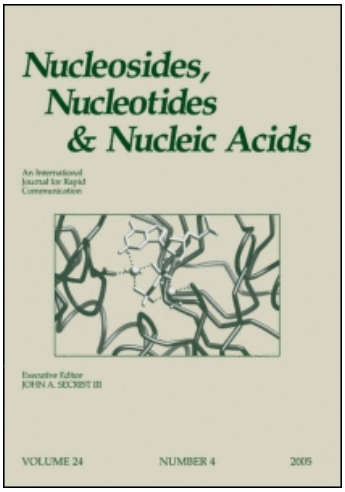

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PROPERTIES AND ANTI-HIV ACTIVITY OF NICKED DUMBBELL OLIGONUCLEOTIDES

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ABSTRACT: We have designed a new type of oligodeoxyribonucleotide. These oligodeoxyribonucleotides form two hairpin loop structures with base pairs (sense and antisense) in the double helical stem at the 3' and 5'-ends (nicked dumbbell oligonucleotides). The nicked dumbbell oligonucleotides are molecules with free ends that are more resistant to exonuclease attack. Furthermore, the nicked dumbbell oligonucleotide containing phosphorothioate (P=S) bonds in the hairpin loops has increased nuclease resistance, as compared to the unmodified nicked oligonucleotide. The binding of the nicked dumbbell oligonucleotide to RNA is lower than that of a single-stranded DNA. We also describe the anti-HIV activity of nicked dumbbell oligonucleotides.

INTRODUCTION

The therapeutic use of oligonucleotides as sense and antisense agents poses several problems, including the problem of molecular stability. Antisense oligonucleotides undergo nucleolytic degradation and are mainly sensitive to exonucleases. The first generation of antisense oligonucleotides, including phosphorothioates, has been used to inhibit viral as well as cellular gene expression.[1-7] However, phosphorothioate oligonucleotides are eventually degraded, primarily from the 3'-end. Recently, several

#This article is dedicated to Professor Yoshihisa Mizuno on the occasion of his 75th birthday.

stabilization methods have been proposed, such as including chemical substituents at the 3'-hydroxyl groups[8], and creating oligonucleotides containing a hairpin loop structure at the 3'-end[9]. A major interest in these oligonucleotides concerns their use as antisense compounds, due to their increased resistance to degradation by cellular exonucleases.

In this paper, we have studied the stability of a new type of nicked dumbbell oligonucleotide, with and without a phosphorothioate (P=S) group in the two hairpin loops at the 3'- and 5'-ends (Fig. 1). These oligomers have increased nuclease resistance as compared to their linear counterparts. However, the binding of these oligomers to the RNA was lower than that of a single-stranded DNA. Furthermore, of particular interest is the nicked dumbbell oligonucleotide containing phosphorothioate bonds in the two hairpin loops, which possessed higher anti-HIV activity than the linear antisense phosphorothioate oligonucleotide.

RESULTS AND DISCUSSION

Nuclease sensitivity of nicked oligonucleotides

Antisense phosphorothioate oligonucleotides complementary to viral RNA inhibit viral replication in cells cultured with Rous sarcoma virus[10], human immunodeficiency virus[11-13], vesicular stomatitis virus[14,15], herpes simplex virus[16], and influenza virus[17,18]. However, Agrawal[19] and Iverson[20] have reported that phosphorothioate oligonucleotides are degraded by only 15% in plasma, stomach, and heart after 24 h, whereas in the kidney and liver, degradation approaches 50% within 48 h. In order to overcome this problem, various modifications at the 3'-ends of the oligonucleotides have been tried.[8,9] In the present study, the oligonucleotides have been stabilized by two hairpin structures with the base pairs (sense and antisense) in the stem at the 3'- and 5'-ends.

Each nicked dumbbell oligonucleotide used in this study has its 3'- and 5'-ends within the base pairs in the stem (sense or antisense), and either phosphodiester (n) or phos-phorothioate (S) bonds (Fig. 1).

The nuclease sensitivities of the nicked dumbbell oligonucleotides (nick-1 (n) or (S) and nick-2 (n) or (S)) were studied. First, the 3'-exonuclease, snake venom phosphodiesterase (SVPD), was used for comparative digestion studies of the nicked dumbbell oligonucleotides, and the rate of digestion was determined by hyperchromicity (Fig. 2A). A comparison of the nuclease sensitivities of the oligonucleotides and the two hairpin oligonucleotides with the base pairs in the stem region at the 3'- and 5'-ends showed that the antisense oligonucleotide (anti-ODN, 5'-GGAGATGCCTAAGGC-3'), with the

35mer HIV-1 *rev* RNA 5'-CACAACAAAAGCCUUAGGCAUCUCCUAUGG-CAGGA-3' (5524, 5558)

sense-ODN 5'-GCCTTAGGCATCTCC-3'

anti-ODN 5'-GGAGATGCCTAAGGC-3'

anti-ODN(S) 5'-GsGsAsGsAsTsGsCsCsTsAsAsGsGsCs-3'

nick-1(n)
```
 CC CC GC CT TA GG CA TC TC CC CC C
C                                  C
 CCCCCGGAATC-5'  3'-CGTAGAGGCCCC
```

nick-2(n)
```
 CC CC GG AG AT GC CT AA GG CC CC C
C                                  C
 CCCCCCTCTACG-5'  3'-GATTCCGCCCC
```

nick-1(S)
```
 CsCs CsCs GC CT TA GG CA TC TC CCs CsCs Cs
Cs                                        Cs
 CsCsCsCsCGGAATC-5'  3'-CGTAGAGGCsCsCsCs
```

nick-2(S)
```
 CsCs CsCs GG AG AT GC CT AA GG CCs CsCs Cs
Cs                                        Cs
 CsCsCsCsCCTCTACG-5'  3'-GATTCCGCsCsCsCs
```

FIG. 1. The structure and sequences of the oligonucleotides used in this study, as described in the text.

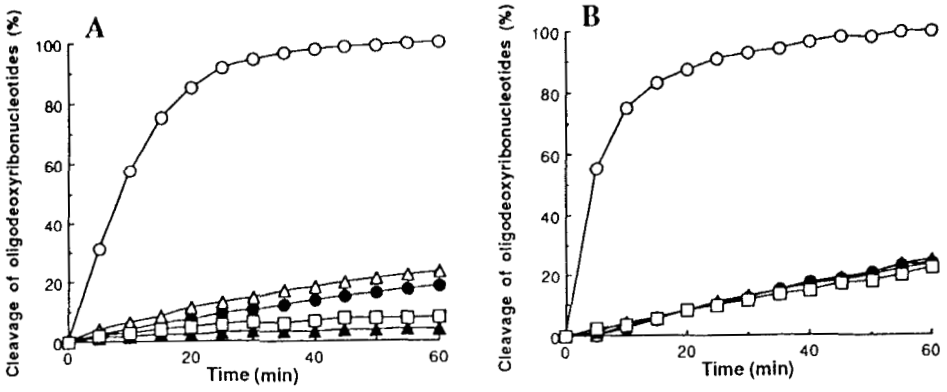

FIG. 2. Digestion of the linear- and nicked dumbbell-oligonucleotides containing phosphodiester (anti-ODN (○), nick-1(n) (●), nick-2 (n) (△)) or phosphorothioate (nick-1 (S) (▲), nick-2 (S) (□)) bonds with snake venom phosphodiesterase (A) and nuclease S1 (B).

sequence complementary to the *rev* splice acceptor site of HIV-1 mRNA[21] was digested extensively by SVPD within 43 min, whereas the nicked dumbbell oligonucleotides (nick-1 (n) and -2 (n)), were more stable. On the other hand, the nicked dumbbell oligonucleotides containing the phosphorothioate group in the two hairpin loops, nick-1 (S) and -2 (S), were considerably more stable than nick-1 (n) and -2 (n).

Similarly, the endonuclease activity of S1 digested the anti-ODN to mononucleotides in 30 min, whereas the nicked dumbbell oligonucleotides were very slowly digested (Fig. 2B). Similar results were obtained when the oligonucleotides anti-ODN, nick-1 (n), and nick-2 (n) were studied for their nuclease sensitivity in fetal bovine serum (Fig. 3). Thus, the nicked dumbbell oligonucleotides containing phosphodiester bonds are more resistant to exo- and endo-nucleases than the linear oligonucleotide (anti-ODN). Furthermore, the nicked dumbbell phosphorothioate oligonucleotides (nick-1 (S) and nick-2 (S)) were found to be more stable to nuclease as compared to their linear counterparts.

The results obtained by incubating the nicked dumbbell oligonucleotides in fetal bovine serum provided additional evidence of their relative stability (Fig. 3). The anti-ODN was digested extensively, whereas the nicked dumbbell oligonucleotides were digested slowly. The stability of the oligonucleotides increased with two hairpin loop structures with base pairs (sense and antisense) in the stem at the 3'- and 5'-ends. The nicked dumbbell phosphorothioate oligonucleotides showed more nuclease resistance than their counterparts containing phosphodiester bonds.

Hybridization between DNA and RNA

Hybridization between DNA and RNA is key to the antisense method, *in vivo* as well as *in vitro*. Even though the sequences confer different physical properties, we supposed that there are some differences between the linear-, the duplex-, and the nicked dumbbell-oligonucleotides containing phosphodiester and phosphorothioate bonds when they form duplexes with the parent strand, especially those with secondary or tertiary mRNA structures. The thermal stabilities of the base-stacked nicked dumbbell oligomer molecules were compared with that of double-stranded DNA (Table 1, Fig. 4). The double-stranded DNA has a Tm = 40 °C, whereas the nicked dumbbell oligonucleotides (nick-1 (n), -2 (n), -1 (S), and -2 (S) give an estimated Tm = 44-48 °C, an increase of about 4-8 °C. These results suggest that the increase in the stability depended on the two hairpin loop structures of the oligonucleotide at the 3'- and 5'-ends.

Next, we measured the melting temperatures of the oligonucleotide bound with the complementary 35 mer HIV-1 *rev* RNA (5'-CACAACAAAAGCCUUAGGCAUCUCC-

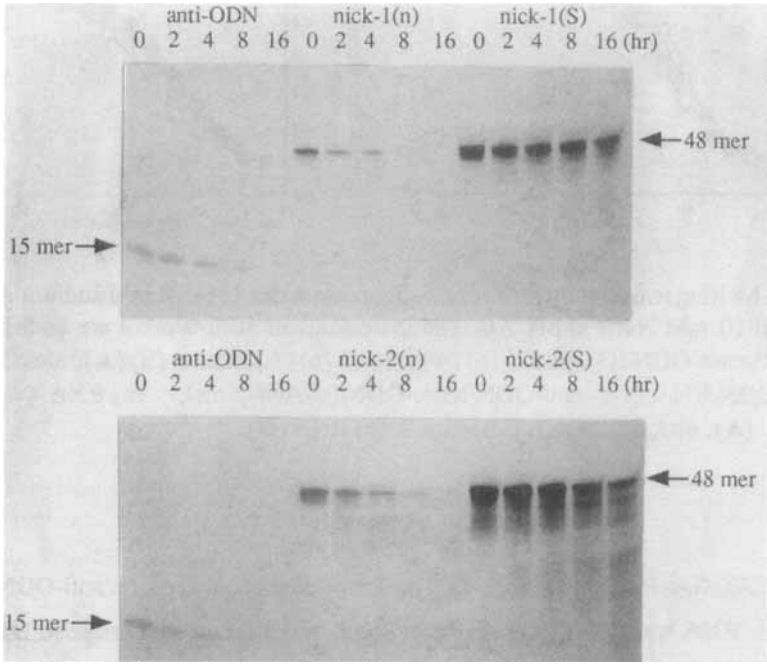

FIG. 3. Digestion of the anti-ODN, nick-1 (n), nick-2 (n), nick-1 (S), and -2 (S) oligonucleotides in the presence of 10% calf serum at 37 °C for 16 h.

TABLE 1. Melting temperatures of oligonucleotides.

Sequences	Tm (°C)[a] -RNA	+RNA
anti-ODN	-	55
double stranded (anti-ODN/sense-ODN)	40	41,53
nick-1 (n)	47	51
nick-1 (S)	48	50
nick-2 (n)	44	46
nick-2 (S)	47	49

a) Values were obtained in 10 mM sodium phosphate buffer and 10 mM NaCl at pH 7.0.

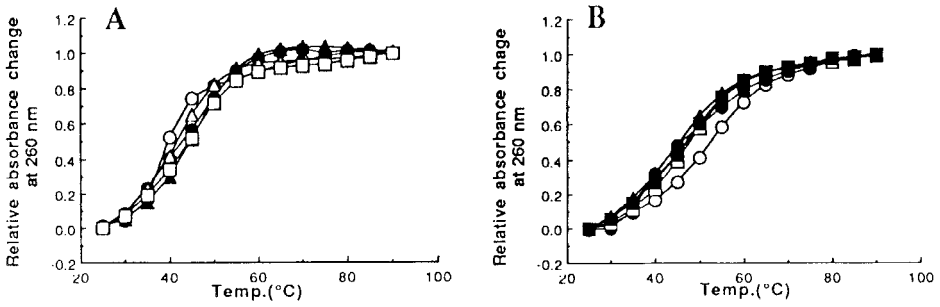

FIG. 4. Melting temperature profiles of oligonucleotides in in 10 mM sodium phosphate buffer and 10 mM NaCl at pH 7.0. The indications of each symbol are as follows: **A**, anti-ODN/sense-ODN (○), nick-1 (n) (●), nick-2 (n) (△), nick-1 (S) (▲), nick-2 (S) (□); **B**, anti-ODN/RNA (○), anti-ODN/sense-ODN/RNA(●), nick-1 (n)/RNA (△), nick-2 (n)/ RNA (▲), nick-1 (S)/RNA (□), nick-2 (n)/RNA (■).

UAUGGCAGGA-3') (Table 1, Fig. 4). The Tm of duplex between the anti-ODN and the 35 mer *rev* RNA was 55 °C. On the other hand, when the double-stranded DNA (anti-ODN/sense-ODN) was mixed with the 35 mer HIV-1 *rev* RNA, two transitions were observed: one, at 53 °C, which was typical of the duplex between the anti-ODN and the 35 mer HIV-*rev* RNA, and one at 41 °C, which coincided with the double-stranded DNA. In contrast, the nicked dumbbell oligonucleotides (nick-1 (n), -2 (n), -1 (S), and -2 (S)) did not undergo the two transitions (see Table 1, Fig. 4). Furthermore, when the 35 mer HIV-1 *rev* RNA was increased up to five molar equiv. under the above conditions, the Tms of the duplexes between the nicked dumbbell oligonucleotides and the 35 mer HIV-1 *rev* RNA approached the Tm of the duplex between the anti-ODN and the 35 mer HIV-1 *rev* RNA (54 °C) (data not shown). These results suggest that the nicked dumbbell oligonucleotides only partially bound to the complementary RNA.

RNase H cleavage.

Antisense oligonucleotide blockage involves the degradation of the RNA fragment bound to the antisense oligonucleotide by the reverse transcriptase-associated RNase H activity. The binding of the oligonucleotides and the nicked dumbbell oligonucleotides to the 35 mer HIV *rev* RNA using the RNase H cleavage reaction was also confirmed by RNase H cleavage. Fig. 5 shows that the specific cleavage of the 35 mer HIV-1 *rev* RNA by RNase H with the anti-ODN and nicked dumbbell oligonucleotides occurred at the different sites. Furthermore, the 35 mer HIV-1 *rev* RNA can be completely cleaved within

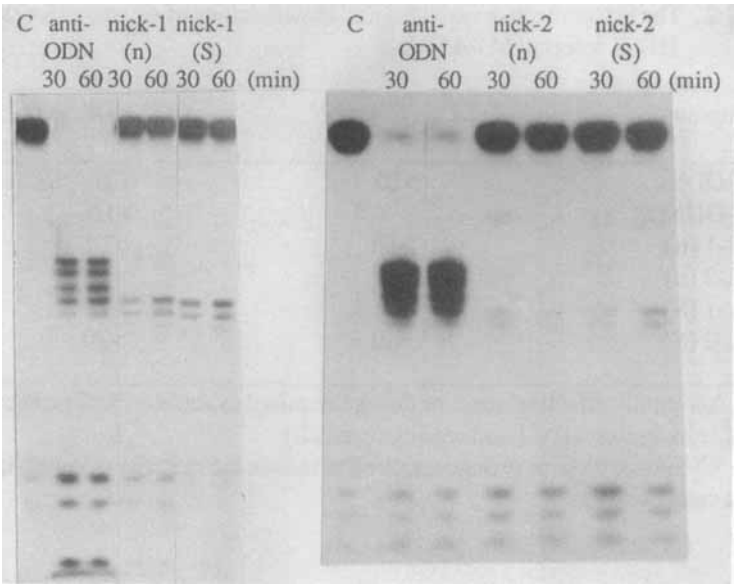

FIG. 5. Specific cleavage of the 35 mer HIV-1 *rev* RNA in the presence of oligonucleotides anti-ODN, nick-1 (n), nick-2 (n), nick-1 (S), and nick-2 (S) by RNase H. Lane C, the 35 mer HIV-1 *rev* RNA treated only with RNase H.

30 min. However, the 35 mer HIV-1 *rev* RNA is not completely cleaved by RNase H in the presence of the nicked dumbbell oligonucleotides within 30 min. The nicked dumbbell structure may influence the binding of oligonucleotides to the complementary RNA. This result was also confirmed by measurement of the melting temperatures of the oligonucleotides with the complementary 35 mer HIV-1 *rev* RNA.

Anti-HIV activity

The effects of the nicked dumbbell oligonucleotide derivatives on the replication of HIV-1 are listed in Table 2. We could not detect any inhibitory effects on virus replication with the nick-1 (n) and nick-2 (n) in HIV-1-infected MT-4 cells. Thus, at a defined concentration, the nicked dumbbell oligonucleotides seemed to remain on the cell surface, and they may be digested by nucleases in an endonucleolytic manner, rather than by exonucleolytic cleavage. In contrast, the antisense phosphorothioate oligonucleotide with the sequence complementary to the *rev* splice acceptor site of HIV-1 mRNA, with a chain length of 15 (anti-ODN(S), 5'-GsGsAsGsAsTsGsCsCsTsAsAsGsGsC-3'), showed activity (EC_{50} of 4.9 μM). However, to our surprise, the nick-1 (S) (EC_{50} of

TABLE 2. The cytoprotective activities and cytotoxicities of oligonucleotides in HIV-1 infected MT-4 cells.

Compound	EC_{50} (μM/ml)[a)]	CC_{50} (μM/ml)[b)]
anti-ODN	>10	>10
anti-ODN(S)	4.9	>10
nick-1 (n)	>10	>10
nick-2 (n)	>10	>10
nick-1 (S)	1.1	>10
nick-2 (S)	0.9	>10

a) 50% Antivirally effective dose, or dosage required to achieve 50% protection of MT-4 cells against HIV-1-induced cytopathicity.
b) 50% Cytotoxic dose or dosage required to reduce the viability of mock-infected MT-4 cells by 50%.

1.1 μM) and nick-2(S) (EC_{50} of 0.9 μM) containing phosphorothioate bonds in the hairpin loops had higher anti-HIV activity than the anti-ODN(S). Furthermore, these compounds exhibited no cytotoxicity at a 10 μM concentration.

Such oligonucleotides may be multifunctional, and could interact with several regions that are not adjacent to the target mRNA. These oligonucleotides may be used to interact specifically with protein factors that have an affinity for certain RNA or DNA sequences.

EXPERIMENTAL

Oligonucleotide Synthesis. The oligonucleotides were synthesized by means of the phosphoramide approach using an Applied Biosystems DNA synthesizer, Model 392. For the oligonucleotides containing a phosphorothioate bond in the two hairpin loops at the 3'- and 5'-ends, the oxidation step was substituted with a sulfurization procedure using Beaucage's reagent.[22] The oligonucleotide derivatives were purified by reverse phase HPLC on a YMCA column. Extinction coefficients of the oligonucleotides were determined by calculating the theoretical extinction coefficients as the sum of the nucleosides and multiplying with the experimental determined enzymatic hypochromicity.[23]

Nuclease Sability of Ncked Oligonucleotides. The 3'-exonucleolytic activity of snake venom phosphodiesterase (SVPD) was measured by hyperchromicity at 260 nm. The oligonucleotide (0.2 A_{260}) was dissolved in 0.7 ml buffer (10 mM Tris/HCl, pH 8.5,

10 mM $MgCl_2$, and 100 mM NaCl), incubated with SVPD at 37 °C in a thermally regulated cell of a UV spectrophotometer, and the A_{260} was recorded against time (Fig.2A). To study the resistance of the oligonucleotides to the endonucleolytic activity of nuclease S1, the oligonucleotide (0.2 A_{260}) was dissolved in 0.7 ml buffer (30 mM CH_3COONa, 280 mM NaCl, 1 mM $ZnSO_4$, pH 4.6), incubated with nuclease S1 at 37 °C in a thermally regulated cell of a UV spectrophotometer, and the A_{260} was recorded against time (Fig. 2B). For studying the resistance of the oligonucleotides to nucleases in fetal bovine serum, the oligonucleotides (0.2 A_{260}) were incubated with 200 μl of culture medium containing 10% fetal bovine serum for 16 h at 37 °C. Aliquots were taken at 0, 2, 4, 8, and 16 h and were analyzed by PAGE (20% polyacrylamide containing 7 M urea). Densitomeric analysis of gels stained with silver nitrate was performed on a Milipore BioImage 60 S (Fig. 3).

Thermal Denaturation Profiles. Thermal transitions were recorded at 260 nm using a Shimadzu UV-2200A spectrometer. The insulated cell compartment was warmed from 25 °C to 90 °C, with increments of 1 °C and equilibration for 1 min after attaining each temperature, using a temperature controller SPR-8 (Shimadzu). Samples were heated in masked, 1 cm path length quartz cuvettes fitted with Teflon stoppers. Each thermal denaturation was performed in 10 mM sodium phosphate buffer (pH 7.0) and 10 mM NaCl, containing 0.5 μM of each strand (35 mer HIV-1 *rev* RNA-anti-ODN, sense-ODN-anti-ODN, nick-1 (n) or nick-2 (n)-35 mer HIV-1 *rev* RNA, and nick-1 (S) or nick-2 (S)-35 mer HIV-1 *rev* RNA). The mixture of duplex and single strands was kept at 90 °C for 10 min, and was then cooled to 4 °C.

RNase H Activity. 5'-^{32}P labeled *rev* RNA (5'CACAACAAAAGCCUUAGGCAU-UCCUAUGGCAGGA3') (1 pmole) was mixed with oligonucleotides (10 pmole) in 30 μl of 20 mM Tris/HCl (pH 7.5), 10 mM $MgSO_4$, 0.1 mM DTT, and 100 mM KCl. RNasin (40 units) and *E.coli* RNase H (0.5 μl, 0.4 units) were added to the mixture, which was incubated at 37 °C. Aliquots were taken at 30 and 60 minutes and were analyzed by PAGE (20% polyacrylamide containing 8.3 M urea) followed by autoradiography (Fig. 5).

Cell Lines. The human T lymphotropic virus type I (HTLV-I)-positive human T cell line, MT-4, was subcultured twice weekly at a density of 3 X 10^5cells/mL in RPMI-1640

medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100IU/mL penicillin, and 100 mg/mL of streptomycin.

Virus. The HTLV-IIIB strain was used in the anti-HIV assay. The virus was prepared from the culture supernatants of MOLT-4/HTLV-IIIB cells, which were persistently infected with HTLV-IIIB. HIV stocks were titrated in MT-4 cells as determined by 50% tissue culture infectious doses ($TCID_{50}$) and plaque forming units, and were stored at -80 °C until use.

Anti-HIV assay. The anti-HIV activity of test compounds in a fresh, cell-free HIV infection was determined as protection against HIV-induced cytopathic effects (CPE). Briefly, MT-4 cells were infected with HTLV-IIIB at a multiplicity of infection (MOI) of 0.01. HIV-infected or mock-infected MT-4 cells (1.5×10^5/mL, 200 μL) were placed into 96 well microtiter plates and were incubated in the presence of various concentrations of test compounds. The dilution ranged from one- to five-fold and nine concentrations of each compound were examined. All experiments were performed in triplicate. After a 5 day incubation at 37 °C in a CO_2 incubator, the cell viability was quantified by a calorimetric assay that monitored the ability of the viable cells to reduce 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to a blue formazan product. The absorbances were read in a microcomputer-controlled photometer (Titertek Multiskan^R; Labsystem Oy, Helsinki, Finland) at two wavelengths (540 and 690 nm). The absorbance measured at 690 nm was automatically subtracted from that at 540 nm, to eliminate the effects of non-specific absorption. All data represent the mean values of triplicate wells. These values were then translated into percentage cytotoxicity and percentage antiviral protection, from which the 50% cytotoxic concentration (CC_{50}), the 50% effective concentration (EC_{50}), and the selectivity indexes (SI) were calculated.[24,25]

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