Cytotoxicological Analysis of a gp120 Binding Aptamer with Cross-Clade Human Immunodeficiency Virus Type 1 Entry Inhibition Properties: Comparison to Conventional Antiretrovirals

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The long-term cumulative cytotoxicity of antiretrovirals (ARVs) is among the major causes of treatment failure in patients infected with human immunodeficiency virus (HIV) and patients with AIDS. This calls for the development of novel ARVs with less or no cytotoxicity. In the present study, we compared the cytotoxic effects of a cross-clade HIV type 1-neutralizing aptamer called B40 with those of a panel of nonnucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs), and the entry inhibitor (EI) T20 in human cardiomyocytes and peripheral blood mononuclear cells. An initial screen in which cell death was used as the end-point measurement revealed that the B40 aptamer and T20 were the only test molecules that had insignificant (0.61 < P < 0.92) effects on the viability of both cell types at the maximum concentration used. PIs were the most toxic class (0.001 < P < 0.00001), followed by NNRTIs and NRTIs (0.1 < P < 0.00001). Further studies revealed that B40 and T20 did not interfere with the cellular activity of the cytochrome P450 3A4 enzyme (0.78 < P < 0.24) or monoamine oxidases A and B (0.83 < P < 0.56) when the activities of the enzymes were compared to those in untreated controls of both cell types. Mitochondrion-initiated cellular toxicity is closely associated with the use of ARVs. Therefore, we used real-time PCR to quantify the relative ratio of mitochondrial DNA to nuclear DNA as a marker of toxicity. The levels of mitochondrial DNA remained unchanged in cells exposed to the B40 aptamer compared to the levels in untreated control cells (0.5 > P > 0.06). These data support the development of B40 and related EI aptamers as new ARVs with no cytotoxicity at the estimated potential therapeutic dose.

The introduction of highly active antiretroviral therapy (HAART) has significantly reduced the rates of morbidity and mortality among patients infected with human immunodeficiency virus (HIV) and patients with AIDS. HAART, however, requires life-long treatment and results in toxicity in up to 50% of patients following 6 to 12 months of therapy (4, 53). Treatment failure arising from toxicity has highlighted the need for close medical supervision and, ultimately, the development of novel, less toxic antiretrovirals (ARVs). This is of even greater concern on the resource-poor African continent, where, unlike in developed countries, monitoring for and diagnosis and management of ARV-associated toxicity are not routinely conducted (54). This problem is likely to increase as the use of ARVs becomes more widespread in resource-poor settings (3).

Mitochondrial toxicity is one of the major complications associated with the long-term use of HAART (6, 7, 37, 38, 67, 68). The nucleoside reverse transcriptase inhibitors (NRTIs) used in HAART inhibit DNA polymerase γ, which is solely responsible for mitochondrial DNA (mtDNA) replication (47). Through this mechanism, NRTIs induce the depletion of mtDNA as well as that of mtDNA-encoded enzymes (8). This results in mitochondrial dysfunction and eventually leads to a range of complications, such as bone marrow suppression and cardiomyopathy (22).

In addition to mitochondrial toxicity, other markers of cytotoxicity caused by ARVs are cell death, modulation of cytochrome P450 (CYP450) and the monoamine oxidase (MAO) A and B enzymes. CYP450 enzymes are mostly amine oxidases and key metabolizers with regard to their catalytic versatility and broad spectrum of oxidative transformation of both exogenous and endogenous molecules (33). This enzyme superfamily plays a vital role in tissue and cardiovascular health (25). On the other hand, MAO enzymes are flavoenzymes that catalyze the oxidative deamination of a large number of biogenic and xenobiotic amines (24, 39). Therefore, any drug that interacts with MAOs, regardless of its function, can lead to a decrease in normal MAO cellular activity. This, in turn, can result in the intracellular accumulation of its natural substrates, such as serotonin, to potentially lethal levels (18).

Because of the toxicity associated with the use of ARVs, we evaluated in the present study the cytotoxic effects of one of the potent and cross-clade HIV type 1 (HIV-1)-neutralizing RNA aptamers, called B40, which was recently isolated and described (17, 21, 42). The B40 aptamer blocks viral entry by binding to core conserved residues on gp120 at the heart of the CCR5 binding site (17, 20). While this is the first study that describes the in vitro toxicity of the experimental HIV-1 gp120 binding RNA aptamer, previous studies have shown that the vascular endothelial growth factor (VEGF) binding aptamer...
did not have any intrinsic toxicity in preclinical assessments (23, 29). Single-dose and repeated-dosing toxicity studies with the anti-VEGF aptamer conducted in rats, rabbits, and theus monkeys showed that there was no observable adverse effect level or dose-limiting toxicity (23, 28, 29).

In the present study, we evaluated the toxicity of the B40 anti-gp120 aptamer using cell viability; caspase 3/7 activity; and CYP450 3A4 (CYP3A4), MAO A and B, and mtdNAs levels as markers of cytotoxicity in human cardiomyocytes and peripheral blood mononuclear cells (PBMCs). Toxicity in human cardiomyocytes was evaluated due to the association of cardio-myopathy with the use of ARVs (34, 49), and toxicity in PBMCs was evaluated because the CD4+ T cells and macrophages found in PBMCs are the backbone of the immune system. HIV-1 predominantly infects and replicates in these cells.

MATERIALS AND METHODS

ARVs. All ARVs were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Four classes of ARVs were tested in this study: the nonnucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine (NVP) and efavirenz (EFV), the NRTIs abacavir (ABC), didanosine (ddI), zidovudine (AZT), stavudine (d4T), tenofovir (TDF), and lamivudine (3TC); the protease inhibitors (PIs) saquinavir (SQV), ritonavir (RTV), nelfinavir (NFV), lopinavir (LPV), tipranavir (TPV), indinavir (IND), amprenivir (APV), and darunavir (DRV); and the entry inhibitor (EI) enfuvirtide (T20).

In vitro transcription and validation of monoclonal aptamers. Plasmid DNA containing the B40 anti-gp120 aptamer was amplified by PCR, as described previously (42). The PCR product was purified with Wizard SV gel and a PCR cleanup system (Promega). The PCR product was digested with BstE II and purified with a Nuclease-free PCR purification system (Zymo Research, CA). The oligonucleotides for the 5′ and 3′ terminal regions of the aptamer were obtained from Integrated DNA Technologies (Coralville, IA). The oligonucleotides were annealed, and the PCR product was ligated to the oligonucleotides using T4 DNA ligase (Fermentas). The DNA was amplified by PCR, and the recombinant plasmid was isolated by electroporation into E. coli strain DH5α. The E. coli DH5α strain was transformed with the recombinant plasmid, and the recombinant plasmid was purified from the E. coli DH5α strain by using a Wizard Plus Miniprep DNA Purification System (Promega). The recombinant plasmid was digested with EcoRI and purified with a Nuclease-free PCR purification system (Zymo Research, CA).

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Isolation of human PBMCs. PBMCs were isolated from heparinized human buffy coats from healthy, HIV-negative donors, as described previously (42). The buffy coats were obtained from the South African National Blood Services. Briefly, the buffy coats were layered into Ficoll-Paque Plus (GE Healthcare), and the PBMCs were isolated by density gradient centrifugation. Following centrifugation at 2,000 rpm for 30 min at 20°C, the PBMC layer was harvested and washed once with ice-cold PBS (Lonza). The cell pellet was then suspended in 30 ml of a hypotonic solution of ice-cold ammonium chloride, and the mixture was incubated at room temperature for 20 min to lyse the remaining red blood corpuscles. The cells were harvested by centrifugation and seeded on tissue culture flasks (Corning) at a density of 2 × 10^5 cells/ml in RPMI containing 20% fetal bovine serum (Sigma-Aldrich), followed by stimulation with phothemagglutinin (PHA) and treatment with interleukin-2 (IL-2), as described previously (42). For comparison, we also cultured PBMCs without mitogen (PHA) and without IL-2 in X-Vivo-10 medium (BioWhittaker) supplemented with 2% autologous serum because we previously showed that this system pro-
duces a slowly proliferating mixed culture of CD4+ T lymphocytes and macrophages that supports a higher level of replication of HIV-1 primary isolates (42).

Cell viability assay. PHA-stimulated, IL-2-treated PBMCs and cardiomyo-
cells in log-phase growth were seeded at a density of 20,000 cells/well in 96-well, white-opaque-well tissue culture plates (Nunc, Thermo Fisher Scientific) in 100 μl growth medium. Unstimulated PBMCs cultured in X-Vivo-10 medium (Bio-
Whittaker) supplemented with 2% autologous serum were also seeded, as described above. The overlay medium in either system was replaced with the relevant culture medium containing the drug at a concentration equivalent to the maximum concentration in plasma (Cmax) in patients at a predetermined therapeuti-
core dose or an equivalent amount of the aptamer (Table 1). Following 24 h of incubation, the overlay medium was removed and replaced with fresh medium. The overlay medium was replaced every 2 days for the duration of the experi-
ment. The assay was done in triplicate and was independently repeated twice. Following the final incubation, the cell viability was measured. The relative light units (RLU) were normalized (i.e., the total number of viable cells in each of the treated samples was divided by the total number of viable cells in the cell control, which was not treated with any drug or test molecule) and expressed as percent cell death. The cell controls, which were not treated, were, on average, more than 95% viable. Wells containing medium alone were used as controls for background luminescence, and the values for those wells were subtracted from the values for the test wells. Dimethyl sulfoxide (DMSO) at a concentration of 0.1% (vol/vol) was used as a solvent for most ARVs; hence, a control for DMSO-initiated toxicity was included whereby cells were treated with 0.1% DMSO for 24 h. The cell viability was measured by use of a count integra-
tion time of 1 s. The results were expressed as RLU from which the value for the blank was subtracted (RLU, blank subtracted).

MATERIALS AND METHODS

ARVs. All ARVs were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Four classes of ARVs were tested in this study: the nonnucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine (NVP) and efavirenz (EFV), the NRTIs abacavir (ABC), didanosine (ddI), zidovudine (AZT), stavudine (d4T), tenofovir (TDF), and lamivudine (3TC); the protease inhibitors (PIs) saquinavir (SQV), ritonavir (RTV), nelfinavir (NFV), lopinavir (LPV), tipranavir (TPV), indinavir (IND), amprenivir (APV), and darunavir (DRV); and the entry inhibitor (EI) enfuvirtide (T20).

Isolation and cultivation of human PBMCs. The cardiomyocytes were cultured in human cardiomyocyte expansion medium (a proprietary medium containing, serum, growth factors, and antibiotics) and in tissue culture flasks containing a proprietary growth matrix at 37°C with 5% CO2. The medium and the flask were also purchased from Celprogen.

The cardiomyocytes were phenotyped by immunocytochemistry with a cardiac protein-specific monoclonal antibody that targets the first 32 amino acids of the C terminus of the human cardiac protein troponin I (R&D Biosystems) and were further incubated at room temperature for 5 min. This was followed by the addition of 1/20 volume of 20X refolding buffer (10 mM HEPES, pH 7.4; 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 2.7 mM KCl [final concentrations]) and incubated at room temperature for an additional 30 min. The refolded aptamer was validated by binding to recombinant gp120 derived from HIV-1on, by using a BIACore 3000 surface plasmon resonance (GE Healthcare), as described previ-
ously (42).

Recombinant HIV-1on gp120. HIV-1in gp120 was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Culture and phenotyping of human cord blood stem cell-derived cardiomyo-
cyes. The human cord blood stem cell-derived cardiomyocytes were purchased from Celprogen. The cardiomyocytes were cultured in human cardiomyocyte expansion medium (a proprietary medium containing, serum, growth factors, and antibiotics) and in tissue culture flasks containing a proprietary growth matrix at 37°C with 5% CO2. The medium and the flask were also purchased from Celprogen.

The cardiomyocytes were phenotyped by immunocytochemistry with a cardiac protein-specific monoclonal antibody that targets the first 32 amino acids of the C terminus of the human cardiac protein troponin I (R&D Biosystems) and were detected by using an anti-goat rhodamine-labeled antibody produced in chickens (Santa Cruz Biotechnology). Briefly, a cell monolayer growing on a chamber slide (Lab-Tek; Nunc) were fixed and permeabilized in 2% paraformaldehyde (PFA) containing 0.1% Triton X-100 (Sigma-Aldrich, Germany) for 30 min. Following this, the primary antibody was added at a concentration of 10 μg/ml in the presence of 1% normal goat serum (Invitrogen), and the mixture was incubated for 1h on ice. The cells were then washed four times with ice-cold phosphate-buffered saline (PBS), prior to the addition of the anti-goat rhodamine-conju-
gated secondary antibody at a concentration of 5 μg/ml. Following incubation for 1 h, the cardiomyocyte monolayer was covered with a glass coverslip in the presence of mounting medium (UltraCruz, Santa Cruz Biotechnology) for im-
munofluorescence analysis.

Isolation and cultivation of human PBMCs. PBMCs were isolated from hepa-
arinized human buffy coats from healthy, HIV-negative donors, as described previously (42). The buffy coats were obtained from the South African National
the luminescent signal was measured in a Modulus microplate reader luminometer (Promega) by use of a count integration time of 1 s. The raw data (in RLU) were measured, and the final results were normalized as described above for the cell viability assay and expressed as the percentage of enzyme activity. A sample with no substrate was included as the control for background activity.

RESULTS

Real-time PCR for quantification of mtDNA toxicity. Cells were exposed to the B40 aptamer T20 or ddC at 2 μM for 7 days. Following exposure to the test molecule for 7 days, the cell monolayer was washed with warm PBS and the cells were lysed in 50 μl of a lysis buffer (Promega) that was compatible with the PCR mixture. A volume of 2 μl of lysate was subjected to PCR for both genomic DNA (nDNA) and mitochondrial DNA (mtDNA) in a LightCycler instrument (Roche, Switzerland). The target nucleic acid was the human polymerase γ accessory subunit (APSOLG), and the target mitochondrial gene was cytochrome c oxidase I (CCO1). The CCO1-specific primer pair consisted of forward primer 5'-TTC GCC GAC GCT TGA CTA TT-3' and reverse primer 3'-AAG ATT ATT ACA AAT GCA TGG GC 5'. PCR products were purified by use of Power SYBR green 5 (Applied Biosystems) in the presence of 5 mM MgCl2 (Promega). The cycling conditions consisted of a single enzyme activation step of 10 min at 95°C, followed by 45 cycles of denaturation at 95°C for 0 s, annealing at 60°C for 10 s, and elongation at 72°C for 5 s, with a temperature transition rate of 20°C/s being used. The relative amounts of mtDNA and nuclear DNA (nDNA) were extrapolated from a standard curve, and the results were calculated as the mean ratio of the amount of mtDNA to the amount of nDNA. Each experiment was carried out in triplicate, and the results represent the averages between two independent experiments. The mtDNA/nDNA ratio for the untreated controls was arbitrarily set at 1.0 and was used as the baseline measurement.

Effect of B40 aptamer in comparison to effects of ARVs on viability of human PBMCs and cardiomyocytes. We evaluated the toxicity of a novel gp120 binding and HIV-1-neutralizing RNA aptamer called B40 in comparison to the toxicities of conventional ARVs in human cord blood stem cell-derived cardiomyocytes (Fig. 1) and human PBMCs. An initial screen by an ATP cell-based assay to measure cell death as the endpoint measurement of cell toxicity showed that the B40 aptamer, which is a novel EI, did not cause the death of either human cardiosomyocytes (1% ± 0%; P = 0.74) or PBMCs (0% ± 5%; P = 0.92) at the maximum concentration used, 2 μM (Fig. 2). The maximum concentration of B40 used in this study (2 μM) was more than 200-fold its IC50. The 50% cytotoxic concentration of B40 could not be obtained even after the use of the aptamer at 50 μM, which had an effect similar to that of the aptamer at 2 μM.
The registered EI, called T20, which also did not cause the death of cardiomyocytes (1% ± 3%; P = 0.74) or PBMCs (0% ± 6%; P = 0.61). All other classes of ARVs tested caused significant cell death of either or both cardiomyocytes and PBMCs (Fig. 2). PIs were generally the most toxic class of ARVs for both cardiomyocytes and PBMCs, causing up to almost 100% cell death (0.0002 < P < 0.00001) at the maximum concentration used (Fig. 2). The two NNRTIs tested, NVP and EFV, caused the death of 80% (0.1 < P < 0.002) and 60% (0.1 < P < 0.002) of PBMCs, respectively, while they each caused the death of about 20% (0.004 < P < 0.002) of the cardiomyocytes (Fig. 2). NRTIs were generally the second least toxic class of ARVs after the EIs, causing the death of less than 20% of the cardiomyocytes and having between 20% and 72% toxicity for PBMCs (Fig. 2).

Cell death was caspase 3/7 independent. To complete the cytotoxicity profile, we further probed the mechanism through which ARVs were inducing cell death by measuring the activity of the executioner caspase 3/7. Despite the significant cell death induced by most ARVs in this study (Fig. 2), no significant increase in caspase 3/7 activity in either cardiomyocytes or PBMCs was observed after treatment with any of the ARVs compared to the activity in untreated control cells (Fig. 3A and B). However, a sharp and statistically significant decrease (P < 0.002) in caspase 3/7 activity was observed at days 4 and 7 for EFV-, ABC-, RTV-, and IND-treated cardiomyocytes (Fig. 3A). There was also a significant decrease in caspase 3/7 activity in PBMCs treated with EFV, ABC, TDF, and RTV for at least 4 and 7 days (Fig. 3B). The marked decrease in caspase 3/7 activity in cardiomyocytes and PBMCs treated with the ARVs directly correlated with the observed decrease in cell viability (Fig. 2). T20- and B40-treated cells were the only cells that had no significant changes in caspase 3/7 activity levels at any of the time points at which activity was measured, again correlating with the cell viability data (Fig. 2). Taken together, these data suggest that cell death was independent of the upregulation of caspase 3/7 activity. The significant decrease in the caspase 3/7 activity of cells treated with the ARVs compared to the activity of untreated control cells simply confirmed that most cells were dead and therefore unable to produce physiological levels of the caspase.

The B40 aptamer did not affect MAO A and B enzyme levels. Since the B40 aptamer and T20 (both EIs) were the only test molecules that had no effect on cell viability and caused insignificant cell death, an end-point marker of cytotoxicity, we selected them for more in-depth cytotoxic analysis at the enzyme and cell metabolism levels. We measured the interaction of the B40 aptamer with the MAO A and B enzymes, because any drug that interacts with MAO A or B, regardless of its function, can lead to a decrease in normal MAO cellular activity. This, in turn, can result in the intracellular accumulation of its natural substrates, such as serotonin, to potentially lethal levels. It is therefore important to screen potential drugs for a
possible interaction with MAO enzymes. In this study, we measured the levels of both the MAO A and the MAO B enzymes in the presence of the B40 aptamer and, for comparison, in the presence of T20. The results clearly indicated that neither of the MAO isozymes was inhibited or induced by the B40 aptamer or T20 in either cardiomyocytes or PBMCs when the effects on treated cells were compared to those on untreated control cells (Fig. 4A to D). However, MAO A was inhibited by clorgyline in a concentration-dependent manner (Fig. 4A and B) and MAO B was inhibited by deprenyl in a similar manner (Fig. 4B and D). Clorgyline and deprenyl, which were used as inhibitors of MAO A and MAO B, respectively, served as good controls, confirmed the presence and the activities of both isozymes in cardiomyocytes and PBMCs, and confirmed that these isozymes are amenable to interference by certain drugs or specific inhibitors.

The B40 aptamer did not affect the level of the CYP3A4 enzyme. CYP3A4 is another key enzyme involved in cell metabolism and commonly affected by the PI class of ARVs. For this reason, we tested if the B40 aptamer modulates the activity of this enzyme in human cardiomyocytes and PBMCs. The effect of the B40 aptamer compared favorably to that of T20 and did not significantly affect the level of CYP3A4 when the effect was compared to that on untreated negative control cardiomyocytes (P = 0.78 for T20 and P = 0.37 for the B40 aptamer) and PBMCs (P = 0.62 for T20 and P = 0.24 for B40 aptamer), even at the highest concentration used (Fig. 5A and B). However, as a positive control, CYP3A4 was significantly inhibited in a concentration-dependent manner by the PI RTV in human cardiomyocytes (P = 0.0001) and PBMCs (P = 0.0008).

The B40 aptamer did not cause mtDNA toxicity. Mitochondrial toxicity is one of the major complications associated with the long-term use of ARVs, specifically NRTIs. To complete the circle, we further tested if the B40 aptamer causes the depletion of mtDNA in human cardiomyocytes or PBMCs. Similar to T20, the B40 aptamer did not cause a significant decrease in the mtDNA levels in either cardiomyocytes or PBMCs compared to the levels in untreated control cells (Fig. 6). The ratio of mtDNA/nDNA in cardiomyocytes treated with the B40 aptamer was 1.17 ± 0.19 (P = 0.3), and that in PBMCs was 1.16 ± 0.11 (P = 0.06), while the ratio of mtDNA/nDNA in cardiomyocytes treated with T20 was 1.01 ± 0.17 (P = 0.5) and that in PBMCs treated with T20 was 1.08 ± 0.10 (P = 0.3). The NRTI ddC was used as a positive control for mtDNA depletion, and treatment with ddC resulted in mtDNA/nDNA ratios of 0.72 ± 0.12 (P = 0.006) in cardiomyocytes and 0.41 ± 0.18 (P = 0.0004) in PBMCs (Fig. 6).
DISCUSSION

This study describes the cytotoxic effects of a novel, cross-clade HIV-1-neutralizing RNA aptamer called B40 and compares the cytotoxic effects of B40 with those of conventional ARVs. We used cell death; caspase 3/7 activation; and MAO A and B, CYP3A4, and mtDNA levels as markers of toxicity in human cardiomyocytes and PBMCs. First, in contrast to most registered ARVs tested, the B40 aptamer did not cause the death of cardiomyocytes and PBMCs even at the highest concentration tested (2 μM). This is encouraging, particularly because the IC_{50} for HIV-1 BaL and several HIV-1 primary isolates of the B40 aptamer in PBMCs is less than 10 nM (17, 21, 42). We have also observed that cell death is independent of caspase 3/7 activation. These are executioner caspases that are involved in all caspase-mediated apoptotic pathways (14, 64). Our data clearly demonstrate that NRTI- and NNRTI-induced apoptosis is caspase independent. These observations are in general agreement with findings described in the literature, in which ARV-induced cell death is frequently reported to be the result of mitochondrial injury (45). Mitochondrial toxicity is one of the major side effects and causes of treatment failures associated with the mid- to long-term use of ARVs, especially NRTIs (45). The severity of mitochondrial toxicity can range from being clinically silent to causing life-threatening conditions such as lactic acidosis (12). One of the most accepted hypotheses of mitochondrial toxicity is that ARVs cause a depletion of mtDNA due to inhibition of the mtDNA polymerase γ (47, 48). This results in the impaired production of mitochondrial enzymes, which participate in oxidative phosphorylation, ultimately leading to diverse organ-specific and/or systemic pathological changes. It was for this reason that we examined the B40 aptamer for its mitochondrial toxicity, and we have unequivocally shown that B40 does not interfere with mtDNA synthesis, ruling out the possibility that potentially deleterious mitochondrial interactions via the known pathways of ARV-induced mitochondrial toxicity are associated with the therapeutic use of this compound. Due to the high incidence of these cases, the NRTI ddC is used with extreme caution in the clinical setting. Furthermore, PI-related toxicity was also caspase independent, as cell death was not preceded by an increase in the level of caspase 3/7 activity (Fig. 3). The sharp decrease in the level of caspase activity induced by most ARVs

![Graph A](attachment://graphA.png)
![Graph B](attachment://graphB.png)
![Graph C](attachment://graphC.png)
![Graph D](attachment://graphD.png)

FIG. 4. MAO A and B enzyme modulation by the B40 aptamer and T20 in cardiomyocytes (A and C) and PBMCs (B and D). Cells were incubated in triplicate with the serially diluted test molecule at a maximum concentration of 2 μM for 2 h. A cell lysate was incubated with MAO A or B substrate for 1 h, followed by the addition of detection reagent, and the resulting luminescence signal was quantified in a luminometer. The no-treatment control was used as the baseline for maximum enzyme activity. No change in enzyme activity was detected for either MAO A or MAO B in the presence of the B40 aptamer and T20 for both cardiomyocytes and PBMCs. However, there was a dose-dependent inhibition of MAO A by clorgyline and MAO B by deprenyl, which are well-documented inhibitors of MAO A and MAO B, respectively, indicating the presence and activity of both enzymes in human cardiomyocytes and PBMCs. Enzyme activity was calculated by relating the RLUs of untreated control cells to that of the treated cells as a percentage. The data on the graphs represent the means, and the error bars represent the standard deviations for three replicates in two sets of independent experiments.
(Fig. 3) was the result of a decrease in the total number of viable cells (Fig. 2). The exact mechanism by which PIIs induce cell death has not been fully dissected (2), but cell cultures treated with RTV showed an accumulation of growth-arrested cells in the G1 phase and high levels of expression of the universal cyclin-Cdk kinase inhibitor p21 (30). It has been speculated that the accumulation of p21 could be due to the inhibition of the cellular proteosome, resulting in cell death (62). Therefore, necrosis due to chemical insult is the most likely cause of cell death, although caspase-independent apoptosis cannot be ruled out (2).

Another favorable property of the B40 aptamer shown in this study is that it did not interact with the MAO A and B enzymes. The two MAO isoforms are present in most tissues, including peripheral tissues and the myocardium (32, 55, 60, 61, 63, 65). Drugs interacting with MAO A or B can cause either the upregulation or the downregulation of the enzymes, leading to the concomitant accumulation or depletion of neurotransmitters, respectively, thus resulting in serious clinical effects. Clorgyline and deprenyl, which were used as positive controls in this study, are well-documented inhibitors of MAO A (35, 43) and MAO B (5, 26), respectively. No ARV currently in clinical use has been shown to interact with either MAO A or MAO B. Notwithstanding this lack of a proven interaction, given the metabolic importance of MAO A and MAO B and their putative interactions with other drugs, it was imperative and prudent to look at the potential interaction of MAOs A and B with the B40 aptamer.

The B40 aptamer also did not affect CYP3A4 activity in either PBMCs and cardiomyocytes, which compared favorably with the findings for T20. Most ARVs, notably, PIIs, are substrates and potent competitive inhibitors of CYP3A4 (27). The CYP450 superfamily of heme-containing enzymes is the major catalyst for the oxidative transformation of most therapeutic drugs and xenobiotics in general, as well as a vast array of endogenous substances (40). The interaction of ARVs with members of the CYP450 enzyme superfamily often causes inactivation of the enzyme, leading to decreased plasma PI levels (40). This effect is often counteracted in the clinical setting by the coadministration of RTV, which is a potent inhibitor of CYP3A4 (36, 56), with other PIIs (70). In this case, RTV plays a dual role both because of its antiretroviral activity and because of its inhibition of the CYP3A4 enzyme to boost the plasma levels of other PIIs. However, this is closely associated with an increased incidence of both metabolic and somatic cardiac changes, which are often silent diseases because they are not readily diagnosed. Lipodystrophy, hyperlipidemia, and insulin resistance are some of the most common such disorders (9–12). Furthermore, the potent inhibition of CYP3A4 by most PIIs, especially RTV (36, 56), seriously restricts the use of other auxiliary drugs that are also metabolized by the same enzyme, as this could lead to life-threatening cardiac events such as arrhythmias. The property of the B40 aptamer to po-
ently and broadly neutralize HIV-1 (21, 42) without inhibiting CYP3A4 therefore makes it an ideal candidate drug as a complement to existing ARVs.

Taken together, these data show that the B40 aptamer does not interfere with any of the major pathways of ARV-related cytotoxicity. These data agree with toxicological observations made with pegaptanib sodium (Macugen), which is the first and only aptamer-based therapeutic agent in clinical use (46). Pegaptanib sodium was approved for clinical use in 2005 and did not exhibit any intrinsic toxicity when it was evaluated in preclinical studies (23, 28, 29). The only side effect reported arises almost exclusively from the injection procedure rather than the drug itself (31).

The cytotoxicological data observed in this study further suggest that B40 in particular and aptamers in general may be safer than most ARVs. This argues for the development of the B40 aptamer and related anti-gp120, HIV-1-neutralizing aptamers (42) as new ARVs (entry inhibitors) without any cytotoxicity. While entry inhibitors such as the B40 aptamer are expected to be less toxic or not toxic because they act extracellularly, the results of this study should be treated with caution because there may be in vitro artifacts. A key challenge now is to evaluate the efficacy, pharmacokinetics, and toxicity of B40 and related aptamers in preclinical studies with a more dynamic in vivo animal model.

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