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Selection and characterization of viruses resistant to the dual acting pyrimidinedione entry and non-nucleoside reverse transcriptase inhibitor IQP-0410

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ABSTRACT

The 1-(2-hydroxyethoxymethyl)-6-(phenylthio)thymine (HEPT)-like compounds with homocyclic moieties at the N-1 of the pyrimidinedione, including the highly potent lead compound IQP-0410, inhibit HIV-1 at sub-nanomolar concentrations primarily through a typical non-nucleoside mechanism involving allosteric inhibition at the hydrophobic binding pocket of the HIV-1 RT. Like all NNRTIs, the pyrimidinediones have no activity against HIV-2 RT. The pyrimidinediones, however, also possess a second mode of action involving inhibition of virus entry at nanomolar concentrations which extends their range of action to include HIV-2. Entry inhibition occurs through recognition of a complex conformational binding site formed upon interaction of the virus with target cells, but does not involve direct inhibition of gp120-CD4 binding. In order to further explore the means by which the pyrimidinediones act, resistant strains of HIV-1 and HIV-2 were selected in cell culture and molecularly and biologically characterized. With HIV-1, three phases of resistance selection occurred which involve an initial appearance of single amino changes in the NNRTI binding pocket, followed by changes in the envelope glycoproteins gp120 and gp41, and subsequent multiple additional changes in the RT, resulting in high level resistance to IQP-0410. With HIV-2, resistance to entry inhibition was achieved with no resistance-engendering mutations detected in the HIV-2 RT. Detailed molecular and biological characterization of IQP-0410-resistant viruses was performed to define the resistance-engendering mutations present in the RT and envelope and to quantify cross-resistance to other HIV inhibitors.

1. Introduction

Approximately 30 different classes of non-nucleoside reverse transcriptase inhibitors (NNRTIs) have been described since the first reports on the HEPT and TIBO compounds (Baba et al., 1989, 1992, 1994; Buckheit et al., 1995b; Danel et al., 1996; Mai et al., 1999; Miyasaka et al., 1989; Tanaka et al., 1991). The NNRTIs are highly potent HIV-1-specific replication inhibitors and act through a noncompetitive, allosteric mechanism to inhibit the HIV-1 reverse transcriptase (RT) through direct binding at a hydrophobic pocket of the p66 subunit of the RT (Cohen et al., 1991; Condra et al., 1992; Grob et al., 1992; Saag et al., 1993). The binding site has been well defined through three-dimensional modeling (Kohlstaedt et al., 1992) as well as direct correlation of compound interactions with amino acid residues via identification of resistance-engendering changes introduced in the RT which map to this NNRTI binding pocket (Mellors et al., 1995).

IQP-0410 (SJ-3366; 1-(3-Cyclopenten-1-ylmethyl)-5-ethyl-6-(3,5-dimethylbenzoyl)-2,4 (1H, 3H)-pyrimidinedione) is a member of the HEPT class of NNRTIs but has distinctive antiviral properties which clearly distinguish the compound from the other members of the inhibitor class (Buckheit et al., 2001). IQP-0410 inhibits HIV-1 replication in established cell lines in cytopathic effect (CPE) inhibition assays with an EC_{50} value at or below 1 nM and possesses an *in vitro* selectivity index (SI) of greater than one million when evaluated against laboratory strains of virus (Buckheit et al., 2001). In addition the compound inhibits laboratory-derived strains of HIV-2 at nanomolar concentration levels with a SI of approximately 100,000. Mechanistic assays have shown that the compound inhibits HIV-1 through two distinct mechanisms,

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including inhibition of both RT and virus entry (Buckheit et al., 2008). IQP-0410 is a mixed type of inhibitor of RT, affecting both the K_m and the V_{max} of the reaction and exhibited a K_i of 3.2 nM against purified HIV-1 RT (Buckheit et al., 2001). As expected for a NNRTI, IQP-0410 has no inhibitory activity against HIV-2 RT, but does inhibit both HIV-1 and HIV-2 replication in cell-based antiviral assays. The entry inhibition mechanism does not prevent infectious virus from attaching to the target cells via interaction of viral gp120 with cellular CD4 and does not yield differential activity on CCR5-, or CXCR4-, or dual-tropic virus strains.

The activity of IQP-0410 and other members of this unique class of NNRTIs have been extensively evaluated in a detailed structure–activity relationship (SAR) analysis (Buckheit et al., 2007, 2008) in order to distinguish the structural features of the molecule which are responsible for the mechanistic activity of the pyrimidinediones against both RT and virus entry. The extended range of action to HIV-2, addition of the entry mechanism of inhibition, and the significant level of potency of the pyrimidinediones appears to derive from the homocyclic substitution (3-Cyclopenten-1-yl) which is linked to the N-1 of the pyrimidinedione through a methyl linker (Fig. 1). Since IQP-0410 is not the most potent inhibitor among the SAR series of compounds with regard to relative activity in RT or entry inhibition assays, but is one of the most potent overall inhibitors of HIV-1 replication, it appears that the structural and chemical features of the molecule may overlap, leading to functionally synergistic antiviral activity of IQP-0410. Four other highly potent pyrimidinediones, including 1-Cyclopropylmethyl-5-ethyl-6-(3,5-dimethylbenzoyl)-2,4 (1H, 3H)-pyrimidinedione (SAR compound 18, IQP-0407), 1-Cyclopropylmethyl-5-isopropyl-6-(3,5-dimethylbenzoyl)-2,4 (1H, 3H)-pyrimidinedione (SAR compound 19, IQP-0528), 1-(1-Cyclopenten-1-ylmethyl)-5-isopropyl-6-(3,5-dimethylbenzoyl)-2, 4 (1H, 3H)-pyrimidinedione (SAR compound 49, IQP-0558) and 1-(3-Cyclopenten-1-ylmethyl)-5-isopropyl-6-(3,5-dimethylbenzoyl)-2, 4 (1H, 3H)-pyrimidinedione (SAR compound 63, IQP-1187) share the antiviral properties of IQP-0410 (Buckheit et al., 2001) and are also being further evaluated as potential therapeutic and microbicide candidates (Buckheit et al., 2008, 2007; Watson Buckheit et al., 2011).

The primary problems associated with the use of NNRTIs in patients are their toxicity and side-effects, which significantly compromise patient adherence to treatment regimens (Blas-Garcia et al., 2011). Lack of compliance and the relative ease of selecting drug resistant virus strains have resulted in few NNRTIs progressing to significant clinical use (Adams et al., 2010; Cane, 2009; de Bethune, 2010). Current research involving the NNRTIs obviously requires the development of new compounds with enhanced potency, better dosing regimens, greater genetic barriers to resistance, and the ability to be utilized in patient populations exposed to the previous generations of NNRTIs such as nevirapine, efavirenz and delavirdine (Fulco and McNicholl, 2009; Prajapati et al., 2009; Sweeney and Klumpp, 2008; Zhan et al., 2009a,b). It is also important that future NNRTIs be amenable for use in women of child bearing age and children. In this report we define the genotypic and biologic changes associated with the selection of resistant viruses to the lead pyrimidinedione IQP-0410 and the cross-resistance profiles of these viruses to other approved anti-HIV compounds in order to define optimal combination therapy regimens for future clinical use.

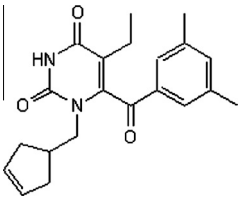

Fig. 1. The chemical structure of IQP-0410 (1-(3-Cyclopenten-1-ylmethyl)-5-ethyl-6-(3, 5-dimethylbenzoyl)-2,4 (1H, 3H)-pyrimidinedione; formerly known as SJ-3366 (Buckheit et al., 2001) or SAR compound 62 (Buckheit et al., 2007, 2008).

2. Materials and methods

2.1. Cells and viruses

The established human cells (CEM-SS and HeLa-CD4-LTR-β-galactosidase) and the laboratory derived virus isolate HIV-1_{IIIB} used in these evaluations were obtained from the NIAID AIDS Research and Reference Reagent Program and have been previously described in detail (Kimpton and Emerman, 1992; Nara et al., 1987; Popovic et al., 1984). HIV-2_{ROD} was a gift from Dr. Luc Montagnier (Pasteur Institut, Paris). Established cells were maintained in RPMI 1640 medium (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Lonza). Fresh human cells were obtained commercially (Biological Specialty Corporation, Reading PA) and mononuclear cells were maintained in RPMI 1640 medium supplemented with 15% fetal bovine serum, L-glutamine, penicillin and streptomycin after isolation by Ficoll-hypaque centrifugation.

2.2. Materials

IQP-0410 was obtained as a dry white crystalline or amorphous powder from Samjin Pharmaceutical Co., Ltd. (Seoul, Korea). Stock materials were stored at −80 °C and solubilized in 100% dimethylsulfoxide (DMSO). All DMSO stock solutions were diluted at least 400-fold prior to performing drug susceptibility assays. ELISA plates for p24 quantification were purchased from Perkin Elmer (Branford, CT). Materials required for the performance of RT inhibition assays, anti-HIV assays, and for the growth and maintenance of established and fresh human cells have been previously described (Buckheit et al., 2001). Antiviral test compounds were obtained from the NCI Developmental Therapeutics Program (Rockville, MD), the NIH AIDS Research and Reference Reagent Program (Rockville, MD), ISIS Pharmaceuticals (Carlsbad, CA), or Sigma–Aldrich (St. Louis, MO).

2.3. Antiviral and cross-resistance assays

The HIV inhibitory activity of the test compound was evaluated as described (Buckheit et al., 1995b) in a microtiter anti-HIV assay using CEM-SS cells, or in fresh human peripheral blood mononuclear cells. These assays quantify the ability of a compound to inhibit HIV-induced cell killing or HIV replication as measured by use of the tetrazolium viability stain XTT or by RT assay, respectively. Quantification of compound toxicity was performed in parallel by measuring cell viability using the tetrazolium dye XTT, which is metabolized to a colored formazan product by viable cells. Antiviral and toxicity data are reported as the concentration of compound required to inhibit 50% of virus-induced cell killing or virus production (EC_{50}) and the concentration of compound required to reduce cell viability by 50% (TC_{50}). The SI of the compound is defined as the TC_{50}/EC_{50}.

2.4. Selection of IQP-0410-resistant viruses

Resistant virus isolates were selected in cell culture by serial passage of the IIIB strain of HIV-1 or the ROD strain of HIV-2 in CEM-SS cells in the presence of antiviral compound (Buckheit

et al., 2001). For serial passage in increasing concentrations of the compound, the initial selection was performed with a compound concentration equivalent to twice the EC_{50} value of the compound as determined in the microtiter anti-HIV assay. With successive passages the compound concentration was increased twofold to enhance the selective pressure on the virus. For selection using fixed concentrations of a test compound, successive passages of the virus were performed in the presence of identical low, intermediate and high concentrations of compound over defined periods of time. Upon selection of a drug-resistant virus isolate, evaluation of the fold-resistance of the selected virus to the selecting compound and cross-resistance testing against other approved and experimental compounds was completed according to the methods described for the performance of antiviral assays. In this study, resistance has been defined as a greater than a fivefold increase in EC_{50} when compared to the activity of the compound against the wild-type (HIV-1_{IIIB} or HIV-2_{ROD}) virus isolates.

2.5. Analysis of mutations in IQP-0410-resistant viruses

Viral RNA was purified using the QIamp Viral RNA Mini Kit (Qiagen) and subjected to RT-PCR amplification using Stratascript First Strand Synthesis System and PiccoMaxx High Fidelity DNA polymerase (Agilent Technologies, La Jolla, CA). Amplified DNA was purified using the UNIQ-10 Column PCR Product Purification Kit (Denville Scientific) and 40 ng of the purified DNA was subjected to amplification with Big Dye Terminator v3.1 Sequencing Kit (Applied Biosystems) and automated dye-terminator sequencing on an Applied Biosystems 3100 Genetic Analyzer according to the manufacturer's recommended methods. The sequence of the RT, gp120 and gp41coding regions of the of the IQP-0410-resistant virus isolates were analyzed using Lasergene DNA and protein analysis software (DNASTAR, Inc.) and aligned with and compared to the respective parental wild-type HIV-1_{IIIB} or HIV-2_{ROD} virus nucleic acid or protein sequence using Clustal W alignment. DNA oligonucleotide primers for amplification and sequencing were purchased from Integrated DNA Technologies (Coralville, IA).

2.6. Virus entry inhibition assays

Serially diluted compound and virus (at a pre-determined titer) were added to HeLa-CD4-LTR-β-galactosidase cells that had been added to a 96-well flat-bottomed plate 24 h prior to assay initiation. Cells, compound and virus were allowed to incubate for 2 h at 37 °C/5% CO_2 and then the cells were washed to remove any unbound virus and test compound. Following the addition of tissue culture medium to the wells and a 48 h incubation period, the cells were lysed and evaluated for β-galactosidase expression using a chemiluminescent substrate (Gal-Screen, Tropix).

2.7. RT inhibition assays

Analysis of the drug sensitivity of purified RT containing defined amino acid substitutions was performed as previously described (Boyer et al., 1993; Buckheit et al., 1995a).

3. Results

3.1. In vitro efficacy of IQP-0410 against wild-type and drug-resistant viruses

IQP-0410 exhibits activity against wild-type clinical strains of HIV-1 at sub-nanomolar concentration levels and against clinical strains of HIV-2 at nanomolar concentration levels. The compound is equally active against viruses representative of all clinical subtypes found throughout the world with the exception of subtype O strains. IQP-0410 possesses a large SI of approximately 1 million against HIV-1 and near 100,000 against HIV-2. A representative dose response cuve for IQP-0410 against HIV-1_{IIIB} is presented in Fig. 2, showing the compounds high selectivity and activity (EC_{50} = 0.18 nM, SI = 2,777,778).

In general, IQP-0410 behaves similarly to a highly potent NNRTI with time of addition assays demonstrating that compound addition can be delayed until 8–10 h post-infection, similar to efavirenz, UC781, and nevirapine (data not shown). Assays performed with a wide variety of viruses possessing defined NRTI- and NNRTI

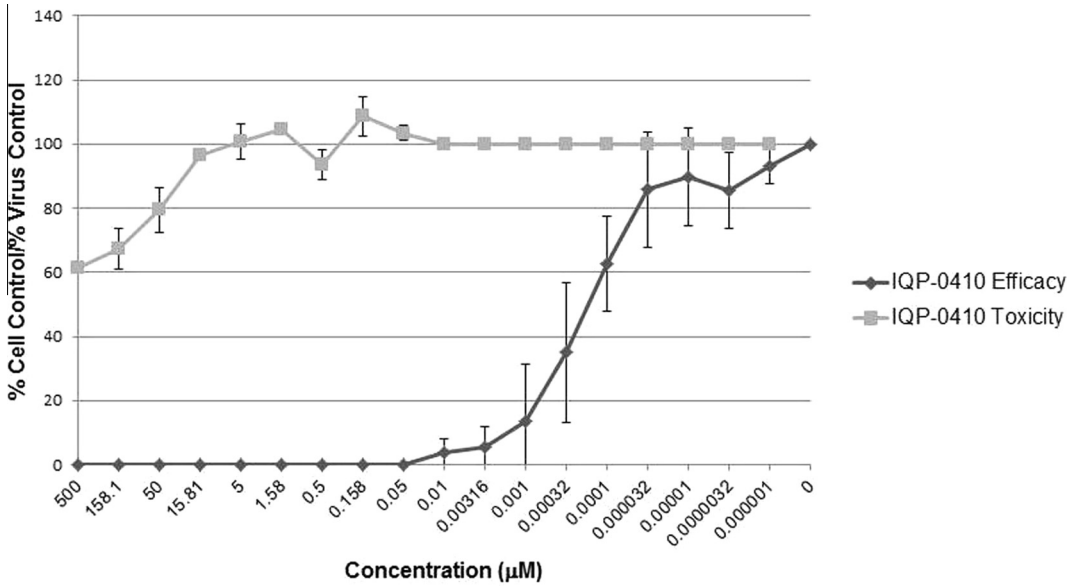

Fig. 2. The antiviral efficacy and toxicity of IQP-0410 was determined using a cytopathic effects inhibition assay using CEM-SS target cells and HIV-1_{IIIB} as described in Section 2. The IQP-0410 dose response curve ranged from a low concentration of 0.000001 μM to a high concentration of 500 μM. The assay defined EC_{50} was determined to be 0.00018 μM while the TC_{50} was determined to be >500 μM, yielding a selectivity index of 2,777,778. Toxicity to the target cells was determined to be due to compound precipitation in the assay wells and not to overt cell killing.

resistance-engendering amino acid changes suggests that amino acids K101E, K103N, V108I, E138K, Y181C, Y188C and P236L in the hydrophobic non-nucleoside binding pocket of RT have direct effects on the antiviral activity of IQP-0410 (Table 1). Mutations specific to nucleoside or nucleotide RT inhibitors, protease inhibitors, or the fusion inhibitor T20, have no effect on antiviral activity of the compound. Viruses possessing the NNRTI mutations exhibit relatively small (relative to the TI of >1 million) reproducible reductions in sensitivity to IQP-0410, yielding from approximately 10-fold to 375-fold increases in EC_{50} values. Laboratory-derived nevirapine-resistant virus (N119) possessing the Y181C amino acid change and pyridinone-resistant virus (A17) possessing both the K103N and Y181C amino acid changes exhibited slightly higher levels of resistance to IQP-0410, ranging from 300-fold to approximately 1000-fold when evaluated in human PBMCs (Table 2). Sensitivity testing in fresh human PBMCs using clinical strains of virus possessing multiple resistance-engendering mutations in the RT and/or protease was also performed (Table 2). A clinical multi-drug resistant virus strain with mutations in both RT and protease (MDR769 with both NRTI and NNRTI mutations, including Y181I rather than Y181C) was found to be 785-fold resistant to IQP-0410. MDRs with mutations in protease alone (Merck 144-44) were found to remain sensitive to IQP-0410. Interestingly, MDRs with mutations in both RT and protease had nearly wild-type sensitivity to IQP-0410.

Table 1
Activity of IQP-0410 against drug-resistant viruses with defined amino acid changes in reverse transcriptase.[a]

Mutation	EC_{50} (μM) versus site-directed mutant viruses[b]	EC_{50} (μM) versus viruses selected for resistance to other NNRTIs[c]
Wild-type HIV-1	0.002	0.002
L74V	0.0002	0.001
A98G	0.0006	NA
L100I	0.0002	0.008
K101E	>0.14	0.05
K103N	>0.14	>0.14
V106I	0.0006	NA
V108I	0.003	0.05
E138K	NA[e]	13
T139I	0.002	0.01
V179D	0.001	NA
Y181C	0.009	0.085 to >0.10
M184V	0.001	0.006
Y188C	0.06	NA
Y188H	NA	0.10
P236L	NA	0.028
4xAZT[d]	0.0006	0.001
4xAZT + L100I	0.0001	NA
4xAZT + Y181C	0.036	NA

[a] The results presented were obtained from a single representative antiviral assay with appropriate control compounds evaluated in parallel selected from a minimum of two antiviral assays. We have demonstrated that the standard error among multiple antiviral assays averaged less than 10% of the respective mean EC_{50}. In each individual assay, mean efficacy values are derived from a minimum of three replicate samples. Fold-resistance has been calculated as the ratio between the activity of IQP-0410 against the resistant virus versus its activity against wild type virus.

[b] Viruses with specific single mutations engendering resistance to compounds of the NNRTI class were engineered into the pNL4–3 backbone. These viruses were subsequently propagated in CEM-SS cells to make a high tittered stock of virus which was subsequently utilized in antiviral efficacy assays.

[c] HIV-1_{IIIB} wild-type viruses were utilized to select for resistance to specific NNRTIs and the RT coding region was sequenced to identify the NNRTI resistance engendering mutations. No other region of these viruses was sequenced. Virus was propagated in CEM-SS cells prior to use in the antiviral efficacy assays.

[d] 4xAZT virus possesses the following amino acid changes: D67N, K70R, T215F and K219Q.

[e] NA; A virus strain with this particular genetic background was not available for evaluation.

3.2. In vitro selection of IQP-0410-resistant strains of HIV-1 and HIV-2

In order to better understand the proposed dual mechanism of action of IQP-0410 as an inhibitor of both reverse transcription and virus entry, IQP-0410-resistant HIV-1 and HIV-2 were selected and characterized to more precisely define the molecular interactions of the compound with the virus and to understand the role of RT, gp120 and gp41. Serial passage of HIV-1_{IIIB} or HIV-2_{ROD} in the presence of IQP-0410 was performed in CEM-SS cells, starting with a sub-optimal effective concentration of the compound (two times the EC_{50} concentration). At each subsequent passage of the virus the concentration of IQP-0410 was doubled with respect to the previous passage. With HIV-1, virus resistant to IQP-0410 appeared after 2 passages in cell culture (passage 3) and selection was continued until a virus that was completely resistant to the antiviral effects of the compound was obtained, requiring approximately 18 passages of virus in culture. The data presented in Table 3 **(HIV-1 Selection 1)** exhibit the fold-resistance for each of the viruses obtained over the course of the 18 passages as determined by evaluating the activity of IQP-0410 in cell-based microtiter anti-HIV CPE inhibition assays. Fold-resistance begins to escalate at passage 3 in the presence of eight times the EC_{50} concentration of the compound and steadily increases until a virus strain that is over two million-fold resistant to the compound is selected at passage 18.

Additional resistance selections with IQP-0410 using serial dose escalation or fixed concentration methodologies were performed in order to further define the resistance potential of the compound. In these replicate selection assays, resistant viruses were consistently selected with profiles similar to that described in Table 3 (data not shown). In all cases where serial dose escalation was employed, resistance to IQP-0410 occurred with steady but slow progression in fold-resistance punctuated by more abrupt large jumps in resistance such as was observed at passages 3, 13, 16 and 18 in Table 3 **(HIV-1 Selection 1).** A representative example of data obtained from a second representative resistance selection is presented in Table 3 **(HIV-1 Selection 2)** which highlights a similar resistance selection profile. In this selection, resistance levels remained somewhat steady until passage 8 when the fold-resistance rapidly increased from approximately 100-fold to >125,000-fold over the course of 2 passages. In selections employing fixed concentrations of compound, virus replication was routinely observed in IQP-0410 concentrations up to approximately 1 μM with approximately 30–45 days in cell culture (data not shown).

Selection of a strain of HIV-2_{ROD} which was resistant to the entry inhibitory activity of IQP-0410 was performed using identical dose escalation methods in CEM-SS cells. In this selection, moderate resistance is observed until passage 4 of the selection at which time the fold-resistance jumps to nearly 8500-fold at passage 7, resulting in an HIV-2 virus which is completely resistant to the antiviral effects of IQP-0410 (Table 3, **HIV-2 Selection**).

3.3. Genotypic changes in the RT and envelope of resistant virus strains

The viruses obtained at each passage of the resistance selections with HIV-1 and HIV-2 were subjected to genotypic characterization. Mutations which appear in the viral genome have been defined in RT, gp120 and gp41 and the specific amino acid change and its time of appearance are presented in Table 3.

3.3.1. Changes in the reverse transcriptase

For the first HIV-1 resistance selection shown in Table 3 **(HIV-1 Selection 1)**, sequencing of the RT from each virus obtained during the selection demonstrates that the Y181C amino acid change in the RT first appears in virus obtained at passage 3 and remains present in every additional virus of the selection. The appearance

Table 2
Activity of IQP-0410 against resistant viruses in PBMCs.

Virus strain	Mutations	IQP-0410 EC_{50} (μM)[a]	Fold-resistance
Clade B (WT) US/92/727	–	0.00068	–
Nevirapine resistant N119	Y181C	0.225	331
Pyridinone-resistant A17R	Y181C, K103N	0.804	1182
Protease inhibitor resistant Merck 1026–60	L10I, M46I, I54V, L63P, A71V, V82A, L90M	0.0009	1.3
Protease inhibitor resistant Merck 1064–52	L10I, I54V, L63P, A71T, V82F, L90M	0.001	1.5
Protease inhibitor resistant Merck 1022–48	L10I, M46I, V82F, I84V	0.0024	3.5
Protease inhibitor resistant Merck 1002–60	L10I, M46I, I54V, L63P, V82F, L90M	0.0023	3.4
Protease inhibitor resistant RF/V82F/I84V	V82F, I84V	0.004	5.9
Multidrug resistant MDR 769	RT mutations = M41L, K65R, D67N, V75I, F116Y, Q151M, Y181I, L210W, T215Y protease mutations = L10I, M36M/V, M46I, I54V, L63P, A71V, V82A, I84V, L90M	0.534	785
Multidrug Resistant Merck 144–44	V32I, M46I, L63P, L90M	0.002	2.9

[a] The results presented were obtained from a single representative antiviral assay with appropriate control compounds evaluated in parallel selected from a minimum of three antiviral assays. We have demonstrated that the standard error among multiple antiviral assays averaged less than 10% of the respective mean EC_{50} and TC_{50}. In each individual assay, mean efficacy and toxicity values are derived from a minimum of three replicate samples.

Table 3
Selection and genetic characterization of HIV-1 and HIV-2 resistant virus strains to IQP-0410.

Passage	HIV-1 Selection 1			HIV-1 Selection 2			HIV-2 Selection	
	Fold-resistance[a]	RT mutations[b]	Env mutations	Fold-resistance	RT mutations	Env mutations	Fold-resistance	Env mutations
1	2	ND	ND	1	L214F	M421I, V696I	1	ND
2	2	ND	ND	1	ND	V696I, I165K, E398K, I418F	1	ND
3	7	Y181C	K490I	1	E169K	I165K, I418F, D669N	1	ND
4	15	ND	ND	12.5	E169K, V108I	I165K, I418F, D669N	607	W750Stop
5	26	ND	K490I, L684L/I, T818I	32.5	E169K, V108I, L234I	I165K, I418F, D669N, V696I, E398K	432	ND
6	41	ND	ND	77.5	E169K, V108I, L234I, M357T	V696I, I165K, E398K, T138I, N672S	3816	ND
7	72	ND	ND	70	E169K, V108I, L234I	V696I, I165K, E398K, R737K	8547	A149T, K185E, K422R, W750Stop
8	150	ND	ND	170	E169K, V108I, K103N, L234I	I165K, E398K, I418F, T138I, V696I, R737K		
9	250	E6K, Y181C	ND	19050	E169K, K103N, L234I	V696I, I165K, E398K, I418F, D669N, T138I, R737K, F383I		
10	500	ND	I165K, T297I, G410R, I423F, K490I, N674N/D, N677N/S, V701I, T818I, L684I	>125000	E169K, K103N, L234I	V696I, I165K, E398K, I418F, D669N, T138I		
11	875	E6K, Y181C, F227L	ND	>125000	E169K, V108I, K103N, L234I	V696I, I165K, E398K, I418F, D669N, T138I		
12	1000	ND	ND	>125000	ND	V696I, I165K, I418F, D669N, T138I		
13	5000	ND	ND	>125000	ND	ND		
14	7833	ND	ND	>125000	ND	ND		
15	11733	E6K, V106I, Y181C, F227L	I165K, T297I, G410R, I423F, K490I, N674D, N677N/S, L684I, V701I, T181I					
16	33333	ND	ND					
17	35500	ND	ND					
18	2500000	ND	ND					

ND = not done.
[a] Fold-resistance was calculated by dividing the experimentally determined EC_{50} concentration value of IQP-0410 tested against the selected resistant virus by the determined EC_{50} concentration value of IQP-0410 tested against wild-type virus.
[b] Amino acid changes in RT and Env identified at passages which were subjected to genetic characterization are presented for virus obtained at that passage.

of the Y181C change is associated with the first escalation in fold-resistance, resulting in a virus with 7-fold loss of sensitivity to IQP-0410, consistent with results reported for the Y181C-possessing viruses shown in Table 1. The Y181C amino acid change is the only selected mutation found in the RT in viruses from passage 3 through passage 8 despite an increase in resistance to IQP-0410

from 10-fold to 150-fold, suggesting that mutations elsewhere in the genome are contributing to the loss of sensitivity to the selecting compound during this passage range. At passage 9, a second amino acid change (E6K) appears in the RT followed by the F227L amino acid change at passage 11. The appearance of the F227L correlates with one of the larger jumps in fold-resistance from passage 9 through passage 13. A fourth amino acid change, V106I, appears in virus obtained at passage 15 and is associated with another large jump in fold-resistance between passages 15 and 18 as this mutation becomes fixed in the RT of the resistant virus. This change is of interest since V106I alone did not appear to induce resistance to IQP-0410 (Table 1), however, in combination with the other existing mutations a large increase in resistance is observed.

The second HIV-1 selection reported in Table 3 was unique compared to the bulk of the resistance selections performed with IQP-0410 in that the Y181C mutation did not appear in the early passage history. In this selection, a L214F amino acid change was detected in the RT following the initial passage, followed by the appearance of E169K at passage 3, V108I at passage 4, L234I at passage 5, and M357T at passage 6. Although each of these amino acid changes was correlated with an increase in resistance, the overall fold-resistance of the IQP-0410-resistant virus (approximately 70-fold) remained relatively small compared to the SI of the compound (approximately 1 million) through passage 7 with these four amino acid changes. Resistance to IQP-0410 was likely caused by the mutations E169K and V108I since the other mutations are not known to cause resistance to NNRTIs and V108I alone did not induce resistance (Table 1). At passage 8, a jump in the viral resistance to 170-fold occurred and was associated with the appearance of the K103N amino acid change in the RT. Based on data obtained with a virus possessing K103N (Table 1), a much larger increase in resistance might have been expected with this amino acid change. At passage 9, the fold-resistance jumped to 19,000-fold, concomitant with the loss of the V108I change, suggesting that V108I may have antagonized the resistance engendered by the appearance of K103N, or that the appearance of other mutations in envelope allowed greater levels of resistance to be achieved. V108I did, however, reappear in the resistant virus obtained at passage 11 without a loss of resistance. Through passage twelve, only one additional amino acid change was detected in the RT (H221Y) but this change was not correlated with an increased level of resistance to IQP-0410.

As would be expected for an NNRTI, sequencing of the RT of HIV-2 (Table 3, **HIV-2 Selection**) did not identify any amino acid changes in the RT which were responsible for the resistance of that virus to IQP-0410.

3.3.2. Changes in the envelope

Evaluation of the sequence of the envelope glycoproteins of the selected HIV-1 and HIV-2 viruses identified approximately 11 mutations in HIV-1 and 4 mutations in HIV-2 (Table 3). These mutations involved changes in both gp120 and gp41. In replicate selections performed with HIV-1, the mutations found in gp120 and gp41 were not consistently associated with specific amino acid loci, but did occur in regions associated with chemokine receptor engagement and fusion. With the first selection of HIV-1 resistant viruses, the initial changes in the envelope were first detected at passage 3 and changes continued through passage 15. Observed amino acid changes in the envelope glycoproteins included a K490I change in gp120 (12 amino acids from the gp120-gp41 junction), N677S in gp41 (adjacent to an N-linked glycosylation site), and V701I in gp41. These three mutations have not been reported as natural genetic polymorphisms in the HIV population and thus are likely associated with resistance to IQP-0410. Three additional changes N674D in gp120 (a glycosylation site), L684I in gp41, and T818I in gp41 (a glycosylation site) appeared in the envelope early in the selection of the HIV-1 resistant virus but these changes are common in the HIV-1 population and thus most likely represent natural genetic polymorphisms or fitness changes in the genome. Continued selection of the HIV-1 resistant virus yielded additional mutations which appeared by passage 10 and these changes included I165K in the V2 loop of gp120, T297I at a glycosylation site in the V3 loop of gp120, and G410R and I423F in the V4 loop of gp120. Each of these mutations were identified as being rare in the HIV population suggesting they may be associated with resistance (or fitness) of the viruses to IQP-0410. No additional late passage appearing changes were detected in gp41.

In the second HIV-1 selection reported in Table 3, six mutations were discovered in the envelope with three changes appearing in each of gp120 and gp41. Coincident with the loss of entry inhibition and the increase in fold-resistance seen in this resistant virus selection after passage 8, the T138I mutation in gp120 and the V696I mutation in the gp41 transmembrane domain appear the most significant. Other mutations which appeared during this selection included amino acid changes which appeared in the envelope and then reverted back to the wild-type amino acid (F383I in gp120, M421I in gp120, N672S in gp41, and R737K in gp41) and thus likely do not contribute to resistance. An additional four amino acid changes were detected in both the selected virus and the control virus passaged in the absence of IQP-0410. These mutations included I165K, E398K, I418F and D669N.

In the HIV-2 resistant virus (Table 3), one mutation appeared early in the selection, introducing a stop codon at position 750 which resulted in the truncation of 100 amino acids from the cytoplasmic tail of gp41. This virus remained replication competent. This mutation has been previously reported as a tissue culture adaptation in HIV-2_{ROD}. Three additional mutations were identified in the resistant viruses but they appeared at passage 7 at a time after the virus had become completely resistant to IQP-0410. One of these mutations was rare in the HIV-2 population (K185E near the V2 loop and adjacent to a glycosylation site) and two were commonly found amino acid changes, including A149T (adjacent to a glycosylation site) and K422R (near the V4 loop). No other changes were found in the envelope glycoproteins of the HIV-2 resistant virus.

3.4. Cross-resistance profiles of IQP-0410-resistant strains

The resistant strains of virus which were obtained at significant passages of the initial selection (Table 3, **HIV-1 Selection 1**) when new amino acid changes appeared (passage 7, passage 13 and passage 15) were utilized to evaluate the cross-resistance of IQP-0410-resistant virus strains to other inhibitors, including nucleoside and non-nucleoside RT inhibitors and protease inhibitors (Table 4). Although cross-resistance of the IQP-0410-resistant viruses was only detected to other NNRTIs, the overall levels of cross-resistance to these other NNRTIs are relatively small (generally less than 100-fold resistant) compared to the TI of IQP-0410. All of the nucleoside and nucleotide RT inhibitors and protease inhibitors remained active against the IQP-0410-resistant viruses at each of the tested passages (data not shown). With the NNRTIs, cross-resistance of the viruses was observed to those compounds which have known sensitivity to the Y181C change in the RT. Detailed evaluation of the data obtained, however, indicates that the pattern of resistance observed with IQP-0410 is specific to this compound and distinct from the resistance patterns that emerge with the other NNRTIs. For example, whereas the fold-resistance to IQP-0410 continues to increase with increasing passage number, resistance to other NNRTIs appears to reach a plateau and remain constant as the IQP-0410 resistance increases. Thus, with each of the three virus passages evaluated (passage 7, 13 and 15),

Table 4
Cross-resistance phenotype of IQP-0410 resistant viruses to other non-nucleoside RT inhibitors – selection 1.

Compound	EC_{50} (μM)/(fold-resistance)[a]			
	IQP-0410 P7 Y181C	IQP-0410 P13 E6K,Y181C, F227L	IQP-0410 P15 E6K,V106I, Y181C,F227L	HIV-1_{IIIB} wild-type EC_{50} (μM)
IQP-0410	0.1 (140)	2.8 (2800)	10 (10,000)	0.001
Costatolide	0.06 (ES)	0.1 (S)	0.3 (S)	0.4
Dihydrocostatolide	0.09 (ES)	0.2 (S)	0.3 (S)	0.6
UC10	0.8 (5.1)	1.4 (9)	1.5 (10)	0.2
NSC 676509	1.4 (24)	2.2 (37)	1.9 (31)	0.06
UC781	0.5 (53)	0.9 (94)	0.7 (70)	0.01
NSC 675187	0.3 (37)	0.5 (60)	0.8 (86)	0.009
Thiazolobenzimidazole	66 (25)	>66 (>25)	>66 (>66)	2.7
Oxathiin carboxanilide	3.7 (S)	9.4 (9)	7.4 (7)	1.1
TIBO	4.3 (43)	2.4 (24)	2.0 (20	0.1
Diphenylsulfone	>100 (>21)	>100 (>21)	>100 (>21)	4.8
α-APA	>5 (>26)	>10 (>53)	>10 (>53	0.2
E-BPTU	0.7 (35)	1.2 (60)	1.02 (51)	0.02
Nevirapine	>5 (>83)	>10 (>167)	>10 (>167)	0.06
AZT	0.003 (S)	0.003 (S)	0.003 (S)	0.007
3TC	0.08 (S)	0.08 (S)	0.003 (S)	0.1

S = Sensitive, ES = Enhanced sensitivity.
[a] The results presented were obtained from a single representative antiviral assay with appropriate control compounds evaluated in parallel selected from a minimum of three antiviral assays. We have demonstrated that the standard error among multiple antiviral assays averaged less than 10% of the respective mean EC_{50} and TC_{50}. In each individual assay, mean efficacy and toxicity values are derived from a minimum of three replicate samples.

Table 5
Cross resistance phenotype of IQP-0410-resistant viruses – selection 2.

	EC_{50} (μM)/(fold-resistant)[a]						
Passage #	IQP-0410	AZT	EFV	UC781	TFV	T20	ISIS 5320
HIV-1_{IIIB} (WT)	0.004	0.007	0.009	0.003	1.0	0.02	0.5
2	0.006	0.007	0.002	0.01	2.3	0.02	<0.08
	(1.5)	(1.0)	(0.2)	(3.3)	(2.3)	(1.0)	(<0.16)
4	0.08	0.005	0.003	0.04	7.9	0.02	1.0
	(20)	(0.7)	(0.3)	(13)	(7.9)	(1.0)	(2.0)
6	0.05	0.003	0.005	0.2	4.0	<0.003	0.8
	(12.5)	(0.4)	(0.6)	(70)	(S)	(<0.15)	(1.6)
8	10.3	0.003	0.3	0.5	5.4	0.018	0.2
	(2575)	(0.4)	(29)	(180)	(5.4)	(0.9)	(0.4)
10	>200	<0.002	0.3	1.05	3.6	0.004	0.4
	(>50000)	(<0.3)	(31)	(350)	(S)	(0.2)	(0.8)
12	>200	0.003	0.3	6.9	2.0	0.005	0.2
	(>50000)	(0.4)	(34)	(2300)	(S)	(0.25)	(0.4)
13	>200	0.004	0.8	>10	4.6	0.003	0.08
	(>50000)	(0.6)	(86)	(>3333)	(S)	(0.15)	(0.16)

Compound abbreviations: AZT: azido-thymidine; EFV: efavirenz; UC781: thiocarboxanilide; TFV: tenofovir; T20: Fuzeon; ISIS 5320: oligonucleotide $T_2G_4T_2$.
[a] The results presented were obtained from a single representative antiviral assay with appropriate control compounds evaluated in parallel selected from a minimum of three antiviral assays. We have demonstrated that the standard error among multiple antiviral assays averaged less than 10% of the respective mean EC_{50} and TC_{50}. In each individual assay, mean efficacy and toxicity values are derived from a minimum of three replicate wells.

resistance to IQP-0410 increases from 140-fold to 2800-fold to 10,000-fold, reflecting the accumulation of amino acid changes in the RT, changes in the sensitivity of the virus to entry inhibition, as well as other potential fitness and replication capacity changes that impact on the ability of IQP-0410 to effectively inhibit virus replication. A similar evaluation of these viruses with five carboxanilide compounds (oxathiin carboxanilide, UC10, NSC 676509, UC781 and NSC 675187), 8-chloro-TIBO, and the HEPT analog E-EBTU yields a much different cross-resistance profile. In these cases, resistance rises to its peak level at passage 7 and no further increases in resistance are observed as the passage number increases, suggesting that the initial mutational changes are NNRTI-specific and the later changes are IQP-0410 specific and likely related to the loss of entry inhibition. All of the IQP-0410-resistant strains remained sensitive to compounds of the calanolide class (calanolide A, costatolide, dihydrocostatolide), with enhanced sensitivity observed with the Passage 7 IQP-0410-resistant virus with the Y181C change. The calanolides are known to exhibit enhanced antiviral activity to viruses with the Y181C amino acid change (Buckheit et al., 1999).

Cross-resistance evaluations were also performed for the viruses obtained at each passage from the second resistance selection (Table 5). These results indicate that the IQP-0410-resistant viruses remain completely sensitive to the entry/fusion inhibitors (T20 and ISIS 5320), and the nucleoside and nucleotide RT inhibitors (AZT, tenofovir). Cross-resistance to other NNRTIs was observed with thiocarboxanilide (UC781) and efavirenz, especially after the appearance of the K103N mutation in the RT. These data suggest that the fusion inhibitor T20 (Fuzeon) and the phosphorothioate oligonucleotide attachment/fusion inhibitor ISIS 5320 may exhibit enhanced sensitivity to IQP-0410-resistant viruses (5–10-fold enhanced sensitivity).

Cross-resistance evaluations to a broader panel of compounds representative of various anti-HIV mechanisms of action was performed utilizing resistant viruses obtained over 14 passages of resistance selection to IQP-0410 (Table 6). These results demon-

Table 6
Cross-Resistance Phenotype of IQP-0410-Resistant Viruses – Selection 3.

Passage #	Fold-resistance of IQP-0410-resistant virus to test compound[a]													
	IQP-0410	AZT	3TC	ddC	EFV	NVP	UC781	SQV	RTV	NFV	IDV	CSB	T20	DS
EC_{50} (μM)	0.004	0.007	0.01	0.05	0.009	0.02	0.003	0.002	0.07	0.02	0.004	0.25	0.004	0.96
1	0.6	0.1	1.3	0.4	0.2	3	0.7	2	2	1.2	2	1.6	<0.5	0.6
2	22.5	0.4	0.4	0.3	0.2	>25	5.3	5	0.1	0.5	1.2	1	<0.5	1.0
3	218	0.4	0.8	1.1	0.2	195	21	<8	0.5	0.8	8	1.7	0.5	0.6
4	223	0.3	<0.1	ND	0.3	ND	3.3	ND	0.5	ND	4.3	ND	0.2	ND
5	243	0.1	0.9	0.54	0.1	>500	10	3	0.5	0.3	1.8	3.6	4.5	0.6
6	255	1.7	0.6	1.2	0.2	>500	43	8	1.4	0.8	3.8	3.6	13.8	1.4
7	280	0.9	0.9	0.9	0.2	>500	20	7	0.4	1.8	2	3.2	0.2	0.7
8	1205	0.3	0.6	0.4	0.2	>500	40	8	0.6	0.8	1	1.2	0.8	0.7
9	1275	2.1	0.6	0.7	0.3	>500	29	7.5	0.4	0.4	1.8	2	0.2	0.5
10	1200	0.3	3.4	1.0	0.4	>500	50	5.5	0.4	0.6	2	2.4	0.2	0.5
11	1300	0.6	1.9	0.6	0.2	>500	20	5	0.6	0.2	4	0.8	0.2	0.4
12	1425	0.6	2.1	2.3	0.4	>500	167	4	1	1.3	3.3	1.6	0.2	0.3
13	1400	0.3	0.8	0.3	0.7	>500	133	4.5	0.6	0.8	2	2	0.2	0.1
14	>50000	0.4	0.7	0.3	1.1	>500	167	3	0.4	0.2	1.8	3.2	0.2	0.08

Compound abbreviations: AZT: azido-thymidine; 3TC: 3′-thiocytidine; ddC: dideoxycytidine; EFV: efavirenz; NVP: nevirapine; UC781: thiocarboxanilide; SQV: saquinavir; RTV: ritonavir; NFV: nelfinavir; IDV: indinavir; CSB: Chicago Sky Blue; T20: Fuzeon; DS: dextran sulfate.

[a] The results presented were obtained from a single representative antiviral assay with appropriate control compounds evaluated in parallel selected from a minimum of three antiviral assays. We have demonstrated that the standard error among multiple antiviral assays averaged less than 10% of the respective mean EC_{50} and TC_{50}. In each individual assay, mean efficacy and toxicity values are derived from a minimum of three replicate wells.

strate the lack of cross-resistance of IQP-0410-resistant virus to nucleoside analogs, protease inhibitors, and entry/fusion inhibitors, and again suggest the potential enhanced sensitivity of the resistant virus to T20 and dextran sulfate. Cross resistance is clearly observed to other NNRTIs. These data suggest that IQP-0410 and efavirenz might be effectively used in combination with each other based on their cross resistance profiles.

4. Discussion

IQP-0410 is a small molecule pyrimidinedione HIV inhibitor characterized by a novel dual mechanism of action resulting in the inhibition of both reverse transcription and virus entry. The mechanism of RT inhibition has been defined at an enzymatic level and entry inhibition has been shown to involve recognition of a pre-fusion conformational epitope which is formed upon co-culture of virus with cells at 4 °C, and does not include direct inhibition of the gp120-CD4 interaction or virus attachment. The studies performed herein have helped to confirm and expand our understanding of the inhibitory mechanism of IQP-0410 against both HIV-1 and HIV-2. A more complete understanding of resistance-engendering mutations and cross-resistance evaluations are important to the analysis and performance of human clinical trials and the selection of appropriate combination therapeutic strategies.

Among the first generation of NNRTIs, single amino acid changes in the RT were determined to have a major impact on their therapeutic use and thus the appearance of the Y181C, L100I and/or the P236L amino acid changes completely abrogated the activity of NNRTIs such as nevirapine, α-APA, or delavirdine (BHAP) (Ding et al., 1995; Milinkovic and Martinez, 2004; Romero et al., 1996; Watson Buckheit et al., 2011). Newer generations of NNRTIs, such as efavirenz, TMC125 (etrivirine) and TMC278 (rilpivirine), require an accumulation of mutations in the hydrophobic NNRTI binding pocket in order to develop high level resistance to the compounds (Vingerhoets et al., 2005; Young et al., 1995) and thus the NNRTIs have found an important niche in the current HAART regimens. The approved NNRTIs still have significant issues with toxicity and side effects which reduce patient compliance and result in the opportunity for high level resistance to evolve (Adams et al., 2010; Blas-Garcia et al., 2011; de Bethune, 2010). The pyrimidinediones represent yet another advance in the development of the NNRTIs by complementing the need to select for multiple mutations in the RT for high level resistance with a second distinct mechanism of antiviral action which introduces an intrinsically high genetic barrier to resistance. Thus, in light of the significantly high SI of IQP-0410 (approximately 1 million), high level resistance to the compound requires mutations in the viral envelope glycoproteins to abrogate entry inhibition prior to the accumulation of mutations in the RT which yield the high level resistance. Combination anti-HIV therapy, best represented by the currently employed HAART regimens, has evolved in order to target multiple steps in HIV replication with different drugs in order to avoid resistance selection, to allow the use of lower concentrations of synergistic drugs, and to lower drug toxicity. HAART regimens have proven that long lasting and durable antiviral responses may be obtained even when targeting only three to four HIV protein targets (Gathe, 2003; Palmisano and Vella, 2011; Portsmouth et al., 2003). The pyrimidinediones allow HAART-like therapy to be employed with one highly potent small molecule possessing two distinct antiviral targets and can be used in combination with all classes of antiretroviral agents (Hartman et al., 2011).

IQP-0410 rapidly selects for both HIV-1 and HIV-2 resistant virus strains when virus is treated with suboptimal doses of the compound. In the HIV-1 selection process, resistance evolves through three distinct phases to eliminate the dual mechanism of antiviral action. Upon culture of HIV-1 in the presence of IQP-0410, a virus possessing a mutation in the NNRTI binding site is initially selected. This initial stage in the selection process, which routinely results in the appearance of a virus with the Y181C change in the RT, yields viruses with 10 to 100-fold levels of resistance to IQP-0410. Following the initial RT mutation (or the accumulation of changes observed in the HIV-1 Selection 2), amino acid changes begin to accumulate in gp41 and gp120. Many of these mutations are at or in close proximity to glycosylation sites. The appearance of these Env mutations increases the level of resistance of the virus over 5–6 passages to approximately 1000-fold. Finally, with the elimination of entry inhibition, additional mutations accumulate in the RT yielding high level resistance to IQP-0410. All of the mutations described in the RT are consistent with IQP-0410 acting as a typical HIV-1 specific NNRTI. The three phase resistance selection process renders the pyrimidinediones quite distinct from other NNRTIs and indicates that the pyrimidinediones possess an intrinsically high genetic barrier to resistance because the compounds act at two distinct sites requiring a

significant accumulation of mutations. Thus, IQP-0410 or related pyrimidinedione might be a highly useful addition to existing HAART regimens.

The amino acid changes in the envelope of the IQP-0410-resistant viruses also provide important clues as to the secondary entry inhibition mechanism of action of the compound. In light of the large number of mutations and site directed mutations that would need to be constructed in order to evaluate the contribution of each mutation, we have limited our evaluation of the mutations to an evaluation of their locations and potential roles in resistance. The cluster of three mutations in V4/C4 include changes in the β20/β21 domains of the bridging sheet are important for co-receptor binding. In addition, mutations in the membrane proximal domain of gp41 and in the transmembrane domain are a component of the binding site of the broadly neutralizing 4E10 monoclonal antibody. This pattern of mutations in gp120 and gp41 is consistent with alterations in coreceptor binding following CD4 engagement and changes in gp41 that may impact gp120 binding, trimer formation, and fusion efficiency. We hypothesize that entry inhibition due to interference with both co-receptor binding and fusion is the most likely mechanism of action, and this interpretation is consistent with the biological results which demonstrate the ability of IQP-0410 to target a tertiary conformational compound binding epitope which is formed upon cocultivation of virus and target cells at 4 °C. The consistent selection of resistance engendering mutations in the membrane proximal domain of gp41 suggests a conserved site for IQP-0410 binding in or near the membrane spanning domain. A small molecule that targets the 4E10 binding site would be expected to have broad activity against clinical HIV isolates. Consistent with the proposed entry inhibitory mechanism of action of IQP-0410 suggested by the mutational analysis, viruses resistant to the compound appear to become more sensitive to inhibition by T20, suggesting that changes in the envelope glycoprotein selected by IQP-0410 which abrogate entry inhibition also sensitize the resistant virus to fusion inhibition. These envelope changes also sensitive the resistant viruses to the V3-loop targeted compounds ISIS 5320 and dextran sulfate but only with viruses obtained at later passages of the selection.

Thus, resistance selection to IQP-0410 confirms the antiviral mechanism of action involving both RT and virus entry and indicates that both gp120 and gp41 play a role in the virus entry inhibition. With HIV-2, the selected resistant strain did not possess any changes in the RT while several mutations were identified in the envelope glycoproteins, confirming the entry inhibitory mechanism of action of IQP-0410 against HIV-2 since the NNRTIs do not target the HIV-2 RT.

The high sensitivity of MDR strains to IQP-0410 suggests that viruses from highly ARV therapy-experienced patients with RT mutations were moderately less sensitive to the activity of IQP-0410 consistent with the appearance of the Y181C change (10- to 100-fold loss of sensitivity). However, viruses with PI-resistance mutations in combination with the RT mutations or PI mutations alone exhibited wild-type levels of activity and in some cases had enhanced sensitivity to the antiviral effects of IQP-0410. Thus, the PI mutations appear to sensitize viruses to the activity of IQP-0410. The data obtained indicated that IQP-0410 became more sensitive to the MDR-PI resistant strains as greater numbers of mutations accumulated in the protease. Further research will be required to confirm and expand this important finding.

The pyrimidinediones represent an attractive class of anti-HIV inhibitors for preclinical and clinical development. IQP-0410 has progressed through the bulk of our IND-directed preclinical development evaluations and has been found to have little to no toxicity at doses ranging up to 1000 mg/kg in small and large animals. Additional studies are being performed to improve upon the solubility of IQP-0410 in formulation in order to enhance pharmacokinetics however concentrations obtained in plasma are 10-fold greater than the EC_{99} concentrations of IQP-0410 as defined by *in vitro* assays. A greater understanding of the resistance potential of the class will be important for evaluation of the results of clinical trials, for selection of appropriate drugs to be utilized in combination, and for the biochemical and mechanistic definition of the entry inhibition mechanism of action. A precise model for the entry inhibitory capability of IQP-0410 might allow the discovery and development of even more potent inhibitors of virus entry since it is clear that the conformational binding site represents a new and novel entry target. The pyrimidinediones are not the first class of small molecules to exhibit a dual mechanism of action involving both RT and entry inhibition (McMahon et al., 1995), however they are as potent as the best NNRTIs and the first NNRTIs in which significant HIV-2 inhibition is observed. In addition, the pyrimidinediones possess a broad spectrum of antiviral activity against drug- and multi-drug resistant viruses which will be further exploited. Continued development of other inhibitors in the pyrimidinedione class with greater entry inhibition potential and/or enhanced activity against problematic NNRTI-resistant viruses, as well as additional mechanism of action studies utilizing biochemical and cell based assay systems are currently being employed to define the best lead compounds for clinical development.

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