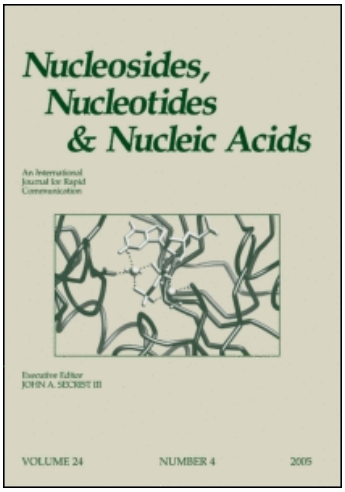

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Novel Guanosine Quartet Structure Binds to the HIV Envelope and Inhibits Envelope Mediated Cell Fusion

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NOVEL GUANOSINE QUARTET STRUCTURE BINDS TO THE HIV ENVELOPE AND INHIBITS ENVELOPE MEDIATED CELL FUSION

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Abstract

We have identified a phosphorothioate oligonucleotide $T_2G_4T_2$, which is a potent inhibitor of HIV infection *in vitro*. The compound was identified by combinatorial screening of a library of all possible octanucleotide sequences. The oligonucleotide forms a parallel-stranded, tetrameric guanosine-quartet (G-quartet) structure which specifically binds to the HIV envelope glycoprotein (gp120) and inhibits both cell-to-cell and virus-to-cell infection at submicromolar concentrations. In the current study we demonstrate that the tetramer inhibits the infection of laboratory-derived isolate of HIV-1 and HIV-2 in a variety of phenotypically distinct established human cell lines and a panel of biologically diverse clinical isolates in fresh human peripheral blood lymphocytes and macrophages. The compound was also active against all drug-resistant virus isolates tested. In combination with AZT, ISIS 5320 exhibits additive to slightly synergistic anti-HIV activity. Cell-based mechanism of action studies demonstrate that the compound inhibits the binding of infectious virus and virus-infected cells to uninfected target cells by binding to the cationic V3 loop of the envelope glycoprotein. The G-quartet structure is a potential candidate for use in anti-HIV chemotherapy.

Discovery

Specific binding of nucleic acids to proteins regulates cellular functions at many levels. Recently the notion of using synthetic nucleic acids as ligands to modulate the activities of proteins for therapeutic purposes has received considerable attention. Several *in vitro* strategies have been developed to selectively screen for nucleic acid sequences that bind to specific proteins[1,2], including proteins that are not naturally bound by nucleic acids.[3] Selection techniques have also been used with libraries of peptides.[4,5]

A selection strategy, **s**ynthetic **u**nrandomization of **r**andomized **f**ragments (SURF)[6], was employed to screen libraries of oligonucleotides for antiviral activity in HIV-infected cell culture. This type of selection technique involves iterative synthesis and screening of

increasingly simplified sets of oligonucleotide pools. Oligonucleotide analogs can be used in the libraries because iterative synthesis and screening techniques do not require enzymes either to synthesize the randomized pools or to screen for the active molecular species. The use of nuclease-resistant oligonucleotide analogs allows screening for functional activities in cell culture where unmodified RNA or DNA would be rapidly degraded. Screening of compound libraries in antiviral assays in the presence of whole viruses and cells provides the opportunity to screen "a library of compounds" against "a library of targets." Any step in the viral life cycle could potentially be inhibited, thus increasing the chances for finding an active antiviral compound. Since functional inhibition of viral infection rather than nucleic acid binding is the selection criterium, the chances are enhanced that a useful compound will be identified. Once a functionally active compound is identified, the structure of the active compound, its molecular target site and mechanism of action must all be determined.

A phosphorothioate oligonucleotide library containing all possible 8 nucleotide sequences divided into 16 sets, each consisting of 4,096 sequences, was screened for inhibition of HIV infection using an acute infection model [7]. In the initial rounds of selection antiviral activity was observed only in the set containing guanosine in two fixed positions (Table 1). Subsequent rounds of selection showed that four consecutive Gs were required for maximum antiviral activity. No strong selection preference was observed for nucleotides flanking the guanosine core. The sequence TTGGGGTT (ISIS 5320) was chosen for further study because the structures of similar sequences have been well characterized [8-11]. The structure and mechanism of antiviral activity of ISIS 5320 are described in Wyatt *et al.* [12].

Structure

G-quartet structures are intra- or inter-molecular four-stranded helices stabilized by planar Hoogsteen-paired quartets of guanosine [14, 15] (Figure 1)*. The structures are stabilized by monovalent ions, preferentially potassium rather than sodium, bound between two planes of G-quartets and coordinated to the carbonyl oxygens. NMR studies of an RNA oligonucleotide UGGGGU[8] and deoxy oligonucleotides with cores of four guanosines [9, 10, 11] showed that these oligonucleotides form four-stranded, parallel helices with *anti* glycosidic torsion angles and equivalent grooves. The oligonucleotides in the combinatorial library pools that show antiviral activity (Table 1) form high molecular weight species as shown by size exclusion chromatography. Although the oligonucleotide ISIS 5320 has a phosphorothioate backbone, evidence suggests that it adopts a four-stranded, parallel helix as do phosphodiester oligonucleotides of similar sequence [12].

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* Figure 1 appears on page 1125.

TABLE 1

Activity of phosphorothioate oligonucleotide pools in acute HIV assay

Combinatorial Pools	IC_{50} (mM)			
	X=A	X=G	X=C	X=T
Round 1				
NNA NXN NN	inactive	inactive	inactive	inactive
NNG NXN NN	inactive	**19.5** (5%)	inactive	inactive
NNC NXN NN	inactive	inactive (0%)	inactive	inactive
NNT NXN NN	inactive	inactive	inactive (0%)	inactive
Round 2				
NNG XGN NN	60.7	**1.8** (36%	55.6	56.2 (3%*)
Round 3				
NNG GGX NN	8.0	**0.5** (94%)	3.1 (19%*)	8.6
Round 4				
NAG GGG XN	0.5	0.5	0.5	0.5 (87%)
NGG GGG XN	0.5	0.6 (99%*)	0.4	0.5
NCG GGG XN	0.7	0.6	0.5 (91%)	0.4
NTG GGG XN	0.4 (82%)	0.5	0.4	0.5
Round 5				
XTG GGG TN	0.2 (94%)	0.6 (89%*)	0.3 (94%)	0.3 (94%)
Round 6				
TTG GGG TX	0.6 (90%)	0.6	0.5	**0.3** (93%)

Phosphorothioate oligonucleotides were synthesized using standard protocols [13]. Random positions, N, are an equimolar mixture of each base. Antiviral data are reported as the quantity of drug (in mM of oligonucleotide strand) required to inhibit 50% of virus-induced cell killing (IC_{50}) in an acute HIV-1 infection assay which measures protection from HIV-induced cytopathic effects [7]. "Inactive" pools showed no antiviral activity at 100 mM strand concentration. The % tetramer, determined by size exclusion chromatography is given in parentheses for selected pools. An asterisk indicates that multiple aggregate species were observed.

The G-quartet structure is responsible for antiviral activity. For eight-residue phosphorothioate oligonucleotides, at least four guanosines in a row are necessary for inhibition of HIV infection and oligonucleotides containing the same bases as ISIS 5320 but with different sequence are inactive (Table 2). Heat denaturation of the tetrameric complex formed by ISIS 5320 before addition to the antiviral acute assay resulted in loss of activity; antiviral activity was recovered upon renaturation. The striking difference in antiviral activity among the initial set of 16 sets of oligonucleotides used for combinatorial screening can be explained by the presence or absence of the G-core and therefore the tetramer structure (Table 1). In the active 5'NNGNGNNN3' pool, approximately 12% of the molecules contain at least four sequential Gs, and size exclusion chromatography showed that 5% of pool formed tetramers (Table 1). In contrast, in the other three round 1 pools where X=G,

TABLE 2

Activity of Analogs of ISIS 5320 in Acute HIV Assay

Sequence	Compound#	IC_{50} (mM)	%Tetramer
TsTsGsGsGsGsTsT	5320	0.3	98
TsTsGsGsGsGsTsT--heat denatured	5320	inactive	0
TsTsGsGsGsGsGsGsTsT	6766	0.6	63
TsTsTsGsGsGsTsT	5543	inactive	0
GsGsGsGsTsT	5952	0.5	94*
GsGsGsGsT	4943	1.4	61*
GsGsGsG	4803	4	29*
TsTsGsGsGsG	5739	13	40*
TsGsGsGsG	5544	inactive	57*
TsGsGsGsGsT	5804	3.4	78
ToToGoGoGoGoToT	5671	inactive	93
TsTsGoGoGoGsTsT	6339	5.0	80
ToToGsGsGsGoToT	6423	inactive	72
a-TsTsGsGsGsGsTsT	7282	0.5	98
a-ToToGoGoGoGoToT	7283	inactive	97

Data from the acute HIV assay (see legend for Table 1) for sequence variants and analogs of ISIS 5320. Chemical modifications of the oligonucleotide are indicated: "s", phosphorothioate backbone; "o", phosphodiester; "a", a-configuration of the glycosidic bond; "B", biotin (incorporated during chemical synthesis using biotin-linked CPG from Glen Research). Inactive oligonucleotides showed no activity at 25 mM concentration. The % tetramer was determined by size exclusion chromatography. An asterisk indicates that more than one aggregate species.

only 0.4% of the molecules contain at least four sequential Gs ,and, in other pools, there are no molecules with four consecutive Gs.

While oligonucleotides containing three or more G's can form parallel-stranded tetramers [16], our studies show that at least four G's are required for antiviral activity (Table 2). Additional G's did not increase activity; when the number of guanosines was increased to six (ISIS 6766), there was a loss of activity roughly equivalent to the decrease in amount of tetramer. The phosphorothioate GGGG (ISIS 4803) shows some antiviral activity; however, addition of two nucleotides on the 3' end of the oligonucleotide were required for nearly optimal activity. Oligonucleotides with the quartet core exposed on either the 3' or 5' end form more than one aggregate making them more difficult to characterize structurally. Superstructures formed by G-rich oligonucleotides with terminal guanosines have been observed by others [17, 18]

The sequence TTGGGGTT with a phosphodiester backbone is inactive in the anti-HIV assay. Thus the G-quartet structure is not sufficient for antiviral activity. Two

hypotheses were considered: the thioate backbone may be mechanistically required, or the modified backbone may prevent nuclease degradation of the oligonucleotide. Oligonucleotide analogs with the glycosidic torsion angle oriented in the a-configuration are resistant to nuclease degradation. Both the phosphorothioate a-analog (ISIS 7282) and the phosphodiester a-oligonucleotide (ISIS 7283) form tetramers. Only the phosphorothioate analog was active against HIV. Thus, the phosphorothioate is structurally required for antiviral activity.

Activity Against Clinical Isolates and Drug Resistant Strains

ISIS 5320 was discovered in the acute HIV-IIIB screen, where the antiviral IC_{50} approximates 0.3 μM and the IC95 is in the 1-3 μM range. It is active against both lymphotropic and monocytotropic strains. The compound has been tested against a panel of clinical HIV isolates (both NSI and SI strains). The compound was active against 5 clinical isolates with an IC_{50} in the 1-2 μM range (Table 3). ISIS 5320 was also active against a panel of drug resistant strains with IC_{50}'s in the sub-micromolar range (Table 4).

Mechanism of Action

The oligonucleotide ISIS 5320 acts at an early stage of the viral infection; ISIS 5320 had no effect on chronically infected cell models that respond only to inhibitors that work at post integration steps (data not shown). In a high multiplicity of infection (MOI) experiment[19], ISIS 5320 inhibited production of intracellular PCR-amplifiable DNA (data not shown), which indicates that the compound inhibits an early step of HIV replication, such as binding, fusion, internalization, or reverse transcription.

We found that ISIS 5320 directly inhibits binding or fusion in two types of assays. Inhibition of syncytium formation was observed between cells expressing viral protein gp120 and cells expressing the cell surface receptor for the virus, CD4. ISIS 5320 inhibits cell-to-cell spread of HIV Infection.

ISIS 5320 also inhibited binding or fusion of infectious virus to CD4+ cells. Cells were incubated for 15 minutes with oligonucleotide at 37°C prior to the addition of virus. After 1 hour, the cells were washed extensively to remove unbound virus and oligonucleotide. During the incubation period, only virus binding and membrane fusion events occur [19]. Extent of infection after 48 hours was determined as previously described[20]. ISIS 5320 inhibited binding; virus production was 50% of control without oligonucleotide at a concentration of approximately 0.4 mM (Figure 2)*. Heat-denatured ISIS 5320 and a phosphorothioate control (ISIS 6071) both showed non-specific inhibition, with

* Figure 2 appears on page 1126.

TABLE 3

Activity of ISIS 5320 Against Clinical HIV-1 Strains in Human Peripheral Blood Mononuclear Cells

Strain	Type	Growth	IC_{50}
WEJO	SI	Rapid/High	1.6
ROJO	SI	Rapid/High	2.0
BAKI	SI	Rapid/High	1.1
VIHU	NSI	Slow/Low	1.8
WOME	SI	Rapid/High	1.7

TABLE 4

Activity of Compounds Against Drug-Sensitive and Drug-Resistant Virus Strains

Virus	Resistance	Cell	IC_{50}
RF	None	CEM	0.96
RF	None	MT2	0.20
A17	Pyridinone	MT2	1.46
G910-6	AZT	MT2	0.27
N119	Nevirapine/AZT	CEM	0.29
HIV-2 ROD	None	CEM	0.84

50% inhibition at about 5 mM. These fusion and binding inhibition experiments strongly suggest that ISIS 5320 inhibits a very early step in viral infection, either during binding of the virion to the cell or during the early events of fusion and internalization of the virion.

Molecular Target

ISIS 5320 binds to the V3 loop of viral envelope protein gp120. The V3 loop of gp120 is considered the principal neutralizing domain of the protein; peptides derived from this region elicit type-specific neutralizing antibodies that block viral infection by blocking fusion [21]. The V3 loop of gp120 (amino acids 303-338) is also the site of action of anionic polysaccharides, such as dextran sulfate, that inhibit viral binding, replication and syncytium formation [22]. Although the V3 loop is a highly variable region of gp120, a high percentage of positively charged amino acids is maintained across HIV strains. The positive amino acid side chains are considered functionally essential for fusion between the virion and cell membranes [22]. Fast replicating syncytium-inducing isolates contain V3 loops with higher density of positive charge than non syncytium-inducing strains [23].

Experimental proof for the V3 binding mechanism includes direct viral binding and fusion experiments, direct envelope binding experiments, V3 loop antibody binding inhibition, and competition experiments with dextran sulfate.

Mechanism and Development of Resistance

Recent experience has shown that some HIV targets are more vulnerable to the development of resistance than others. The basis for the antiviral activity of ISIS 5320 is primarily its interaction with the cationic amino acid side chains in the V3 loop of the viral envelope protein gp120. The positively charged nature of the V3 loop is believed to be essential for mediation of fusion between the virion and cell membrane. Thus, resistance to ISIS 5320 may be linked to the development of less or non-infectious virions.

To date ISIS 5320 has been active against every strain of HIV that has been tested (Table 3 and 4). This includes a panel of 5 clinical isolates (both NSI and SI strains) and strains resistant to AZT, nevirapine, pyridinone and strains of HIV-2. Experience has shown that *in vitro* resistance to other viral binding inhibitors (with the exception of V3 loop antibodies) has been difficult to develop. Repetitive passage experiments to determine if HIV can develop resistance to ISIS 5320 were initiated in September, 1993 and are currently in progress. No resistance has been developed in eight passages.

Specificity of ISIS 5320 for HIV

ISIS 5320 was discovered using an iterative *in vitro* selection strategy (SURF) which tailors the molecule for optimal activity against the target. This was followed by a medicinal chemistry structure-activity optimization in which approximately 100 variants were synthesized and tested before selection of the 8-mer ISIS 5320. The antiviral activity of ISIS 5320 is specific for HIV. Other viruses tested were not inhibited in the sub-micromolar range (Table 5).

Synergy with AZT

To evaluate the activity against HIV of ISIS 5320 in combination with AZT, drug combination assays were performed according to the method of Prichard and Shipman [24]. Combination antiviral assays were performed with CEM-SS cells utilizing the III_B strain of virus.

Combination antiviral assays indicated that ISIS 5320 and AZT synergistically or additively inhibit HIV replication. *No antagonism or enhanced cytotoxicity was detected in any of these assays.* Moderate synergistic inhibition was noted at all concentrations of AZT tested in combination with ISIS 5320 at 0.54 mM. At lower concentrations of ISIS 5320, combination with AZT yielded additive anti-HIV activity.

TABLE 5

Activity Against Several RNA and DNA Viruses

Virus	IC_{50} (mM) ISIS 5320 (TTGGGGTT)	ISIS 6071 (TGTGTGTG)
RNA Viruses		
HIV	0.4	>50
Influenza Virus	40	>100
DNA Viruses		
HSV-2	4	>100
VZV	>100	>100
Adenovirus	>100	>100
Vaccinia Virus	>100	>100

Conclusions

The SURF combinatorial strategy was effective in identifying an *in vitro* inhibitor of HIV. This is the first example that we are aware of in which a combinatorial screening strategy selected an intermolecular noncovalently assembled complex. The G-quartet structure was selected because of the presence of the intermolecular structure in the active pool and its exclusion from the inactive pools based on sequence constraints. Neither the structure of the active oligonucleotide, nor its mechanism of action, nor the molecular target site on HIV was anticipated. Combinatorial screening techniques using novel diverse chemical structures coupled with relevant assay strategies hold great promise in drug discovery.

The tetrameric form of phosphorothioate $T_2G_4T_2$ blocks cell-to-cell and virion-to-cell spread of HIV infection by binding to the gp120 V3 loop. The tetramer provides a rigid, compact structure with a high thio-anionic charge density that may be the basis for its strong interaction with the cationic V3 loop. Although the V3 loop is a hypervariable region, the functional requirement for cationic residues in the V3 loop may limit the virus' ability to become resistant to dense polyanionic inhibitors. Compounds derived from the G-quartet structural motif are potential candidates for use in anti-HIV chemotherapy.

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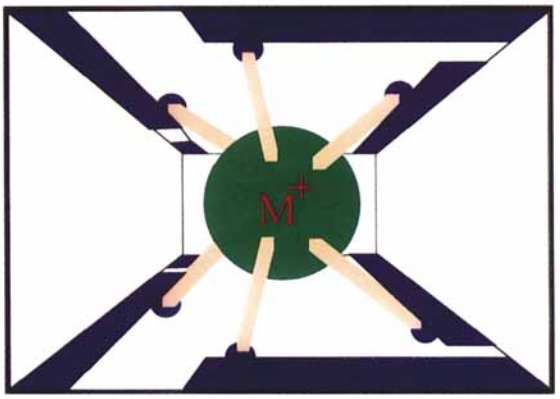
(a)



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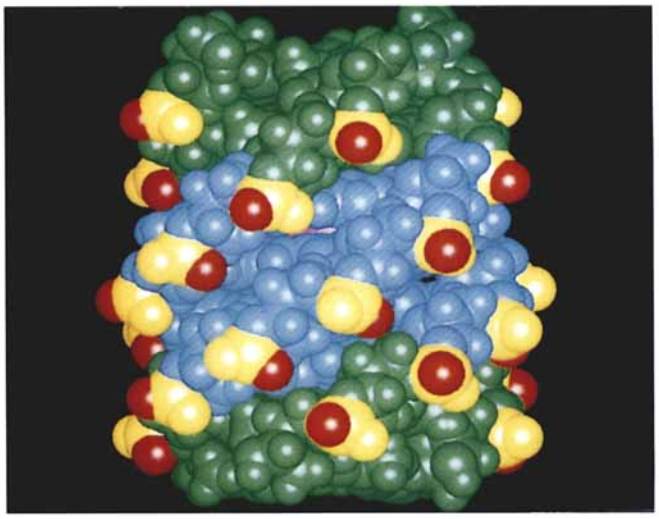

(c)

Figure 1 (a) The G-quartet is a cyclic hydrogen bonded array of four guanine bases. G-quartets may be stacked upon each other to form quadruple helices. (b) Monovalent cations, preferably potassium, are bound between the quartets. ISIS 5320 is a four-stranded parallel G-quartet structure. This illustration was adapted from Williamson (1990)[15]. (c) ISIS 5320 model. Thymidine residues are shown in green, guanosine residues are shown in blue and the phosphorothioate groups are shown in yellow and red (oxygen).

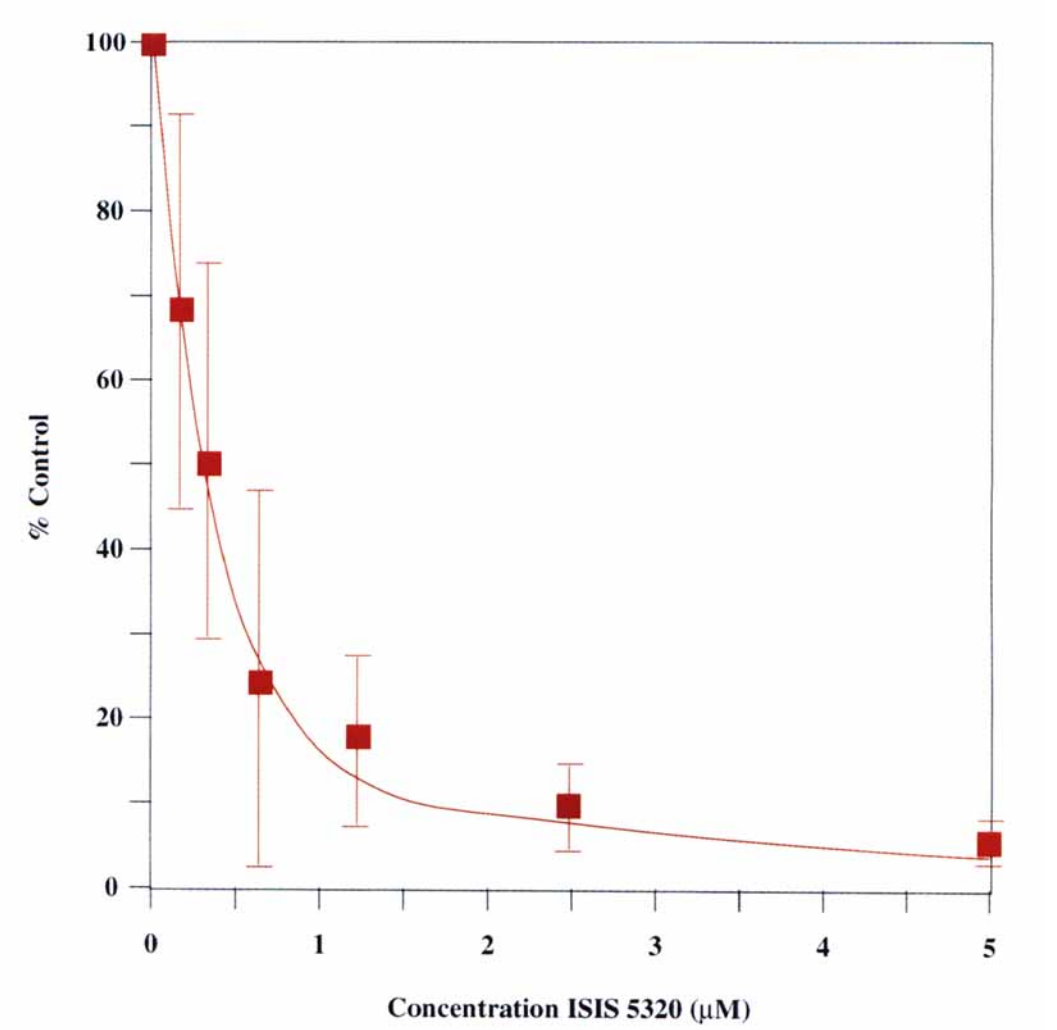

Figure 2 ISIS 5320 inhibits the binding of infectious virus to a CD4-expressing cell. Inhibition of binding was quantitated using the HeLa-CD4-LTR-b-gal cell line. 1 x 10^4 cells were plated in a 96-well microtiter plate and allowed to grow overnight. Drug was added at the appropriate concentration for 15 minutes prior to the addition of infectious HIV-1. The MOI of infection was predetermined to provide between 50 and 100% syncytium formation in virus control wells. After a one hour incubation, the cell monolayer was washed extensively to remove all unbound virus and extracellular compound. At 48 hours, the number of syncytia were quantitated by microscopic observation and ß-galactosidase ELISA.

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