Supporting Information

Specific Targeting of Hepatitis C Virus NS3 RNA Helicase. Discovery of the Potent and Selective Competitive Nucleotide-mimicking Inhibitor QU663. †

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Experimental details for the new compounds (molecular modelling, chemistry, HIV-1 RT enzymatic assays, anti-HIV tests, toxicity experiments), Tables 1-4, Figure 1 SI and Schemes 1 and 2.

Molecular Modelling.

All molecular modelling studies were performed on SGI Indigo II R10000 and SGI Octane 2XR10000 workstations.

Analysis of the chemical-physical properties of nucleotides and the newly designed compounds

QX432, **QX449**, **QU494**, and **QU663** were built using the Insight 2000.1 Builder module, the molecular structures of guanine, adenine, cytosine, thymine, and uracil were extracted from the Fragment databank (Accelrys, San Diego). Apparent pK_a values of the newly designed compounds in their tautomeric forms, were calculated by using the ACD/p*K*a DB version 7.00 software (Advanced Chemistry Development Inc., Toronto, Canada), accordingly percentage of neutral/ionized forms were computed using the Handerson-Hasselbach equation. Quinoline moiety of **QU663** was considered protonated in all calculations performed, as a consequence of the estimation of apparent pK_a values. **QU663** charges were manually assigned by comparing partial charges assigned by CVFF force field (*1*) with those calculated by a MNDO (*2*) semi-empirical 1 SCF calculations performed on the neutral and the ionized forms.

All the structures were subjected to conformational search through 50 cycles of Simulated Annealing (Tripos force field, Sybyl software, Tripos, San Louis). A starting temperature of 700 K was applied to surmount torsional barriers, the structure was held at that temperature for 1000 fs, then the temperature was reduced to 0 K by a decrement of 0.5 K/fs. Resulting structures were subjected to energy minimization within the Insight2000.1 Discover module (CVFF force field (*1*), Conjugate Gradient algorithm; ε=80*r) until the maximum RMS derivative was less than 0.001 kcal/Å, and subsequently ranked by their conformational energy values.

In order to properly analyze the electronic properties of **QX432**, **QX449**, **QU494**, **QU663**, and nucleotides, their most stable conformers were subjected to a full geometry optimization by semi-empirical calculations, using the quantum mechanical method AM1 in the Mopac (*2*) 6.0 package in Ampac/Mopac module of Insight2000.1. GNORM value was set to 0.5. To reach a full geometry

optimization the criteria for terminating all optimizations was increased by a factor of 100, using the keyword PRECISE. Connolly surfaces were computed and colored by AM1 charge distribution. Obtained structures were superimposed by fitting their electronic distribution.

Modeling of NS3 helicase

NS3 helicase (NS3h) available crystal structures were downloaded from the PDB data bank (http://www.rcsb.org/pdb/; PDB IDs: 1A1V, 8OHM, 1HEI and 1CU1). Hydrogens were added to all the PDB structures considering a pH value of 7.2.

The X-ray structure of NS3h in complex with the inhibitor deoxyuridine octamer (dU_8) was selected to perform subsequent docking calculations (PDB ID: 1A1V). Since amino acids 415-417 were lacking in 1A1V structure, the protein loop 414-420, which resulted highly flexible by comparing all available NS3h PDB files, was modeled using the standard homology building procedure provided by the Homology module of Insight2000.1 (Accelrys, San Diego). A structural comparison between 1A1V and the other NS3h X-ray structures resulted in the choice of 1HEI as the reference template for model construction (lowest RMSD value on 413 and 421 backbone atoms). Moreover, the side chains of the residues A195, A244, A357, A360, A372, A530, A541, A580 and A583, changed to alanine in the structure of 1A1V due to poor side chain density were replaced with the ones actually present in the amino acid sequence of the enzyme.

All subsequent structural calculations were performed using the CVFF forcefield to parameterize protein residues and water molecules, while applying to the ligand the above mentioned manual charges assignment derived from semi-empirical methods.

Generation of the starting structure for the docking calculations

The docking procedure formally requires a reasonable starting structure, nevertheless during the subsequent calculation protocol the ligand is subjected to an exhaustive conformational search (torsional angles, rotation, translation) within the fully flexible active site of the protein by means of Monte Carlo and Simulated Annealing methods.

To introduce **QU663** into NS3h structure, the crystal ligand dU_8 was removed, by using the unmerge command in the Biopolymer Module of Insight2000.1 (Accelrys, San Diego). The lowest energy conformer of **QU663** was positioned into the NS3h dU_8 binding site on the basis of its inhibitory activity and mutagenesis data available on the enzyme (*3-8)* (being a competitive inhibitor which does not affect ATPase functioning). All the water molecules present in 1A1V were maintained, with the exception of those that would sterically overlap with the ligand. Obtained complex was subjected to preliminary energy minimization (Steepest Descent algorithm; ε=1) until the maximum RMS derivative was less than 0.5 kcal/Å, to generate roughly docked starting structure, as required by Affinity docking procedure.

Docking calculations

Flexible docking was achieved using the Affinity module in the Insight2000.1 suite, setting the SA_Docking procedure (*9*), and the Cell_Multipole (*10*) method for non-bond interactions. The binding domain area was defined as the logical union of: i) a subset including the ligand plus all residues and water molecules having at least one atom within 10 Å radius from any given ligand atom, and, ii) a subset including all the residues and water molecules having at least one atom within 5 Å radius from any original crystal ligand (dU_8) atoms. All the atoms of the residues included in the above defined binding domain area (71 aminoacids, 15 water molecules and the ligand), were left fully free to move during the entire docking calculations, whereas a tethering restraint was applied on the 15 water molecules included in the binding domain area, using a force constant of 10.0 kcal/mol/Å, to avoid unrealistic water escape during the subsequent Simulated Annealing procedure.

A Monte Carlo/minimization approach for random generation of a maximum of twenty structures was used, with an energy tolerance of 10^6 Kcal/Mol to ensure a wide variance of the input structures to be minimized (2500 iterations; ε=1). During this step the ligand is moved by a random combination of translation, rotation, and torsional changes (Flexible_Ligand option, considering all rotatable bonds), to sample both the conformational space of the ligand and its orientation with

respect to the enzyme. Van der Waals (vdW), Coulombic and tether terms were scaled to a factor of 0.1 to avoid large energy fluctuations and adverse effects on the running of Monte Carlo/minimization calculations. The Metropolis test, at a temperature of 310 K, and a structure similarity check (RMS tolerance = 0.3 kcal/Å), were applied to select acceptable structures. 50 stages of Simulated Annealing (100 fs each) were applied on the resulting complexes. Over the course of the Simulated Annealing, system temperature was linearly decreased from 500 K to 300 K, concurrently the Van der Waals, Coulombic and tether scale factors were similarly decreased from their initial values (defined above as 0.1) to their final value (1.0). A final round of 10^6 minimization steps was applied to on the at the end of the molecular dynamics. After this procedure, the resulting docked structures were ranked by their conformational energy. The lowest energy complex was selected.

Chemistry.

The quinolines **QU663** and **QU494** were synthesized by standard methods starting from *m(o)*-phenetidines as shown in Scheme 1. Reagents for organic synthesis were purchased from Aldrich. TLCs were performed on silica gel plates (Riedel-de-Haen; Art.37341). Merck silica gel (Kieselgel 60) was used for the flash chromatography (230–400-mesh) column. Yields refer to purified products and are not optimized. Melting points were determined using a Buchi melting point B-540 apparatus and were uncorrected. ^{1}H-NMR spectra were recorded on a Bruker 200-MHz spectrometer with TMS as internal standard; the values of the chemical shifts (δ) are given in ppm, and coupling constants (*J*) are given in Hz. Elemental analyses were performed on a PerkinElmer Life Sciences 240C elemental analyzer. Mass spectra were measured on a double focusing mass spectrometer VG 70–250S (VG Analytical Ltd., Manchester, UK) under electron ionization conditions (70 eV and accelerating voltage of 8 KV).

4-Chloro-8-methoxy-2-methylquinoline (2a)

A mixture of *o*-phenetidine (1.30 g, 10.0 mmol) and ethyl acetoacetate (1.56 g, 12.0 mmol) was heated to 100°C for 48h. After cooling to room temperature the condensation product was cyclized in diphenylether under reflux using a Dean-Stark trap for 1.5h. After solvent removing the residue was crystallized from ethanol to afford 1.65 g (82%) of 8-methoxy-4-hydroxy-2-methylquinoline (mp 270-272 °C). The latter compound was treated with phosphorus oxychloride (2.5 mL) at 135°C for 1h. After cooling to room temperature the reaction mixture was poured into a saturated solution of sodium carbonate. The resulting precipitate was collected and crystallized from ethanol to afford the desired compound (1.86 g, 80%) as colourless prisms (mp 96-98 °C). ^{1}H-NMR ($CDCl_3$) δ 8.19 (d, 1H, *J* = 9.4 Hz), 7.31-7.16 (m, 3H), 3.84 (s, 3H), 2.96 (s, 3H). Anal Calcd for ($C_{11}H_{10}ClNO$): C, H, N.

4-Chloro-7-ethoxy-2-methylquinoline (2b)

A mixture of *m*-phenetidine (1.37 g, 10.0 mmol) and ethyl acetoacetate (1.56 g, 12.0 mmol) was heated to 100°C for 48h. After cooling to room temperature the condensation product was cyclized in diphenylether under reflux using a Dean-Stark trap for 1.5h. After solvent removing the residue was crystallized from ethanol to afford 1.8 g (86%) of 7-ethoxy-4-hydroxy-2-methylquinoline (mp 273-275 °C). The latter compound was treated with phosphorus oxychloride (2.5 mL) at 135°C for 1h. After cooling to room temperature the reaction mixture was poured into a saturated solution of sodium carbonate. The resulting precipitate was collected and crystallized from ethanol to afford the desired compound (1.8 g, 89%) as colourless solid (mp 102-103 °C). ^{1}H-NMR ($CDCl_3$) δ 8.03 (d, 1H, *J* = 9.4 Hz), 7.31 (d, 1H, *J* = 2.6 Hz), 7.22-7.16 (m, 2H), 4.16 (q, 2H, *J* = 6.9 Hz), 2.66 (s, 3H), 1.48 (t, 3H, *J* = 6.9 Hz,). Anal Calcd for ($C_{12}H_{12}ClNO$): C, H, N.

N-(8-methoxy-2-methylquinolin-4-yl)hydrazine hydrochloride (3a)

A equimolar mixture of 4-chloro-8-methoxy-2-methylquinoline and hydrazine hydrate in ethanol (20 mL) was heated under reflux for 5h. After cooling to room temperature a fine precipitate was formed. The precipitate was collected and recrystallized from ethanol to afford 8-methoxy-2-methyl-4-hydrazinoquinoline hydrochloride (90%) as pale yellow prisms (mp 266-268 °C). . ^{1}H-

NMR (CD_3OD) δ 8.72 (s, 1H), 8.23 (d, 1H, *J* = 8.2 Hz), 7.27-7.10 (m, 2H), 3.92 (s, 3H), 3.28 (s, 3H). Anal Calcd for ($C_{11}H_{14}ClN_3O$): C, H, N.

N-(7-ethoxy-2-methylquinolin-4-yl)hydrazine hydrochloride (3b)

A equimolar mixture of 4-chloro-7-ethoxy-2-methylquinoline and hydrazine hydrate in ethanol (20 mL) was heated under reflux for 5h. After cooling to room temperature a fine precipitate was formed. The precipitate was collected and recrystallized from ethanol to afford 7-ethoxy-2-methyl-4-hydrazinoquinoline hydrochloride (95%) as pale yellow crystalline solid (mp 272-273 °C). . ^{1}H-NMR (CD_3OD) δ 8.07 (d, 1H, *J* = 8.9 Hz,), 7.14 (dd, 1H, *J* = 2.5, 8.9 Hz), 7.07-7.02 (m, 2H), 4.18 (q, 2H, *J* = 6.8 Hz), 2.61 (s, 3H), 1.42 (t, 3H, *J* = 6.8 Hz). Anal Calcd for ($C_{12}H_{16}ClN_3O$): C, H, N.

N'-(Pyrazinecarbonyl)-N''-(8-methoxy-2-methylquinolin-4-yl)hydrazine (QU494)

To a suspension of pyrazine carboxylic acid (0.36 g, 2.53 mmol) in anhydrous dichloromethane (12 mL) were added, portion-wise within 1h, triphenylphosphine (1.3 g, 5.2 mmol) and 2,2'-dipyridildisulfide (Aldrithiol-2) (1.15 g, 5.2 mmol) and the resulting suspension was stirred at room temperature for 3h. Then a solution of *N*-(8-methoxy-2-methyl-4-quinolyl)hydrazine hydrochloride (650 mg, 2.53 mmol) and triethylamine (7 mL) in anhydrous dichloromethane (45 mL) was added and the reaction mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure and the residue was flash-chromatographed (dichlorometane 4: methanol 1) to yield a yellow solid which was crystallized from ethanol to give 500 mg (78%) of QU494 as pale yellow prisms (mp: 242-244 °C). ^{1}H-NMR (DMSO) δ 11.26 (br s, 1H), δ 10.73 (bs, 1H), 9.08 (s, 1H), 8.66 (m, 1H), 8.51 (s, 1H), 8.00 (d,1H, *J* = 9.1 Hz), 7.16-7.00 (m, 2H), 6.33 (s, 1H), 3.88 (s, 3H), 2.54 (s, 3H). MS (EI) *m/z* 310 (M^{+1}, 100) . Anal Calcd for ($C_{15}H_{13}N_5O_2$): C, H, N.

N'-(Pyrazinecarbonyl)-N''-(7-ethoxy-2-methylquinolin-4-yl)hydrazine (QU663)

To a suspension of pyrazine carboxylic acid (0.24 g, 1.97 mmol) in anhydrous dichloromethane (10 mL) were added, portion-wise within 1h, triphenylphosphine (1.0 g, 3.9 mmol) and 2,2'-dipyridildisulfide (Aldrithiol-2) (0.87 g, 3.94 mmol) and the resulting suspension was stirred at room temperature for 3h. Then a solution of *N*-(7-ethoxy-2-methyl-4-quinolyl)hydrazine

hydrochloride (500 mg, 1.97 mmol) and triethylamine (5 mL) in anhydrous dichloromethane (30 mL) was added and the reaction mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure and the residue was flash-chromatographed (dichlorometane 4: methanol 1) to yield a yellow solid which was crystallized from ethanol to give 520 mg (82%) of **QU663** as yellow prisms (mp: 250-252 °C). ^{1}H-NMR (DMSO) δ 10.85 (bs, 1H), 9.20 (s, 1H), 8.89 (d, 1H, *J* = 1.9 Hz), 8.78 (s, 1H), 8.09 (d,1H, *J* = 9.0 Hz), 7.06-6.98 (m, 2H), 6.29 (s, 1H), 4.12 (q, 2H, *J* = 6.8 Hz), 2.34 (s, 3H), 1.36 (t, 3H, *J* = 6.8). MS (EI) *m/z* 324 (M^{+1}, 100). Anal Calcd for ($C_{17}H_{17}N_5O_2$): C; H, N.

N-(Pyrrolo[1,2-a]quinoxalin-4-yl)hydrazine (5a). A solution of **4a** (1.04 g, 5.6 mmol) and a catalytic amount of *N*,*N*-dimethylaniline in phosphorus oxychloride (16.9 mL, 184 mmol) was refluxed under argon for 5 h. After cooling, the excess phosphorus oxychloride was distilled off, and the residue was partitioned between dichloromethane and sodium hydrogencarbonate solution. The organic solution was washed with brine, dried, and concentrated. The residue was purified by column chromatography (dichloromethane/EtOAc, 9:1) and recrystallized from hexanes to give the 4-chloro intermediate (0.67 g, 59%) as colorless prisms: mp 262-264 °C. This chloro derivative was dissolved in methanol (15 mL), and the solution was cooled at 0 °C. Then a solution of hydrazine monohydrate (140 μL) in methanol (3 mL) was slowly added. After the mixture was stirred for 2 h at room temperature, the solid was filtered off and the solution was concentrated to give **5a** as a white solid which was recrystallized from methanol (590 mg, 86%): mp 159-160 °C; IR (Nujol) 3300 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ4.53 (br s, 2 H), 6.68 (m, 1 H), 6.89 (m, 1 H), 7.30 (m. 2 H), 7.51 (d, 1 H, *J* = 7.6 Hz), 8.05 (d, 1 H, *J* = 7.6 Hz), 8,20 (m, 1 H), 8.71 (br s, 1 H). Anal. Calcd for $C_{11}H_{10}N_4$: C, H, N.

N-(Imidazo[1,2-a]quinoxalin-4-yl)hydrazine (5b). Compound **5b** was prepared following a synthetic procedure as for **5a**, starting from imidazo[1,2-*a*]quinoxalin-4(5H)-one **4b**; mp 133-135 °

C; IR (Nujol) 3300 cm^{-1}; ^1H NMR (DMSO-d_6) δ 4.23 (br s, 2 H), 6.60 (d, 1 H), 6.93 (d, 1 H), 7.30 (m, 2 H), 7.81 (m, 2 H), 8.56 (br s, 1 H). Anal. Calcd for $C_{10}H_9N_5$: C, H, N.

N-(Pyrazinecarbonyl)-N'-(pyrrolo[1,2-a]quinoxalin-4-yl)hydrazine (QX432). To a magnetically stirred suspension of pyrazinecarboxylic acid (31 mg, 0.25 mmol) in anhydrous dichloromethane (1 mL) were added, portionwise within 1 h, triphenylphosphine (132 mg, 0.5 mmol) and 2,2'-dipyridyl disulfide (Aldrithiol-2) (111 mg, 0.5 mmol), and the disappearance of the starting material was monitored by TLC (EtOAc). Then a solution of **5a** (50 mg, 0.25 mmol) in anhydrous dichlometane (3 mL) was added, and the reaction mixture was stirred overnight at room temperature. The solvent was removed, and the residue was taken up in a mixture of EtOAc and 5% hydrochloric acid. The organic phase was washed with 1 N sodium hydroxide and brine, dried, and concentrated. The residue was chromatographed (dichlorometane and 10% methanol and 1% ammonium hydroxide) to give a yellow solid which was recrystallized (EtOAc) to give 45 mg of **QX432** as yellow prisms: mp 216-218 °C; IR (Nujol) 3420, 1653 cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.83 (s, 1 H), 7.20-7.30 (m, 3 H), 7.44 (m, 1 H), 8.13 (m, 1 H), 8.34 (s, 1 H), 8.87 (s, 1 H), 8.98 (s, 1 H), 9.25 (s, 1 H), 9.65 (br s, 1 H), 10.96 (br s, 1 H). Anal. Calcd for $C_{16}H_{12}N_6O$: C, H, N.

N-(Pyrazinecarbonyl)-N'-(imidazo[1,2-a]quinoxalin-4-yl)hydrazine (QX449).

Compound **QX449** was prepared following a synthetic procedure as for **QX432**, starting from *N*-(imidazo[1,2-*a*]quinoxalin-4-yl)hydrazine **5b**; mp 194-196 °C; IR (Nujol) 3400, 1650 cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.63 (d, 1 H), 7.01 (d, 1 H), 7.24-7.35 (m, 3 H), 7.48 (m, 1 H), 8.30 (d, 1 H), 9.06 (s, 1 H), 9.15 (d, 1 H), 9.75 (br s, 1 H), 10.66 (br s, 1 H). Anal. Calcd for $C_{15}H_{11}N_7O$: C, H, N.

HIV-1 RT RNA-dependent DNA Polymerase Activity Assay. Inhibition Assay (*11*). RNA-dependent DNA polymerase activity assay was assayed as follows: a final volume of 25 μM contained reaction buffer (50 mM Tris-HCl pH 7.5, 1 mM DTT, 0.2 mg/mL BSA, 4% glycerol), 10

mM $MgCl_2$, 0.5 μg of poly(rA)oligo$(dT)_{10:1}$ (0.3 μM 3'-OH ends), 10 μM [^{3}H]-dTTP (1 Ci/mmol), and 2-4 nM RT. Reactions were incubated at 37 °C for the indicated time. A total of 20 μL aliquots were then spotted on glass fiber filters GF/C which were immediately immersed in 5% ice-cold TCA. Filters were washed twice in 5% ice-cold TCA and once in ethanol for 5 min and dried, and acid-precipitable radioactivity was quantitated by scintillation counting. Reactions were performed under the conditions described for the HIV-1 RT RNA-dependent DNA polymerase activity assay. Incorporation of radioactive dTTP into poly(rA)/oligo(dT) was monitored in the presence of increasing amounts of the inhibitors to be tested.

Secondary Tests (*11*). Compound **QU663** in enzymatic assays was evaluated for its ability to inhibit syncytia formation on C8166 cells infected with wild-type HIV-1_{IIIB} virus. Furthermore, MTT assays were also conducted as a measure of the cytotoxic effect of the new compound on four different cell lines. Test were carried out as in reference 11.

In Vitro Anti-HIV Assays. Cell Culture Assay (*11*). The ability of the test compound to protect HIV-1 infected T4 lymphocytes (C8166 cells) from cell death was determined following the reported procedure. C8166 is a $CD4^+$ T-cell line containing an HTLV-I genome of which only the tat gene is expressed. A laboratory lymphocyte-tropic strain of HIV-1 (HIV-1-IIIB) was used to infect C8166.

Toxicity Tests (*11*). The cytotoxic potential of **QU663** was measured by screening it in a MTT assay by using NSO murine, Daudi human, 3T3 fibroblast murine cell lines and normal human lymphocytes (NHL). TC_{50}s for **QU663** are: NSO, 0.93 mM; Daudi, 0.95 mM; 3T3, 0.91 mM; NHL, 0.93 mM.

1. Cell Lines. All cell lines were obtained from ATCC. The cells were cultured in RPMI 1640 supplemented with 5% FCS, 0.1 mM glutamine, 1% penicillin, and streptomycin. Cells were grown in Nunc clone plastic bottles (TedNunc, Roskilde, Denmark) and split twice weekly at different cell densities according to standard procedure. 3T3 cells were grown as a monolayer and were split by using tripsin. Perypheral blood mononuclear cells (MNC) were separated from heparinized whole

blood obtained from a healthy donor on a Fycoll-Hypaque gradient as previously described (*7*). MNC thus obtained were washed twice with RPMI 1640 supplemented with 10% FCS, glutamine, and antibiotics, suspended at 200.000 viable cells/mL in medium containing, as mitogen, 5 μg/mL PHA (Sigma) and used in toxicity tests.

2. Chemicals. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Aldrich. It was dissolved at a concentration of 5 mg/mL in sterile PBS at room temperature, and the solution was further sterilized by filtration and stored at 4 °C in a dark bottle. SDS was obtained from Sigma. Lysis buffer was prepared as follows: 20% w/v of SDS was dissolved at 37 °C in a solution of 50% of each DMF and demineralized water; pH was adjusted to 4.7 by adding 2.5% of an 80% acetic acid and 2.5% 1 N HCl solution.

3. Toxicity Experiments. Cells were plated at different concentrations on flat bottom 96-well microplates (0.1 mL/well). Lymphocytes were plated out at 20 000 cells/well. 3T3 cells (murine fibroblast line) were plated at 10 000 cells/well. NSO cells (plasmocytoma murine cell line) were plated out at 3000 cells/well, and Daudi cells (human lymphoblastoid cell line) were plated at 300 cells/well. Twelve hours after plating, different concentrations of each compound were added to each well. After 48 h, MTT assay was performed to analyze cytotoxicity of the different compounds. Some experiments were performed by using confluent cells: compounds were added to the 3T3 monolayer 3 days after plating. Tests were then run as described above.

MTT/Formazan Extraction Procedure. A total of 20 μL of the 5 mg/mL stock solution of MTT was added to each well; after 2 h of incubation at 37 °C, 100 μL of the extraction buffer was added. After an overnight incubation at 37 °C, the optical densities at 570 nm were measured using a Titer-Tech 96-well multiscanner, employing the extraction buffer as the blank.

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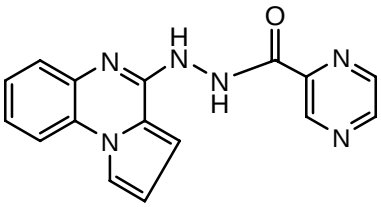
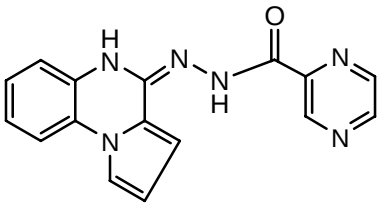
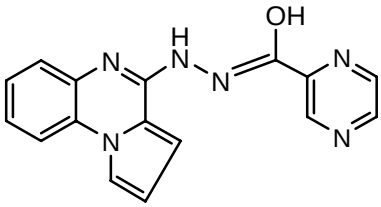
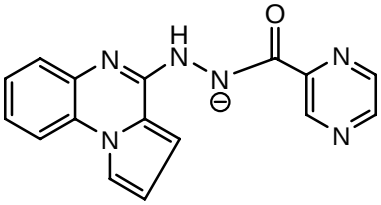
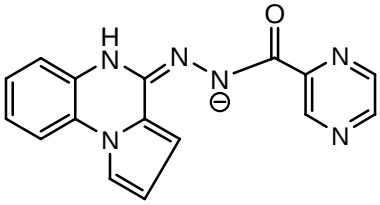
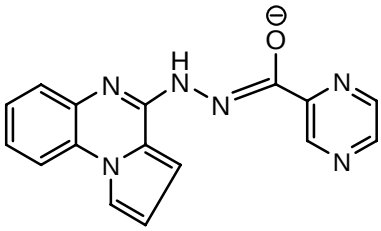
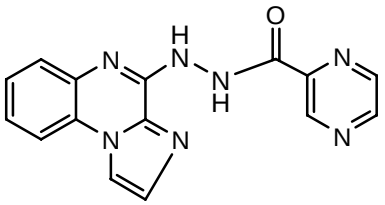
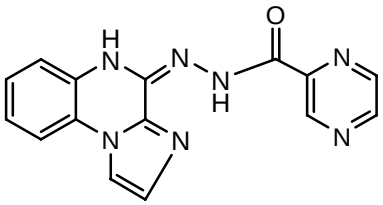
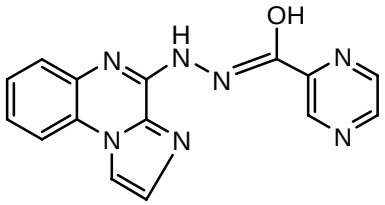
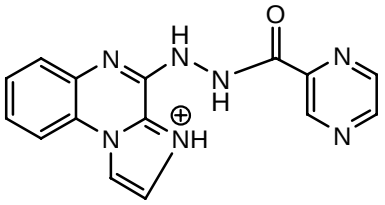
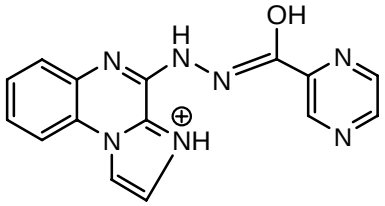
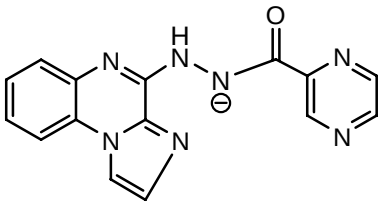
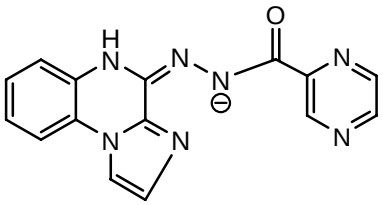
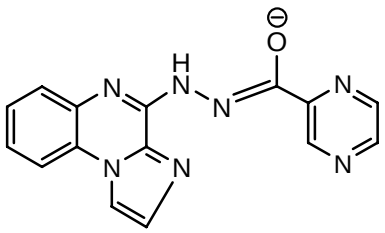
Table 1. Percentage of neutral/ionized forms of **QX432** calculated from apparent pK_a values[a]

	A Tautomer	B Tautomer	C Tautomer
Percentage of neutral form[b]	 98,5%	 42%	 93%
Percentage of anionic form[b]	 1,5%	 58%	 7%

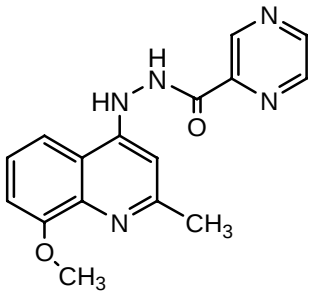
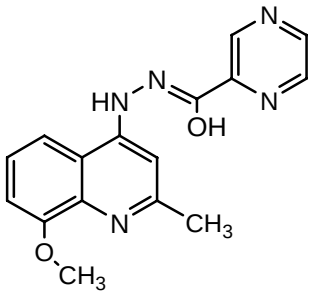
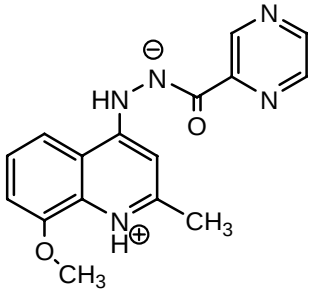
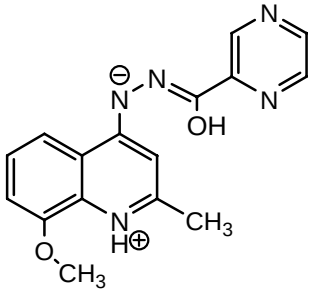
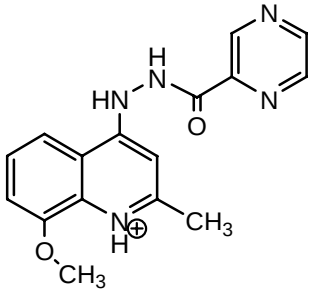
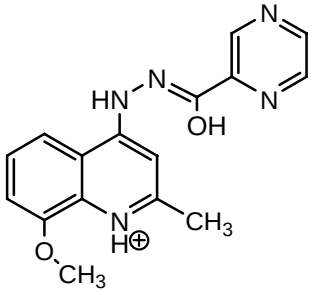
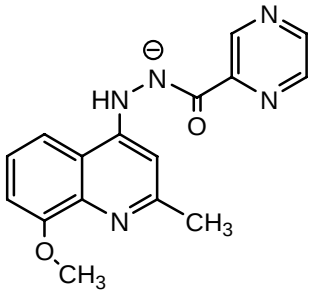
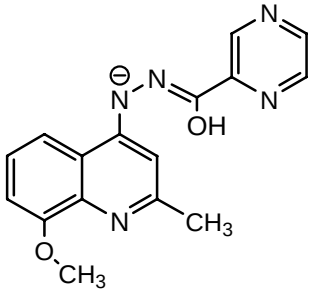
[a] Calculated by using ACD/p*K*a DB 7.0 software (Advanced Chemistry Development Inc., Toronto, Canada); [b]Calculated on each tautomer.

Table 2. Percentage of neutral/ionized forms of **QX449** calculated from apparent pK_a values[a]

	A Tautomer	B Tautomer	C Tautomer
Percentage of neutral form[b]	 96%	 17%	 60%
Percentage of protonated form[b]	 <0.5	N.P.[c]	 <0.5%
Percentage of anionic form[b]	 4%	 83%	 40%

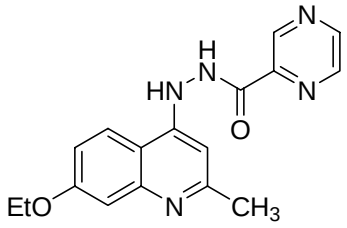
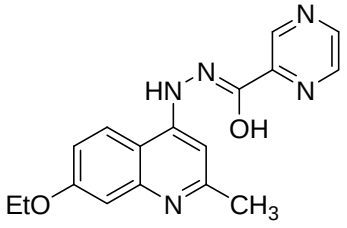
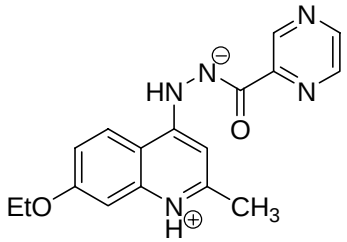
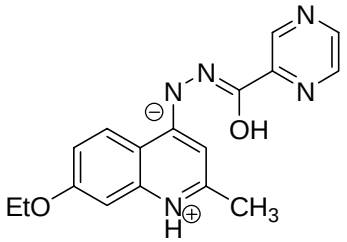
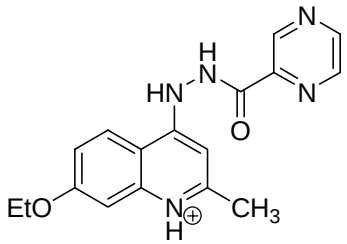
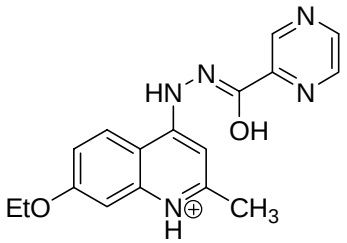
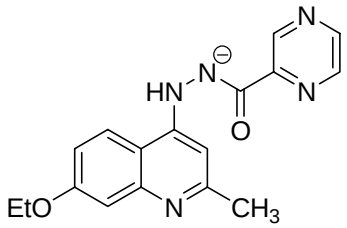
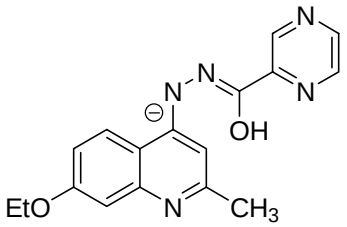
[a] Calculated by using ACD/p*K*a DB 7.0 software (Advanced Chemistry Development Inc., Toronto, Canada); [b]Calculated on each tautomer; [c]Not present.

Table 3. Percentage of neutral/ionized forms of **QU494** calculated from apparent pK_a values[a]

	A Tautomer	B Tautomer
Percentage of neutral form[b]	 97%	 31 %
Percentage of zwitterionic form[b]	 <0.5%	 6%
Percentage of protonated form[b]	 2%	 4%
Percentage of anionic form[b]	 1%	 59%

[a]Calculated by using ACD/p*K*a DB 7.0 software (Advanced Chemistry Development Inc., Toronto, Canada); [b]Calculated on each tautomer.

Table 4. Percentage of neutral/ionized forms of **QU663** calculated from apparent pK_a values[a]

Percentage of forms	A Tautomer	B Tautomer
Percentage of neutral form[b]	 29%	 2 %
Percentage of zwitterionic form[b]	 4%	 80%
Percentage of protonated form[b]	 67%	 13%
Percentage of anionic form[b]	 <0.5%	 5%

[a]Calculated by using ACD/p*K*a DB 7.0 software.(Advanced Chemistry Development Inc., Toronto, Canada); [b]Calculated on each tautomer.

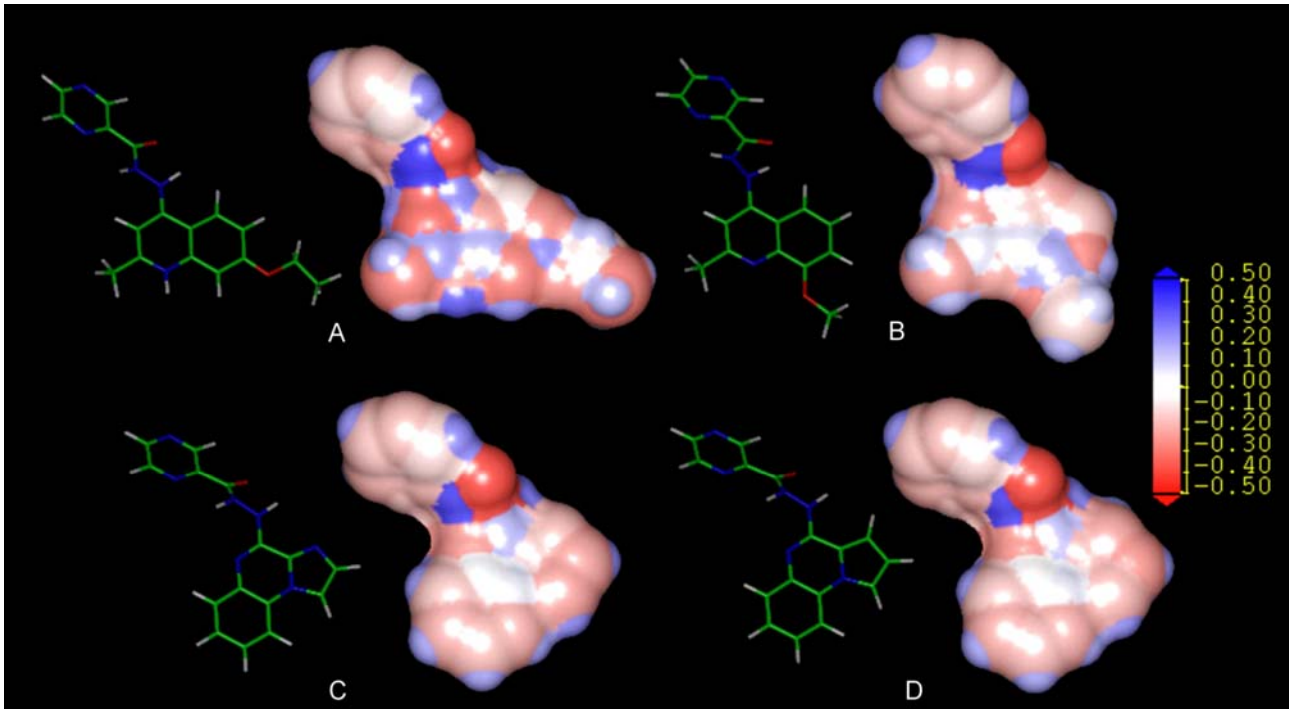
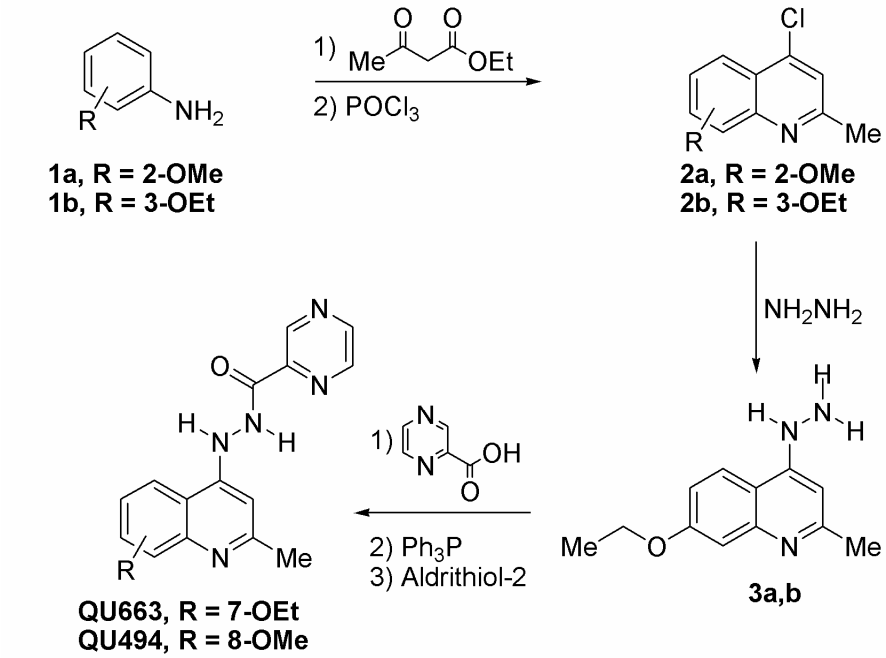


Figure 1 S1. Molecular models of the lowest energy conformer of **QU663** (A), **QU494** (B), **QX449** (C) and **QX432** (D). Left: molecules are represented in capped sticks and coloured by atom type. Right: molecules are represented by their Connolly surface and coloured by partial charges derived from the quantum mechanical method AM1.

Scheme 1



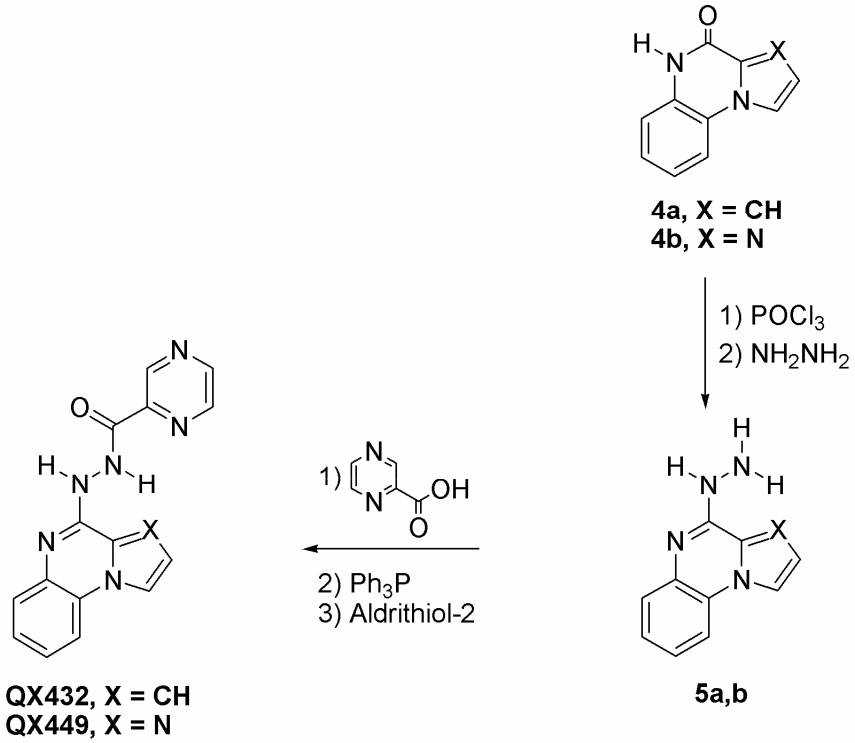
1a, R = 2-OMe
1b, R = 3-OEt

2a, R = 2-OMe
2b, R = 3-OEt

3a,b

QU663, R = 7-OEt
QU494, R = 8-OMe

Scheme 2



4a, X = CH
4b, X = N

5a,b

QX432, X = CH
QX449, X = N