al., 1995; Park and Surh, 1996). Oral feeding of CHLN to humans reduced aflotoxin-DNA adduct formation significantly with no side effects (Egner et al., 2001). It has been recommended for controlling body, fecal and urinary odors in geriatric patients and as an accelerant in wound healing (Young and Bergei, 1980). As antiviral compound, it was shown that CHLN protected HEp-2 cells from nuclear fragmentation induced by poliovirus (Botelho et al., 2004).

Rotavirus (RV), a member of the family *Reoviridae*, is a nonenveloped icosahedral particle, constituted with six proteins organized into three concentric layers and a genome of 11 segments of double-stranded RNA (Estes, 2001). Human rotaviruses are the major etiologic agents of severe dehydrating gastroenteritis in children worldwide (Kapikian et al., 2001). The global mortality associated to rotavirus infections has been estimated in 325 000 – 592 000 deaths annually (median 440 000 deaths) (Parashar et al*.,* 2003).

Bovine herpesvirus (BHV), a member of the family *Herpesviridae* and subfamily *Alphaherpesvirinae*, possess a double-stranded DNA genome, which is involved by an icosahedric capsid and a lipidic envelope with glicoproteins spikes on it (Hinkley et al., 1998). BHV causes economically important diseases of cattle. The diseases are known as infectious bovine rhinotracheitis/infectious pustular

vulvovaginitis (IBR/IPV) (Köppel et al., 1997). BHV has been associated with respiratory, ocular, reproductive, central nervous system, enteric, neonatal and dermal infections. The distribuition of the virus is worldwide (Gibbs and Kweyemamu, 1977; Kahrs, 1977).

Poliovirus (PV), is a member of the genus *Enterovirus* of the family *Picornaviridae*, a large family of small (Latin, *pico*) nonenveloped, plus stranded RNA viruses. It is the causative agent of poliomyelitis. Although no longer a major public health threat in the developed world, PV continues to be one of most thoroughly studied and best understood model of virus to date. The very stable capsid and ease of virion purification, along with high virus titers and the low bio-safety level requirements make PV a favored target for investigations. (Mueller et al., 2005).

In spite of the antimutagenic and anticarcinogenic potential of CHLN against several genotoxic agents has been demonstrated, a few studies on its antiviral activity have been carried out (Dunham, 1954; Mekler et al., 1969; Drzeniek, et al., 1971). For instance, underlying genoprotection process, nuclear fragmentation was preserved after PV infection (Botelho et al., 2004). Therefore, the aim of the present study was to determine the effect of CHLN in the replication of poliovirus, rotavirus and herpesvirus.

2 MATERIALS AND METHODS

2.1 Cell lines and viruses

HEp-2 (human larynx cells-ATCC CCl-23) and MA-104 (monkey kidney cells-ATCC CRL-2378.1) cell lines were grown in DMEM (Gibco, BRL, USA), supplemented with 10% fetal bovine serum (Gibco, BRL, USA), 100µg/ml of streptomycin (Sigma Chem. Co., USA), 100UI/ml of penicillin (Sigma Chem. Co., USA), and 2.5µg/ml of amphotericin B (Bristol Myers-Squibb, Brazil). The strain of bovine herpes virus type 1 (BHV-1) was supplied by Prof. A.A. Alfieri (DMVP/UEL). Poliovirus type 1 was obtained from American Type and Culture Collection (ATCC, VR-58), and rotavirus, strain SA-11, belonged to the collection of the Laboratório de Virologia (DM/UEL). Virus strains were propagated in HEp-2 cells (poliovirus and herpesvirus), and MA-104 cells (rotavirus), and stored at -80°C.

2.2 Citotoxicity assay

Cell lines grown in 96-well plates for 48 hours, at 37°C at 5% CO_2 were treated with varying concentrations (450µg/ml, 380µg/ml, 300µg/ml, 200µg/ml, 100µg/ml, 50µg/ml e 20µg/ml) of CHLN (Sigma Chem Co., USA). The cell viability was evaluated by MTT kit assay, (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma Chem Co., USA) according to the manufacturer's recommendation.

2.3 Selectivity Index

The selectivity index (SI) of CHLN is represented by the ratio of the drug cytotoxic concentration 50% (CC_{50}) for MA-104 or HEp-2 cell cultures by the drug inhibitory concentration 50% (IC_{50}).

2.4 Drug treatment

Three protocols were used: A) Cultured cells were treated with CHLN for 1h and 2h, at 37ºC before infection (prophylactic activity -1h and -2h); B) Virus strains were treated with the drug for 1h at 37ºC before infection (virucide activity), and C) CHLN was added to the culture medium at the moment the infection (therapeutic zero h), 1h after infection (therapeutic 1h), and 2h after infection (therapeutic 2h).

2.5 Plaque forming unit (PFU)

Cell lines were grown for 48 h in 24-well culture plates to 95% confluence, approximately. Cells were treated with varying drug concentrations and infected (therapeutic and prophylactic activities) at a multiplicity of infection (MOI) of 1, and incubated at 37ºC for 1h. Cells were overlaid with nutrient agarose (2x DMEM/1,5% agarose, vol:vol), additionally supplemented with 25mM $MgCl_2$, for poliovirus assay, and incubated at 37ºC for 48h. The nutrient agarose was removed and cells were fixed with 10% formalin and stained with 1% crystal violet. The antiviral activity was defined as the percentage of plaque inhibition, as follows: % Plaque inhibition = [1 – (number of plaque in test/ number of plaque in control) x 100].

For virucide activity, approximately 10^8 PFU of poliovirus and 10^7 PFU of BHV-1 were incubated for one hour at 37 ºC with the drug at varying concentrations and plaque reduction calculated, as previously described.

2.6 Indirect immunofluorescence assay (IFA)

Cell cultures grown in Leighton tubes were infected and treated according to the protocols, previously cited. After 24 h, the cultures were collected, washed with PBS and fixed with cold acetone (-20°C) for 20 minutes. Briefly, cell cultures were overlaid with polyclonal bovine anti-HBV antibodies (supplied by DMVP/UEL); rabbit anti-poliovirus antibodies (supplied by INCQS/FIOCRUZ, RJ) and mouse anti-pig rotavirus policlonal antibodies (supplied by DMVP/UEL), at appropriate dilutions, and incubated at 37°C for 30 minutes. After washings, goat anti-bovine IgG; goat anti-rabbit IgG or sheep anti-mouse IgG conjugated with FITC (Sigma Chem. Co., USA) were used at appropriate dilutions and maintained at 37°C for 30 minutes. Cover glasses were mounted in slides with 50% buffered-glycerol and cells were observed in a UV light microscope. Experiments were done in quadruplicates and 50 cells were counted by cover glass.

2.7 Quantification of rotavirus RNA

MA-104 cell cultures grown in 13X100 tubes were infected with rotavirus at MOI of 1 and treated with the drug, according to previous protocols. After 24 h, approximately, cultures were submitted to three cycles of freeze and thaw, and virus RNA extracted according to Herring et al. (1982). The extracts were treated with dissociation buffer (0,065M Tris/HCL, pH 6.8; 5M urea; 5% 2-mercaptoethanol; 3% SDS; 0,01% bromophenol blue and 10% glicerol) at 60ºC for 30 minutes, and submitted to polyacrylamide gel electrophoresis (PAGE) (Herring et al., 1982). Lambda phage DNA HindIII digest (Gibco BRL, USA) was used as quantitative standard. After staining with silver nitrate, RNA quantification was performed with Pharmacia Image Master VDS 1D Prime software.

2.8 Virus strains inhibition by interferon and cytosine arabinoside (Ara-C)

Poliovirus, BHV-1 and rotavirus were submitted to antiviral assay with 1 000 U/ml, 10 000 U/ml and 100 000 U/ml human alfa-2 B interferon (Meizler Com. Intern. SA, Brazil). Additionally, BHV-1 was also assayed with Ara-C (Pharmacia NV/SA-Belgium) at the concentration of 50μg/ml. Both antiviral compounds were also monitored by plaque assay, except for rotavirus that IFA was used.

2.9 *Statistics*

The data were analysed by ANOVA followed by Dunnett´s test. $P \leq 0,05$ values were considered significant. The CC_{50} and IC_{50} were calculed by linear regression analysis of the dose-response curves generated. All experiments were performed in quadruplicate.

3 RESULTS

Cytotoxicity of CHLN evaluated by cellular viability demonstrated that concentrations equal to 285 µg/ml and 180µg/ml of the drug reduced HEp-2 and MA-104 cell viability in 50%, respectively.

Interferon inhibited 100% PFU of poliovirus and BHV-1, and ninety-eight percent of rotavirus-infected fluorescent cells. Ara-C inhibited 60% PFU of BHV-1.

Fig. 1 illustrates the activity of CHLN in the replication poliovirus, at the indicated concentrations. It is shown that when CHLN was added, at the concentration of 2.5µg/ml, two hours and one hour prior infection (prophylactic protocol-2h and -1h, respectively) the inhibition of virus replication, monitored by plaque assay, was 13.7% and 1.8%, respectively. At the same concentration, and time zero (therapeutic protocol 0h), the addition of the drug at the moment of the infection resulted in inhibition of 12%. However, when the drug was added 1h and 2h post-infection (therapeutic 1h and 2h) the inhibitions were none and 3.2%, respectively. Prophylactically, at the concentration of 5.0µg/ml, the inhibitions were 14% and 13.1% -1h and -2h, respectively. In therapeutic protocol at the time zero the inhibition was 36.6%, however, the addition of the drug 1h and 2h after infection resulted in none and 15.2% inhibition, respectively. At the drug concentration of 10µg/ml, under prophylactic treatment -1h and -2h, the inhibitions detected were 11% and 16%, respectively. Therapeutically, at time zero, 34% inhibition was found, while, under the same protocol at 1h and 2h, inhibitions of 13.9% and 32% were observed, respectively. When CHLN was used at the concentration of 20µg/ml, maximum inhibition of 51% was observed, under the therapeutic protocol, at time zero and 2h. Similarly, 1h post-infection an inhibition of 33.9% was observed. While, in

prophylactic treatment, -1h and -2h, there were inhibitions of 18.4% and 13%, respectively.

The inhibition of poliovirus-infected fluorescent cells is shown in fig. 2. At CHLN concentration of 2.5µg/ml in prophylactic treatment, -1h and -2h, inhibitions of 25.2% and 30% were observed, respectively. In therapeutic protocol, 1h and 2h, inhibitions of 34.6% and 30.9% were detected, respectively, while at zero hour it was 24%. At 5.0µg/ml, the inhibition determined in the prophylactic treatment, -1h and -2h, were 44% and 39.6%, respectively. Under therapeutic protocol (1h and 2h) the values found were 51% and 35%, respectively, while, at the time zero hour the value was 26.6%. For 10µg/ml, the inhibition of the viral replication, in prophylactic treatments, -1h and -2h, were 32% and 42%, respectively. While, in therapeutic treatment, 1h and 2h, the inhibitions were 59.1% and 42%, respectively, however, at time zero it was 51.1%. The maximum percent of virus inhibition in this assay was also found in the therapeutic treatment 1h, at the drug concentration of 20µg/ml, where the inhibition was 70%. At time zero the inhibition was of 64.4% and, still, at 2h the inhibition was 43.6%. Under prophylactic protocol, -1h and -2h, at the same drug concentration, the inhibitions were 53% and 33%, respectively.

The direct effect of CHLN in poliovirus particle (virucide protocol), monitored by IFA (inhibition of the number of fluorescent cells – FC) and plaque assay (PFU) is demonstrated in fig. 3. The inhibition of FC at the drug concentration of 2.5µg/ml was 8.8%, while, for PFU it was 34%. At 5.0µg/ml, the inhibitions were 37.7% and 38.7% to FC and PFU, respectively. For 10µg/ml, the inhibitions were 53.3% and 44% for CF and PFU, respectively. The highest percentage of inhibition for CF and PFU occurred at the drug concentration of 20µg/ml, 57.7% and 62%, respectively.

The effect of CHLN in rotavirus replication was evaluated by IFA and data are presented in fig. 4. It was found that at the concentration of 2.5µg/ml, in prophylactic protocol -1h and -2h, inhibitions of 17% and 10.5% were observed, respectively. For therapeutic treatment, in the time 0h the inhibition was 18%, while, 1h and 2h the values were 20% and 12.9%, respectively. When the concentration tested was 5.0µg/ml, the inhibitions in prophylactic tests, -1h and -2h, were 20% and 16.8%, respectively. However, for the therapeutic treatment, 0h, 1h and 2h, the inhibitions were, respectively, 35%, 30% and 32%. At the concentration of 10µg/ml, the inhibition of the viral replication in prophylactic protocol, -1h and -2h, were 20% and

15.7%, respectively. Therapeutically, 0h, 1h and 2h, there were respective inhibitions of 37%, 27.5% and 37.1%. The inhibition of 60% was the highest score found in this assay, in therapeutic protocol 0h, at the concentration of 20µg/ml, while, at the time 1h and 2h were 24.2% and 37%, respectively. At the same concentration, inhibition of the viral replication, in prophylactic tests -1h and- 2h were 42% and 29.4%, respectively.

The fig. 5 illustrates the inhibitions of rotavirus nucleic acid and rotavirus-infected fluorescent cells when virus was submitted to drug treatment for 1h, before infection (virucide activity). At the concentration of 2.5µg/ml the inhibitions were 45.3% for RNA and 41% for FC, while, at 5µg/ml 49.3% (RNA) and 44% (FC). With 10µg/ml the inhibitions were 56.6% (RNA) and 45% (FC). At 20µg/ml, the value of inhibition was so high that virus RNA could not be quantified, therefore, being considered inhibition of 100%, while, 70,8% (FC).

The fig. 6 shows the inhibitory effect of CHLN in BHV-1 replication demonstrated by IFA. At 2.5µg/ml, under prophylactic protocol, -1h and -2h, inhibitions of 12% and 30% were observed, respectively. Therapeutically, in time 0h the inhibition was null (0%), while, at 1h and 2h were 11.8% and 27.1%, respectively. When drug was used at 5.0µg/ml, the inhibition found under prophylactic protocol, -1h and -2h, were 30% and 34%, respectively. For therapeutic treatment, 0h, 1h and 2h, the inhibitions were 25%, 21.5% and 23%, respectively. At the concentration of 10µg/ml, prophylactic test, -1h and -2h, presented inhibitions of 28.7% and 30.9%, respectively. However, for therapeutic protocol, 0h, 1h and 2h, the inhibitions were, respectively, 30%, 22.5% and 38%. When drug concentration was increased to 20µg/ml, the highest grade of inhibition was observed, 77%, under the prophylactic test -2h, and 43% for -1h. For therapeutic treatment, 0h, 1h and 2h, the inhibitions were 45%, 23.6% and 36%, respectively.

The effect of CHLN in the replication of BHV-1 (virucide activity) evaluated by IFA and PFU is demonstrated in fig. 7. The inhibition of FC, at 2.5µg/ml, was 16%, while, for PFU the inhibition was 28%. At the concentration of 5.0µg/ml the inhibition of FC was of 37.2% and for PFU 32.5%. However, for 10µg/ml, the inhibition of FC reached 45.2% and for PFU 42%. When drug concentration was increased to 20µg/ml maximum reduction for both FC and PFU was detected, 66.6% and 66.4%, respectively.

The effect of the drug in the replication of BHV-1, under prophylactic and therapeutic protocols, by PFU, is illustrated in fig. 8. For 2.5µg/ml in prophylactic tests, -1h and -2h, inhibitions were 4.1% and 5.6%, respectively. But, for therapeutic protocol, 0h, 1h and 2h, there were values from 5.7%, 18.2% and 15.7%, respectively. For 5.0µg/ml, the inhibition demonstrated in the prophylactic protocol, -1h and -2h, were 10.3% and 13.2%, respectively. However, for therapeutic treatment, 0h, 1h and 2h, the inhibitions were, respectively, 33.5%, 36.5% and 13.2%. At 10µg/ml, in the prophylactic situation, -1h and- 2h, the inhibitions were 12.4% and 22.2%, respectively. At the time 0h, 1h and 2h in therapeutic treatment there were inhibitions of 56.6%, 33.5% and 45%, respectively. At 20µg/ml, the most expressive values were found in therapeutic process, 0h, in that inhibition was 66,5%. Similarly, prophylactic protocol, -1h and -2h, presented inhibitions, respectively, of 21% and 35.5%, while, for therapeutic test, 1h and 2h, inhibitions of 45% and 46% were found, respectively.

The SI was established at maximum antiviral activity, respectively to the protocol used, and data are shown in table 1. For poliovirus the highest SI, 57, was demonstrated for therapeutic protocol 1h by IFA. For the same assay, virucide treatment resulted in a SI of 32.1. Under therapeutic time zero and virucide protocols the SI were, respectively, 15.2 and 22.4, by PFU. For BHV-1 the highest SI were attained for therapeutic time zero (PFU) and virucide (IFA) protocols similarly, 33.1. For prophylactic -2h (IFA) and virucide (PFU) treatments, SI were 18.7 and 22, respectively. Concerning rotavirus, the highest SI found was 33.6 for virucide protocol, evaluated by the inhibition of nucleic acid, but, for therapeutic time zero and virucide protocols (IFA) the SI were 11.8 and 22.5, respectively.

4 DISCUSSION

Chlorophyll and its derivatives are widely found in nature mainly in green plants, however, green algae and some bacteria also possess them. These compounds have been studied for their protecting activity against DNA molecule damage by physical and chemical agents (Sarkar et al., 1994; Negishi et al., 1997). Nevertheless, due to chlorophyll chemical structure it is soluble in lipids only (Allinger et al., 1978) precluding even studies on bioavailability. Alternatively, a synthetic

derivative of chlorophyll, CHLN, was developed with increased water solubility and stability. It is also known that CHLN possesses antimutagenic/anticarcinogenic activities against several natural and synthetic compounds (Young and Bergei, 1980; Ong et al., 1986; Guo et al., 1995; Hasegawa et al., 1995; Park and Surh, 1996; Waters et al., 1996; Chernomorsky et al., 1997; Egner et al., 2001), however, almost nothing is known about its antiviral effect.

Presently, it was demonstrated *in vitro* that CHLN inhibited poliovirus, BHV-1 and rotavirus.

Poliovirus replication was inhibited at highest score in therapeutic and virucide protocols. This inhibitory effect is strengthened by SI for therapeutic 1h and virucide treatments, respectively, 57 and 32.1, by IFA. Since prophylactic protocol demonstrated low activity it is suggested that drug effect in poliovirus receptor sites, in the host cell, might not be the case. Rather, drug effect was most effective on virus particles (virucide activity) and during virus replication process (therapeutic activity).

Concerning rotavirus, CHLN was an efficient virucide. Total inhibition was observed at 20 μg/ml when monitored by virus nucleic acid synthesis, with SI of 33.6. Under the same protocol and the same drug concentration, albeit, by IFA, inhibition was also high, and a SI 22.5. This result is in accordance with therapeutic protocol, time zero, in that inhibition was also high, however, a SI of 11.8 was found. Under this circumstance, virus particles are placed in contact with the drug at the moment of infection. Less efficiency was demonstrated in therapeutic and prophylactic treatments, meaning little effect on virus receptor and during virus replication.

The action of the drug in BHV-1 demonstrated efficiency in prophylactic and virucide treatments. At the concentration of 20 μg/ml the inhibition was circa of 70% in virucide treatment, when monitored by PFU and IFA, with SI 22.0 and 33.1, respectively. Under therapeutic 0h (PFU), where virus is placed in contact with the drug, at the moment of infection, the inhibition was equal, 70% (SI=33.1). Similarly, in prophylactic treatment -2h, at the same concentration, maximum inhibition of 77% (SI=18.7) was demonstrated. In this case, it is speculated that drug inhibited BHV-1 replication mostly interfering on virus particles itself and on virus-binding cell receptor. Therapeutically, drug demonstrated insignificant results. The summation of the results demonstrated different possibilities. For poliovirus, virucide and therapeutic treatments were more efficient, however, for rotavirus and BHV-1 virucide

and prophylactic protocols were most significant. It seems that, independently of the virus, virucide effect is a common event, reflecting the action of the drug on virion itself, but, whether virion receptor sites, on the host cells, and the effect in the replication process are involved depends on different mechanisms. It was demonstrated elsewhere that CHLN binds noncovalently to mutagens and carcinogens to protect cells from harmful effects of related compounds (Negishi et al., 1997; Dashwood et al., 1998; Olvera et al., 2000). Additionally, Torres-Bezauri et al. (2002) demonstrated that without CHLN interaction with those compounds CHLN protecting effect is also absent. Experimental data on the activity of CHLN in virus infections is almost inexistent (Dunham, 1954; Mekler et al., 1969; Drzeniek, et al., 1971). However, Botelho et al. (2004) demonstrated that, underlying the genoprotecting effect, CHLN protected cells from DNA fragmentation induced by poliovirus. Moreover, protection was best attained in virucide treatment, secondly by therapeutic protocol, and lesser in prophylactic protocol. Although, in the present work different tools were used to evaluate CHLN antiviral activity, there was an agreement between the results. Therefore, drug complexation with virion or receptor on host cells seems to be one of the mechanisms of action, nevertheless, interference on virus replication can not be ruled out. It shows, therefore, that CHLN is a promising compound with such versatile effects, however, much has to be understood even on the scope of virus infections.

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Figures Captions

Fig. 1

Fig. 1. The effect of CHLN in the replication of poliovirus monitored by plaque assay in HEp-2 cell cultures. The drug was used at the indicated concentrations added before (prophylactic protocol, -2h and -1h) and after (therapeutic protocol, 0h, 1h and 2h) the infection. Percent of virus inhibition is indicated with the respective standard deviations from experiments performed in quadruplicate (n=4). $P \leq 0.05$, except for prophylactic protocol (-1h), 2.5µg/ml, prophylactic protocol (-2h), 2.5µg/ml and 5.0 µg/ml and therapeutic protocol (1h), 2.5µg/ml.

Fig. 2

Fig. 2. The evaluation of CHLN in poliovirus-infected HEp-2 fluorescent cells treated with the drug, at the indicated concentrations by immunofluorescence assay (IFA). The values represent inhibition in the number of treated cells in relation to control nontreated cells, with the respective standard deviations. The experiments were performed in quadruplicate (n=4). $P \leq 0.05$.

Fig. 3

Fig. 3. The direct effect of CHLN in poliovirus particles (virucide protocol) monitored by IFA (inhibition of the number of virus-infected fluorescent cells -CF) and plaque assay (PFU) in HEp-2 cell cultures. Virus was placed in contact with the drug at the indicated concentrations for 1 hour at 37ºC, before the infection. The values with the respective standard deviations represent: a) the inhibition of the number of fluorescent cells in comparison to control nontreated cells, and b) inhibition of the number of plaques. (n=4). $P \leq 0.05$.

Fig. 4

Fig. 4. The effect of CHLN in the replication of rotavirus in MA-104 cell cultures monitored by IFA. CHLN was used at the indicated concentrations and added before (-2h and -1h) and after (0, 1h and 2h) infection. The values represent the inhibition of the number of virus-infected fluorescent cells in comparison to control nontreated cells (n=4) and their respective standard deviations. $P \leq 0.05$.

Fig. 5

Fig. 5. The virucide action of CHLN in the replication of rotavirus monitored by the synthesis of virus RNA and IFA. Virus was treated at the indicated concentrations, during 1 h at 37ºC, before the infection. Virus RNA was resolved by polyacrylamide gel electrophoresis. The values represent inhibition of the number of virus-specific fluorescent cells in comparison to nontreated cells (n=4) with the respective standard deviations and inhibition of viral nucleic acid. P≤0.05

Fig. 6

Fig. 6. The effect of CHLN in BHV-1- infected HEp-2 cell cultures monitored by IFA. CHLN was used at the indicated concentrations and added before (-2h and -1h) and after (0h, 1h and 2h) infection. The values represent inhibition of the number of virus-specific fluorescent cells in comparison to nontreated cells (n=4) with the respective standard deviations. P≤0.05.

Fig. 7

Fig. 7. The action of CHLN in BHV-1-infected HEp-2 cell cultures (virucide activity) monitored by IFA (inhibition of the number of fluorescent cells - FC) and plaque assay (PFU). Virus was submitted to drug treatment at the indicated concentrations, during 1 hour at 37ºC, before infection. The values, with the respective standard deviations (n=4), represent: a) Inhibition of the number of virus-specific fluorescent cells in comparison to nontreated cells, and b) Inhibition of the number of plaques. P≤0.05.

Fig. 8

Fig. 8. The effect of CHLN in the replication of BHV-1 monitored by plaque assay in HEp-2 cell cultures. The drug used at the indicated concentrations was added before (-2h and -1h) and after (0, 1h and 2h) infection. The values correspond to inhibition of the number of plaques with the respective standard deviations (n=4). P≤0.05, except for prophylactic protocol (-1h), 2.5 µg/ml and 5.0µg/ml and prophylactic protocol (-2h), 2.5µg/ml.

Table caption

Table 1

Table 1: Cytotoxic concentration (CC_{50}) and virus inhibitory concentration (IC_{50}) of CHLN for poliovirus and BHV-1 in HEp-2 and for rotavirus in MA-104 cell cultures.

FIGURE 1

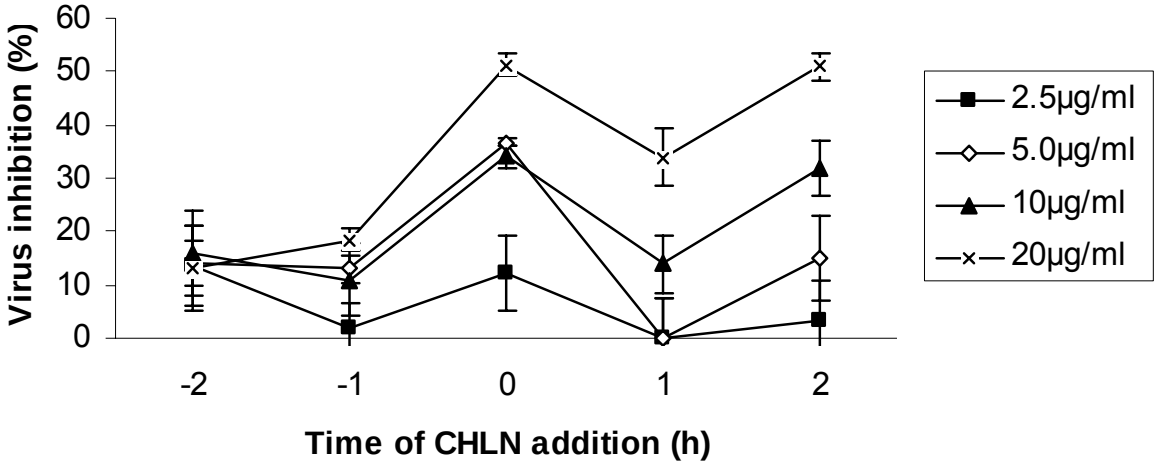

FIGURE 2

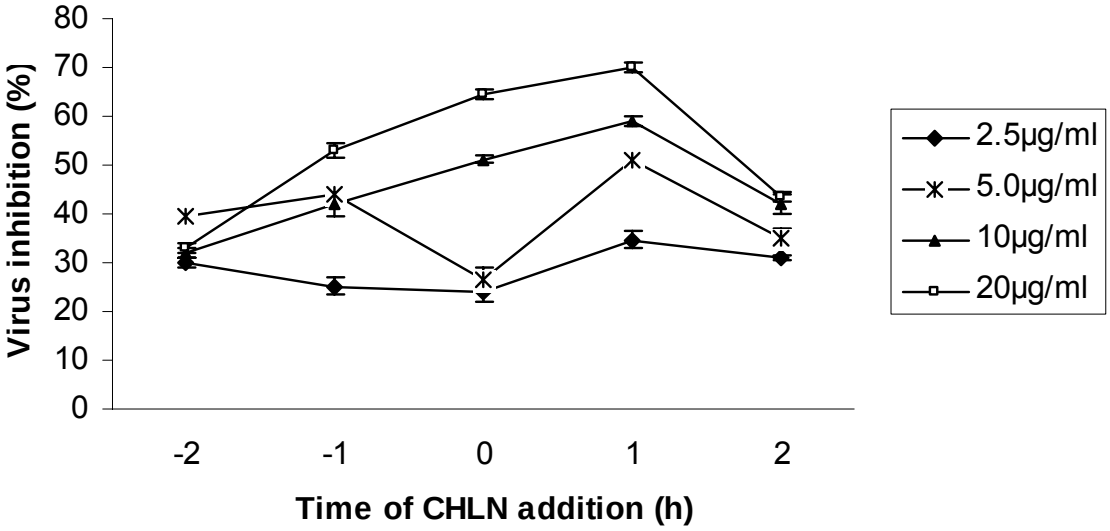

FIGURE 3

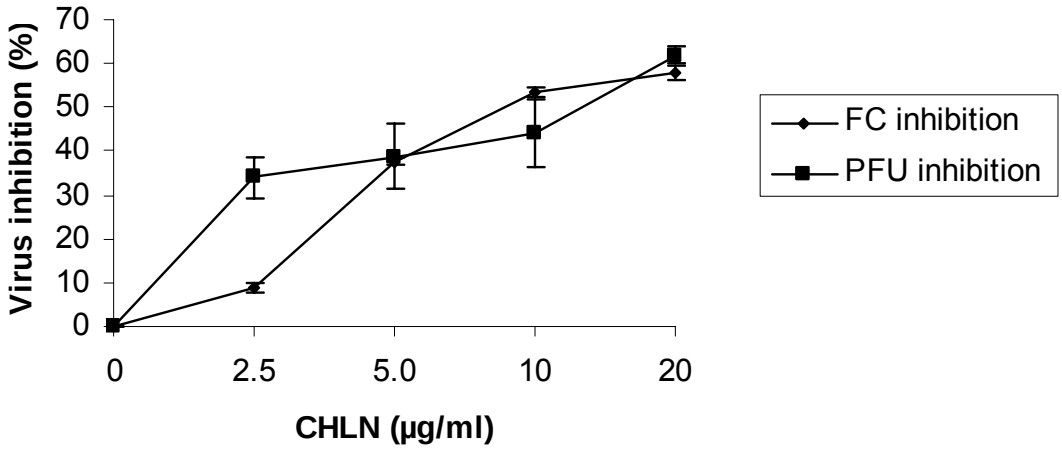

FIGURE 4

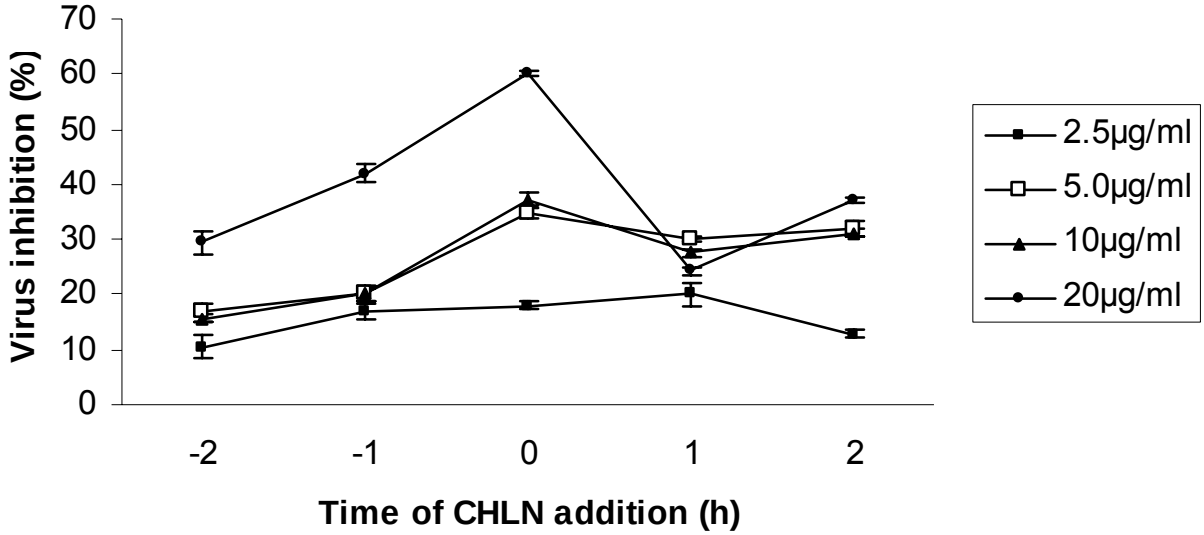

FIGURE 5

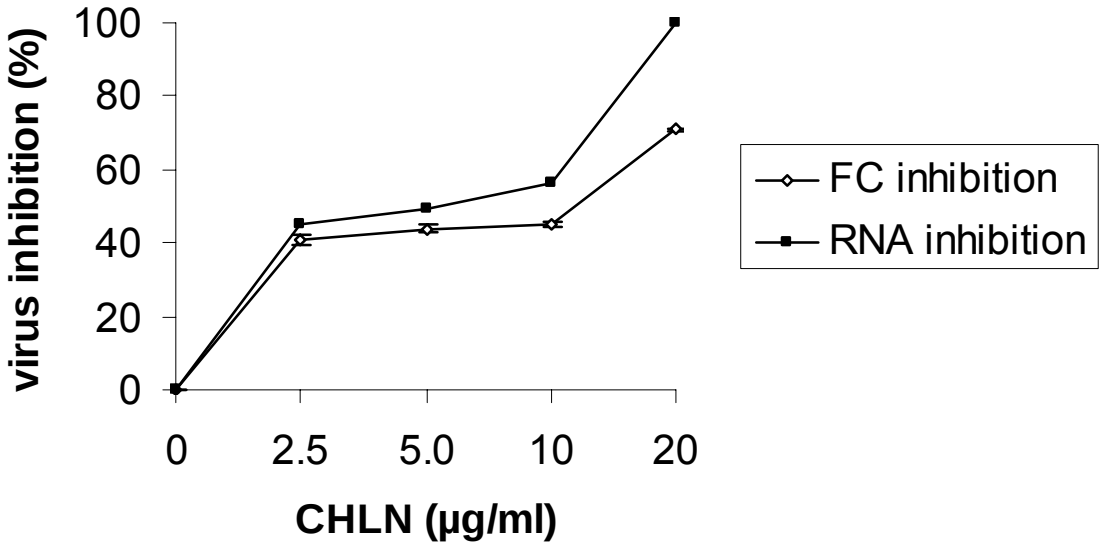

FIGURE 6

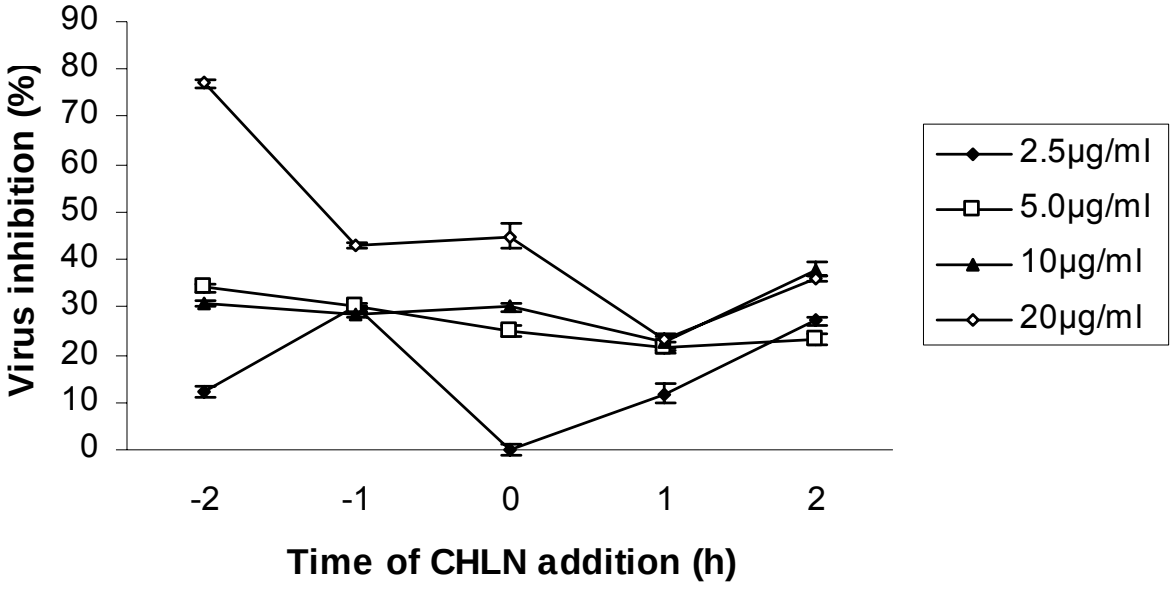

FIGURE 7

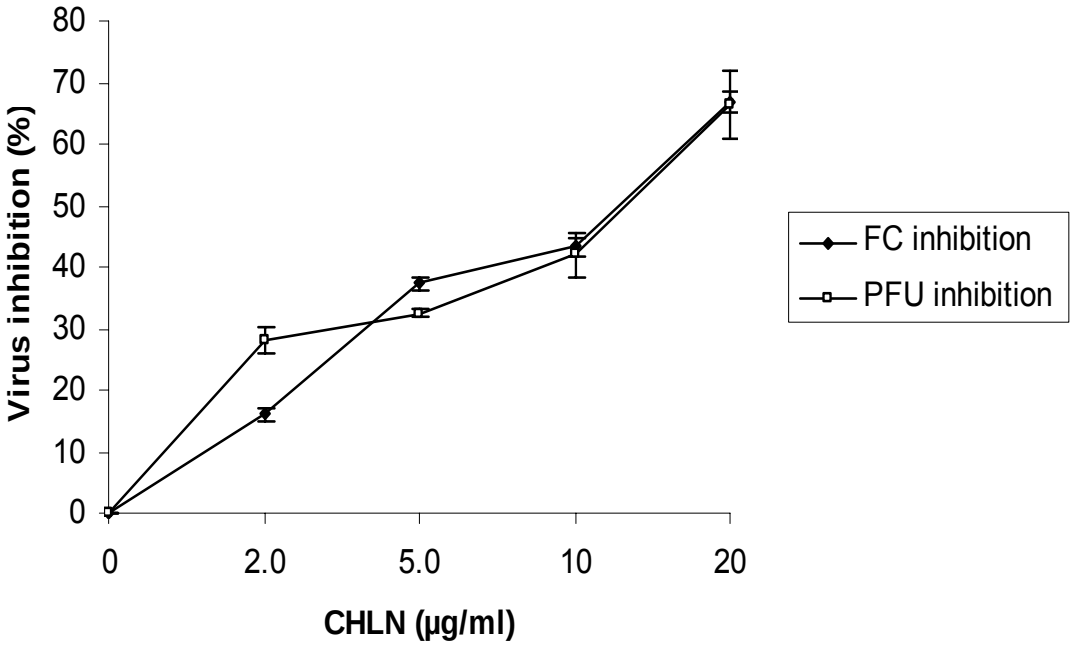

FIGURE 8

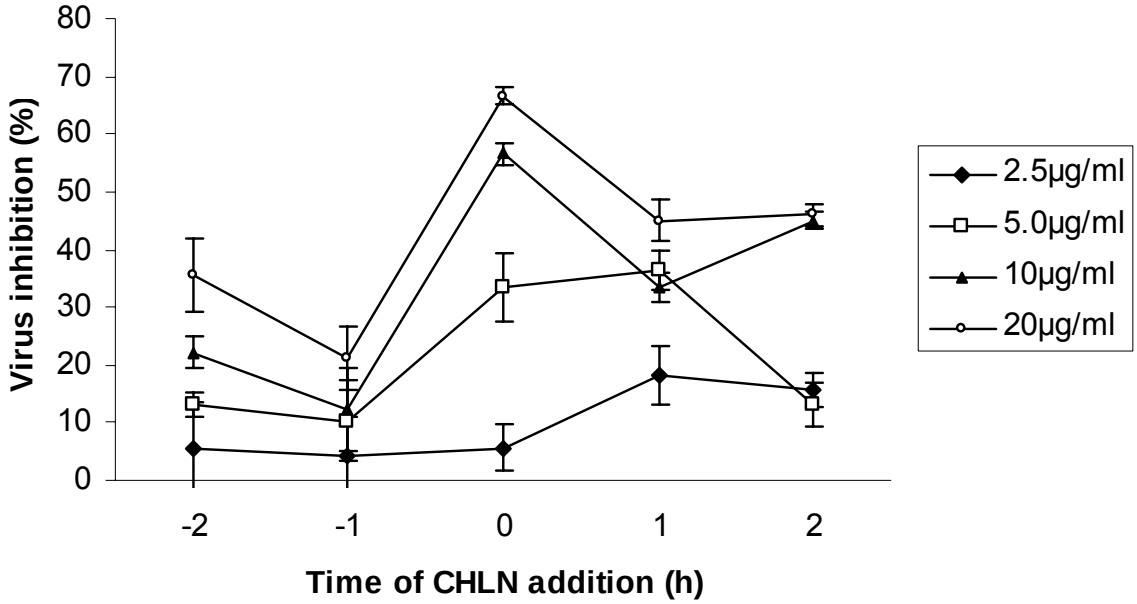

TABLE 1

Virus	Cell	Protocol	CC_{50}[a]	IC_{50}[b]	SI[c]
Poliovirus	HEp-2	T 2h (PFU)	285µg/ml	18.7µg/ml	15.2
		T 1h (IFA)	285µg/ml	5.0µg/ml	57.0
		Virucide (IFA)	285µg/ml	8.8µg/ml	32.1
		Virucide (PFU)	285µg/ml	12.7µg/ml	22.4
BHV-1	HEp-2	P-2h (IFA)	285µg/ml	15.2µg/ml	18.7
		T 0h (PFU)	285µg/ml	8.6µg/ml	33.1
		Virucide (IFA)	285µg/ml	8.6µg/ml	33.1
		Virucide (PFU)	285µg/ml	12.9µg/ml	22.0
Rotavirus	MA-104	T 0h (IFA)	180µg/ml	15.2µg/ml	11.8
		Virucide (IFA)	180µg/ml	8.0µg/ml	22.5
		Virucide (PAGE)	180µg/ml	5.3µg/ml	33.6

[a] CHLN cytotoxic concentration (CC_{50}) was determined by MTT kit assay and represents the minimum toxic concentration capable to inhibit cell viability in 50%.

[b] CHLN antiviral concentration (IC_{50}) was evaluated by plaque assay (PFU), immunofluorescence assay (IFA) or polyacrylamide gel electrophoresis (PAGE), and represents the minimum inhibitory concentration 50%.

[c] Ratio CC_{50}/IC_{50}