Supplementary Data and Detailed Methods

Tight-binding Hydroxypyrazole HIV-1 Nef Inhibitors Suppress Viral Replication in Donor Mononuclear Cells and Reverse Nef-mediated MHC-I downregulation

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***Contents*:**

Figure S1. Plots of Nef inhibitor efficacy against HIV-1 infectivity vs. affinity and residence time. Each of 216 unique Nef inhibitor analogs was tested for inhibition of HIV-1 infectivity using the TZM-bl reporter cell line and for direct interaction with recombinant purified HIV-1 Nef by surface plasmon resonance (SPR; see Materials and Methods and main text for experimental details). Each analog is represented by a blue dot. A) Plot of percent infectivity inhibition vs. affinity. Active analogs were defined as exhibiting at least 10% inhibition of infectivity at 1.0 µM and a K_D value of at least 100 nM (orange box). B, C) Plot of percent infectivity inhibition vs. residence time (defined as the inverse of the dissociation rate constant; $T = 1/k_{off}$). Active analogs inhibited infectivity by at least 10% at 1.0 µM and displayed a residence time of at least 60 minutes (orange boxes). D) Venn diagram illustrating the overlap in active compounds defined by each plot, with 2 analogs unique to the affinity plot (K_D), 7 unique to the residence time plot (T), and 40 common to both plots.

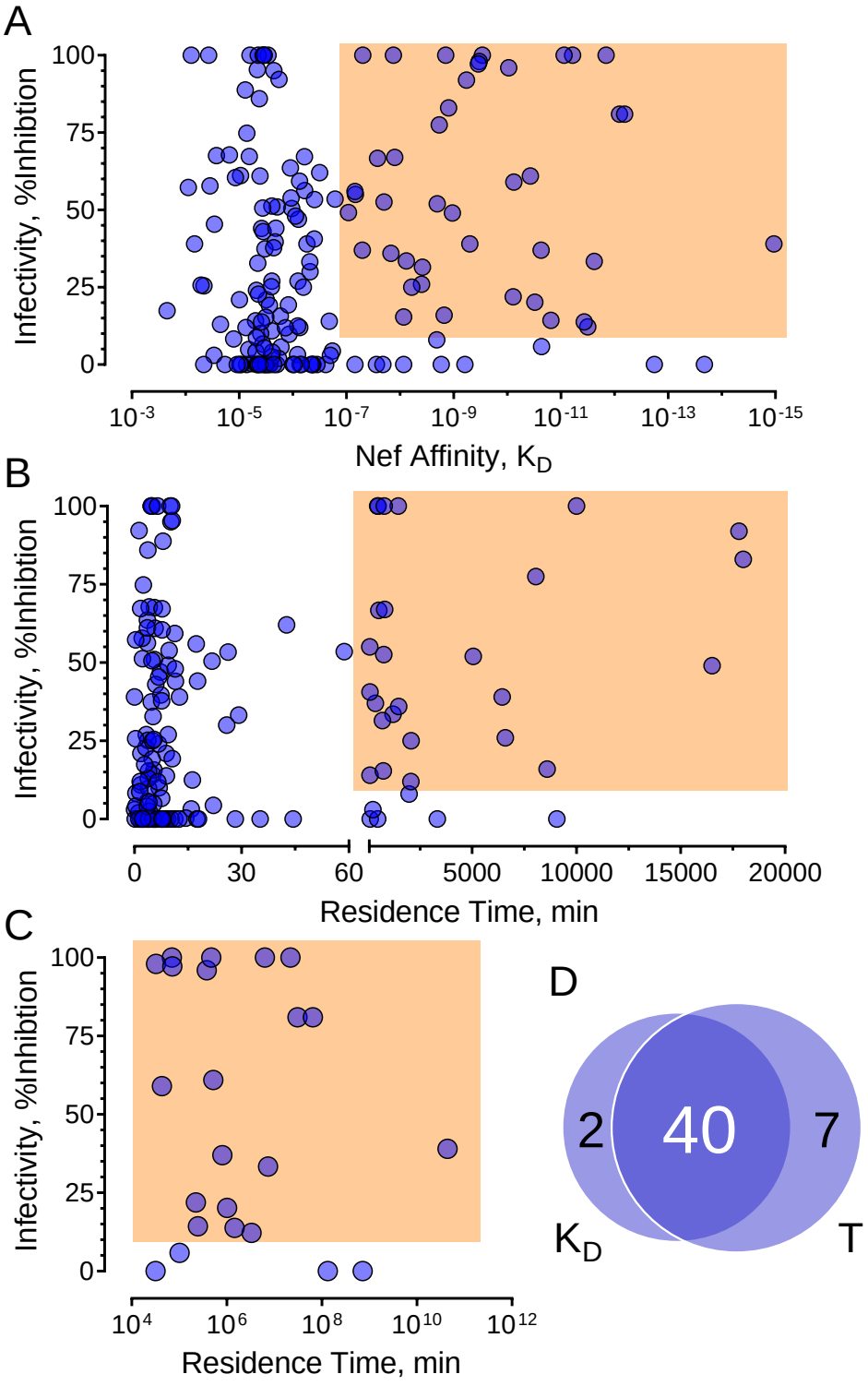


Figure S2. Structures and SPR data for top-scoring Nef inhibitor analogs unique to ranking based on either affinity or residence time. Each of the analogs shown was assayed for interaction with HIV-1 Nef_{NL4-3} by SPR as described in the legend to main Figure 2. Compounds were tested over the range of concentrations shown, and the resulting sensorgrams were best-fit by a two-step induced-fit model. The SPR data for each analog concentration are shown in color, and the fitted curves are superimposed in black. The resulting K_D values are shown below each analog number. SPR results for analog **FC-7943**, a top-scoring compound, are also shown for reference.

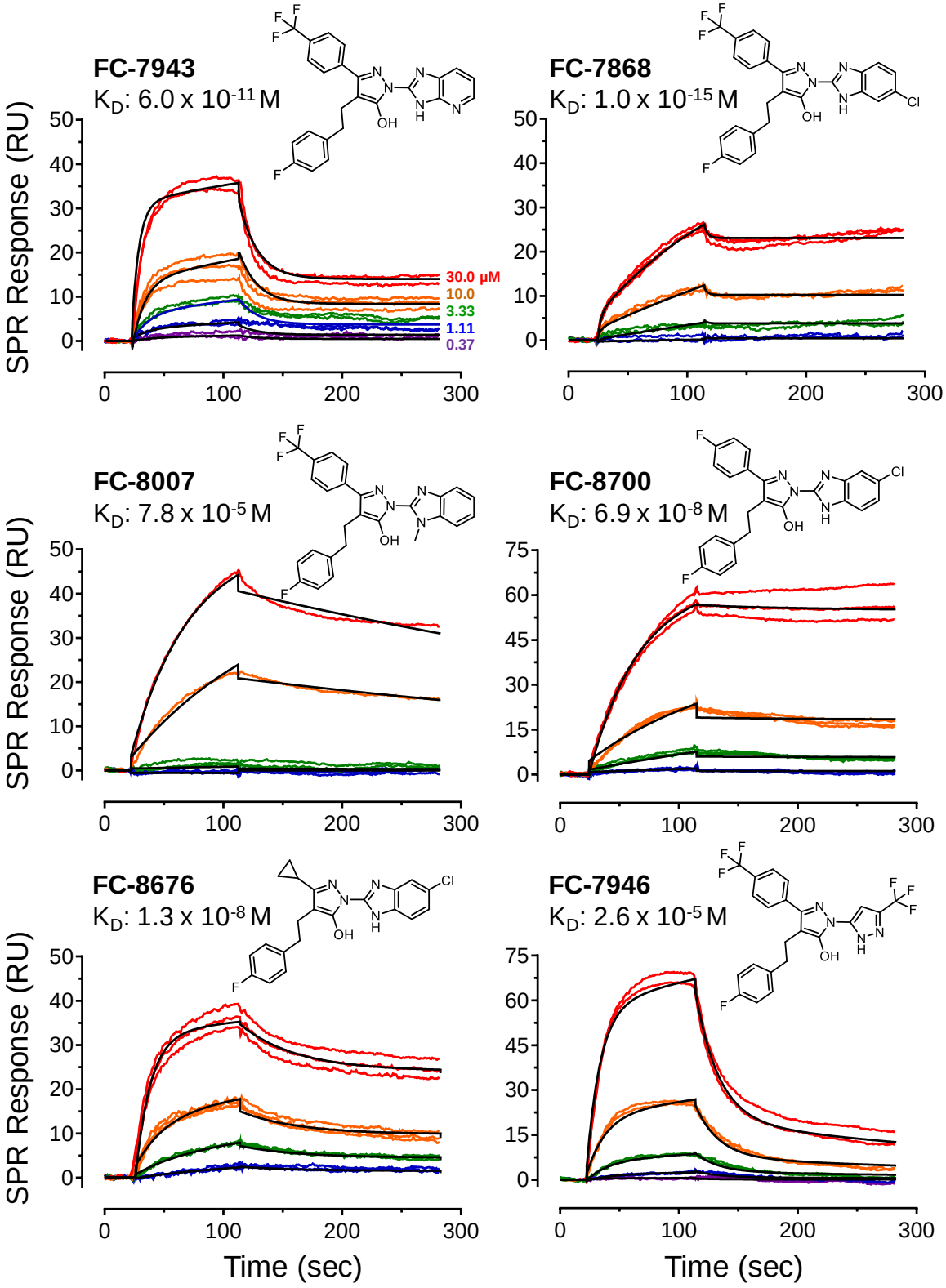


Figure S3. Structures and SPR data for analogs with modifications to the central pyrazole hydroxyl group or lacking the A or B substituents. Each of the analogs shown was assayed for interaction with HIV-1 Nef_{NL4-3} by SPR as described in the legend to main Figure 2. The highest concentration tested in each case was 30 µM (red traces). A) Analogs of active compounds in which the pyrazole hydroxyl group is replaced with a carbonyl (**FC-8006**), a primary amine (**FC-9120**), or a hydrogen atom (**FC-10690**). B) Analogs of active compounds lacking the **A** (**FC-7697**; replaced with a methyl group) or **B** substituents (**FC-7700**; replaced with a hydrogen atom). Core structure shown at right for reference (box; *n* = 1 or 2 carbon linker). C) Analogs of active compound **FC-8517** in which group **A** is replaced with the bulky substituents shown in red.

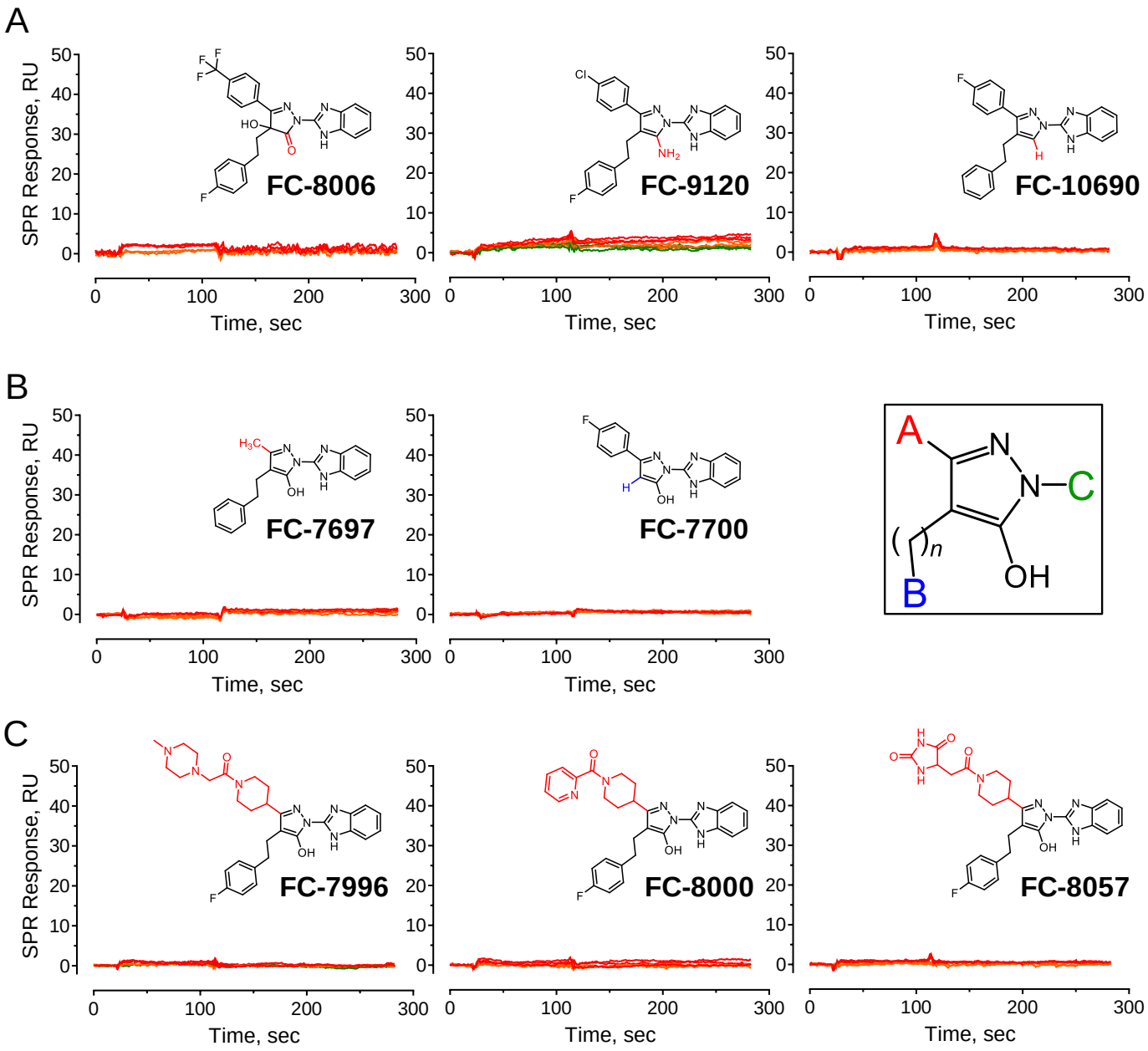


Figure S4. The benzimidazole moiety at position C is essential for Nef inhibitor action. Each of the analogs shown was assayed for interaction with HIV-1 Nef_{NL4-3} by SPR as described in the legend to main Figure 2. The highest concentration tested in each case was 30 µM (red trace). SPR sensorgrams and K_D values for active compounds **FC-7943**, **FC-8052**, and **FC-8698** are shown for reference (left panels). SPR data for the corresponding analogs lacking the aza-benzimidazole (**FC-10567**) or chlorobenzimidazole groups (**FC-10558**, **FC-10580**) are shown on the right. Percent inhibition of HIV-1 infectivity in the TZM-bl reporter cell assay are also shown (final concentration of 1.0 µM in each case).

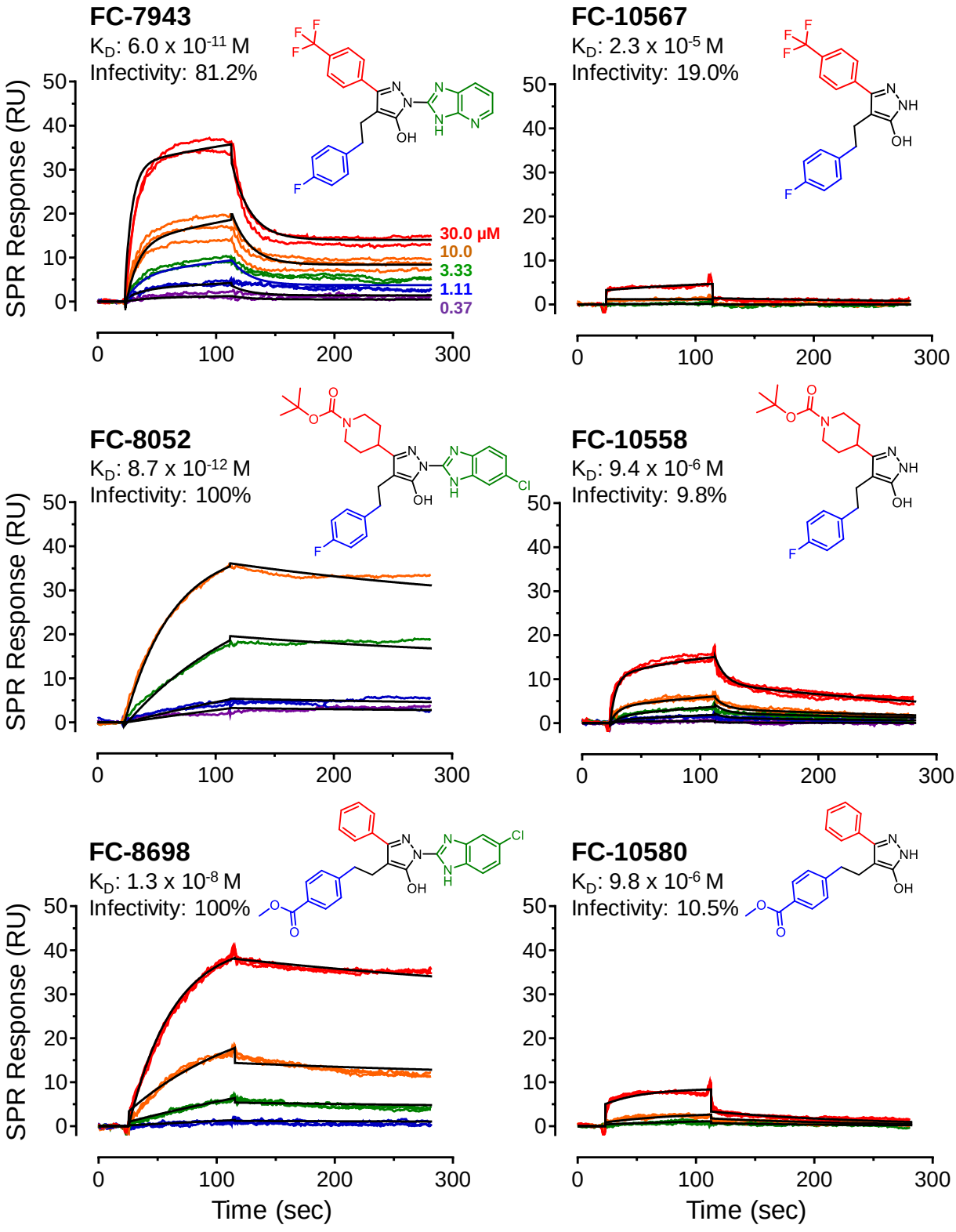
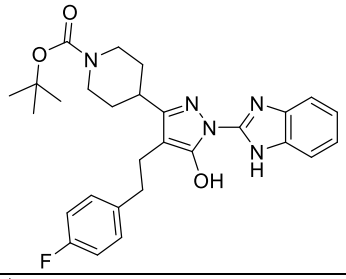
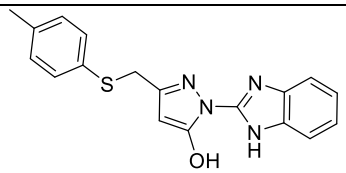
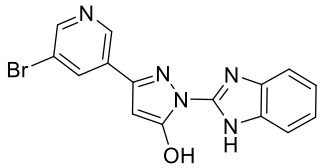
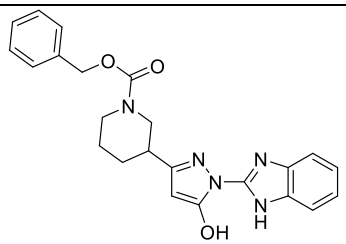
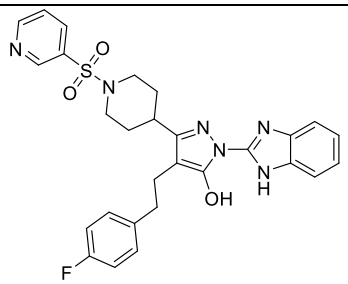
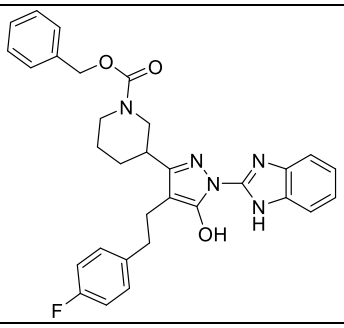
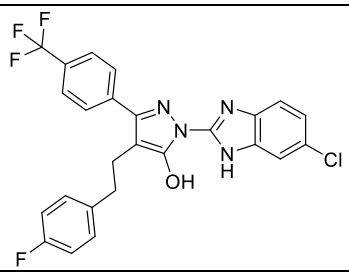
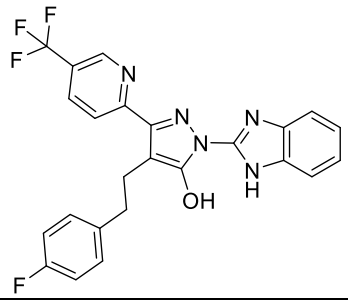
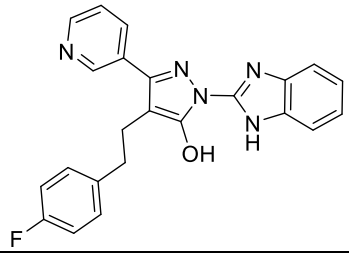
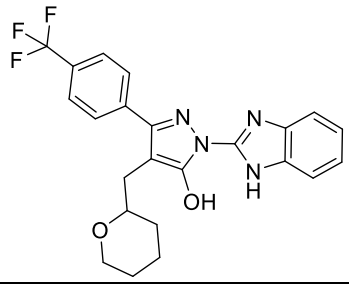
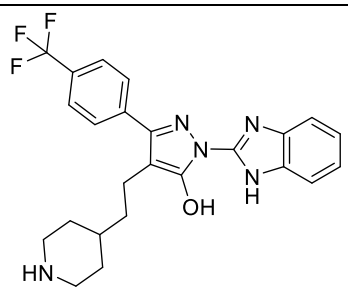
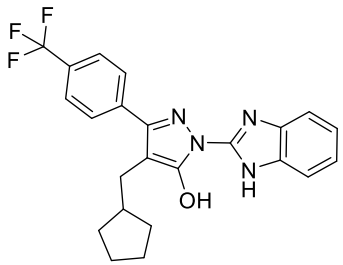
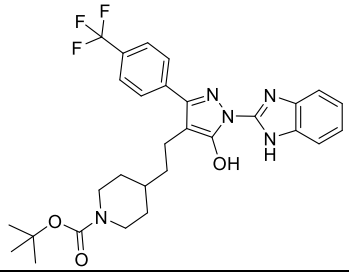
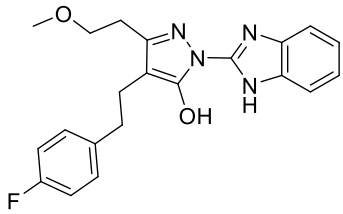
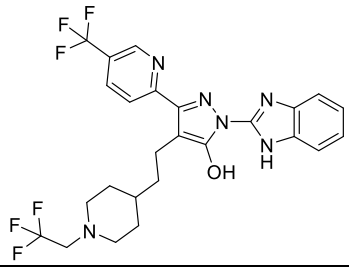
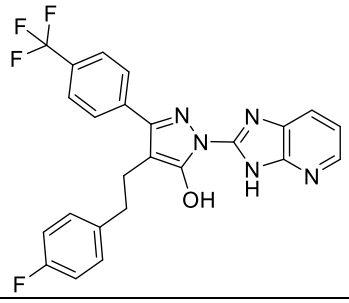
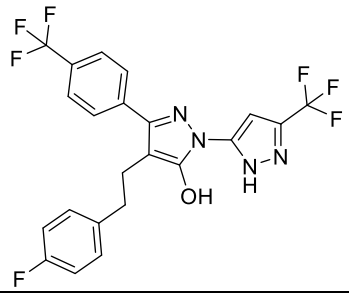
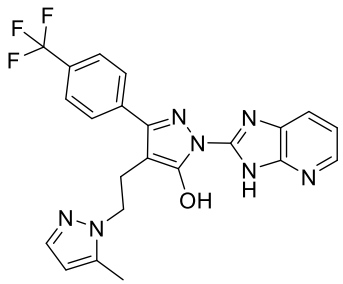
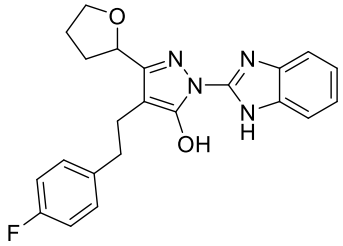
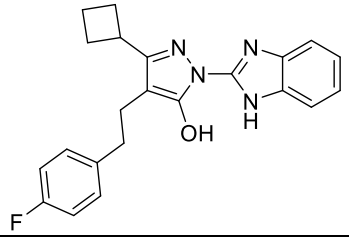
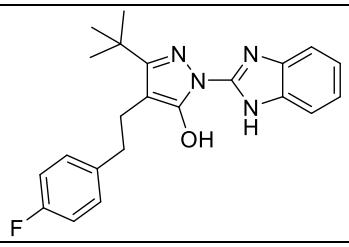
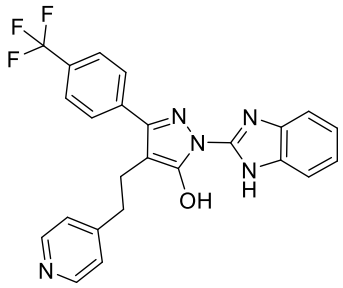
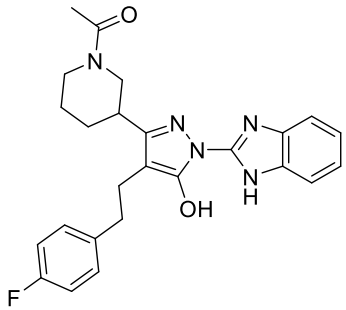


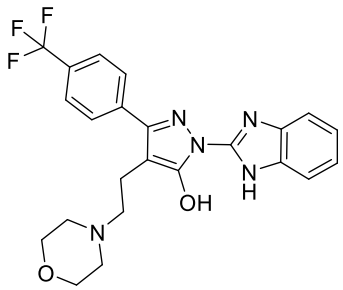
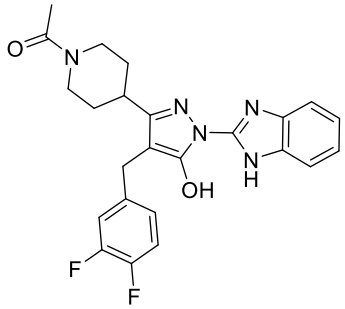
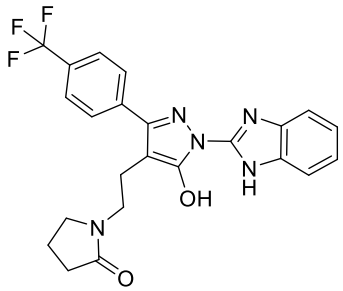
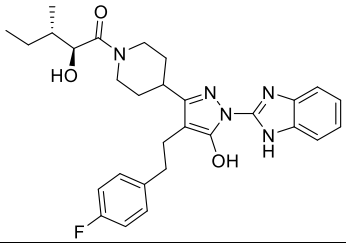
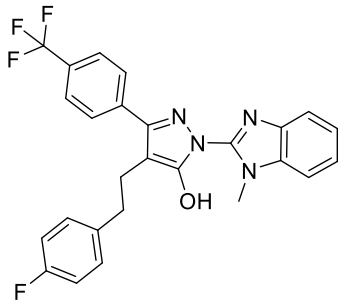
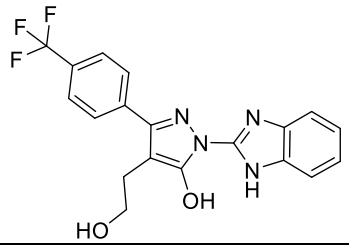
Table S1. Hydroxypyrazole Nef inhibitors: Structures, SPR data, and HIV-1 infectivity inhibition. Each of 216 unique Nef inhibitor analogs were tested for inhibition of HIV-1 infectivity at 1.0 µM using the TZM-bl reporter cell line and for direct interaction with recombinant purified HIV-1 Nef_{NL4-3} by SPR (see Materials and Methods and main text for experimental details). Uninfected TZM-bl cell viability at 1.0 µM is also shown (CellTiter-Blue Assay; Promega). SPR data were best-fit by either a 1:1 Langmuir model ($K_D = k_{off}/k_{on}$) or by a 2-step, induced fit model ($K_D = k_{off}/k_{on} \times k_{off2}/(k_{off2} + k_{on2})$ as indicated. Residence time (T) is defined as $1/k_{off}$ for the 1:1 fit and $1/k_{off2}$ for the 2-step induced fit model. Extent of binding is defined as the ratio of the response units observed at 30 µM (RU_{30}) to the amount of Nef immobilized on the SPR chip (RU_{Nef} values varied by less than 2-fold across all of the SPR experiments). Inhibitor analogs that did not interact with Nef by SPR are not included in the Table (53 analogs).

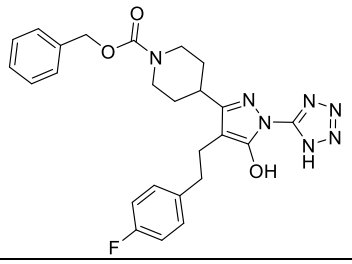
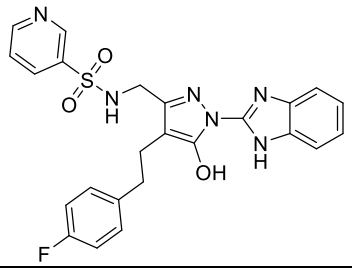
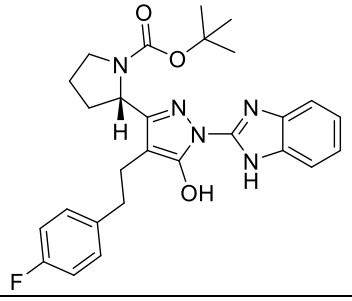
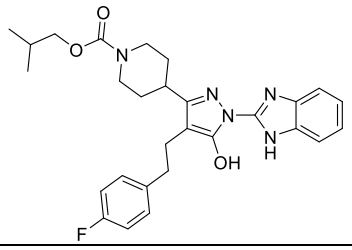
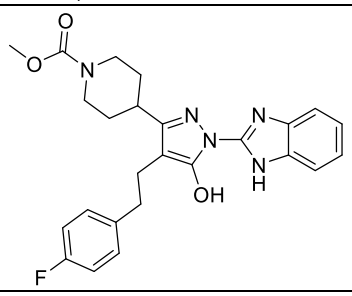
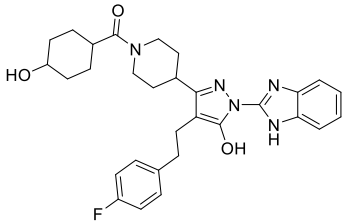
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
7098		6.05E-12	6.36E+06	4.09E-03	2-Step	100	79.2
7698		4.20E-07	1.13E+01	7.23E-04	1:1	0	100
7719		2.90E-06	5.81E+00	2.72E-03	1:1	0	100
7793		2.97E-06	6.11E+00	6.26E-03	1:1	0	100
7817		1.43E-12	2.20E+07	2.76E-03	2-Step	100	77.3

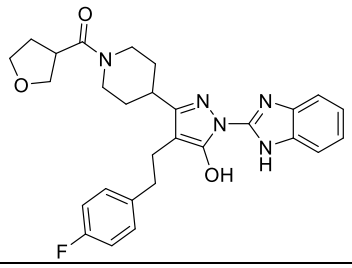
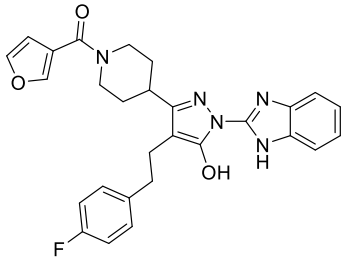
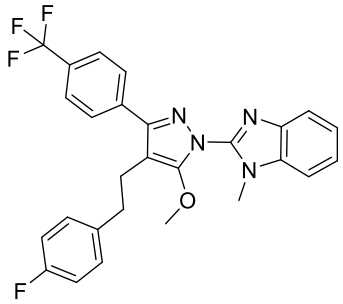
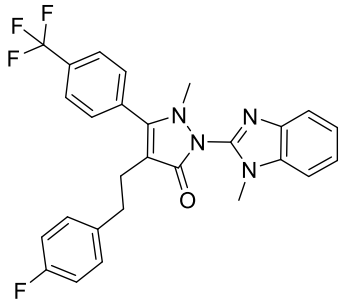
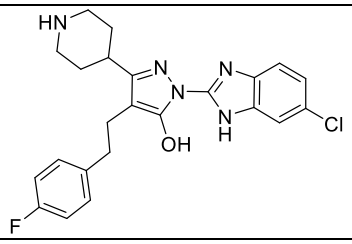
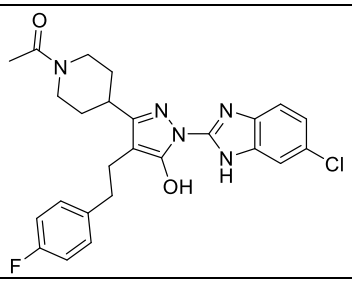
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
7840		2.38E-12	7.41E+06	1.74E-02	2-Step	33.4	93.7
7868		1.06E-15	4.41E+10	1.44E-02	2-Step	38.7	89.9
7877		8.08E-13	3.09E+07	7.52E-03	2-Step	80.7	77.1
7899		4.74E-07	2.58E+01	7.40E-03	2-Step	29.6	97.8
7900		2.25E-06	1.02E+01	1.07E-02	2-Step	95.4	75.2
7901		1.04E-06	2.17E+01	1.04E-02	2-Step	50.5	91.4

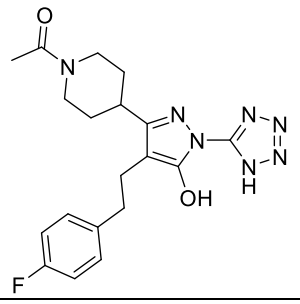
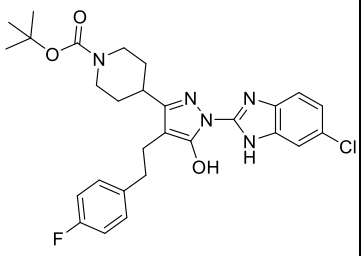
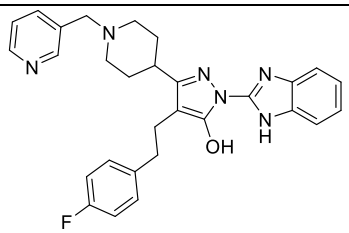
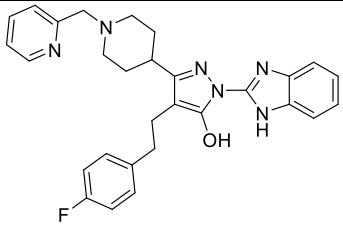
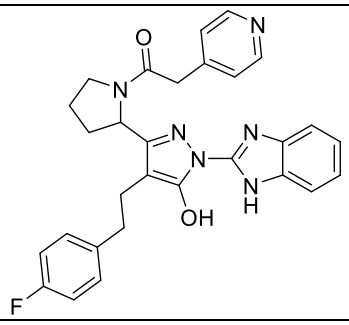
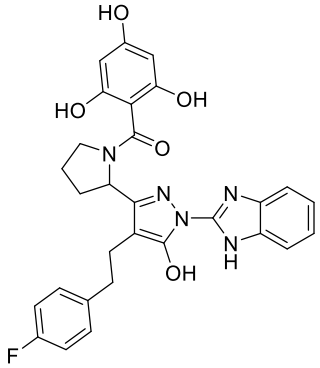
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
7902		9.36E-11	3.79E+05	2.57E-02	2-Step	96.1	85.3
7904		2.29E-11	1.02E+05	8.83E-03	2-Step	5.8	95.1
7919		3.13E-06	8.87E+00	2.86E-03	2-Step	21.2	100
7942		1.24E-09	1.80E+04	5.08E-03	2-Step	83.1	85.2
7943		6.00E-11	6.49E+07	1.34E-02	2-Step	81.2	90.7
7946		2.64E-05	5.59E+00	4.19E-02	2-Step	67.6	100

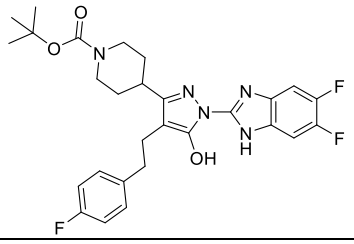
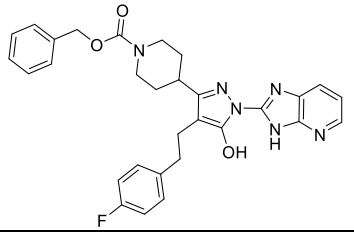
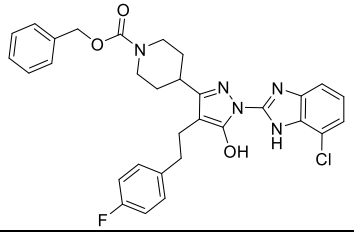
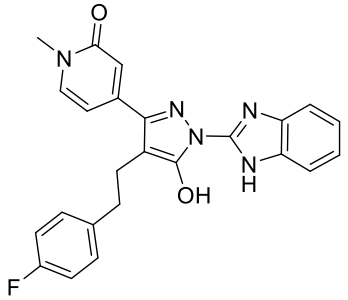
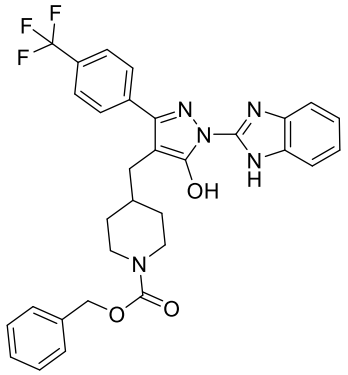
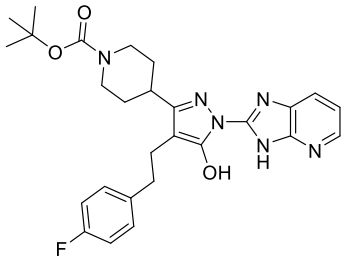
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
7948		7.64E-09	1.20E+03	5.56E-03	2-Step	33.5	97.9
7958		2.46E-06	3.32E+00	5.11E-03	2-Step	26.5	99.9
7959		3.71E-11	5.19E+05	1.28E-02	2-Step	60.9	99.3
7960		3.80E-06	1.15E+01	9.49E-03	2-Step	43.6	98.7
7976		2.91E-10	7.00E+04	1.33E-02	2-Step	100	76.3
7977		2.33E-11	8.05E+05	5.14E-03	2-Step	37.4	97.2

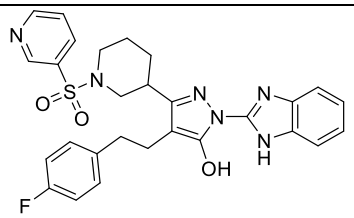
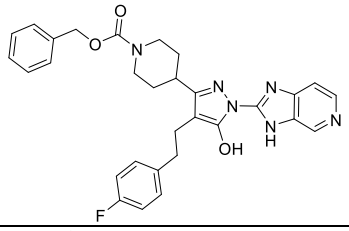
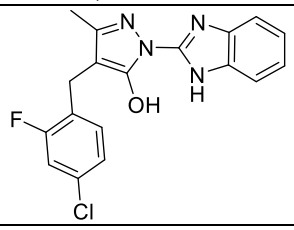
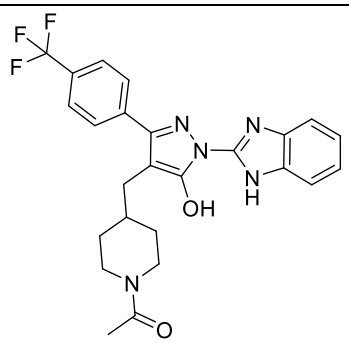
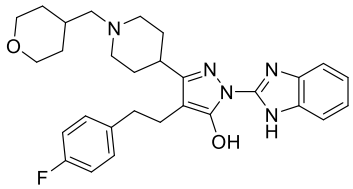
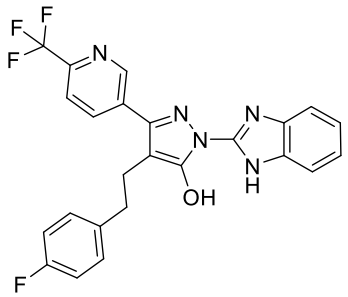
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
7978		4.93E-06	5.16E+00	1.06E-02	2-Step	14.2	96.4
7979		1.95E-06	5.61E+00	4.92E-03	2-Step	51.2	98.2
7980		1.70E-06	5.46E+00	5.62E-03	2-Step	15.7	96.5
8002		4.21E-06	3.78E+00	3.09E-03	2-Step	85.5	91.0
8007		7.84E-05	1.45E+03	2.01E-02	2-Step	99.6	86.4
8011		6.88E-08	9.31E+00	9.06E-04	1:1	0	99.9

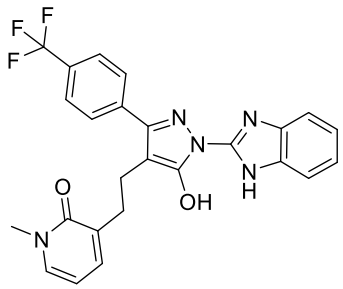
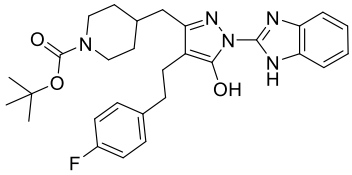
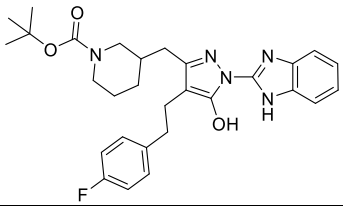
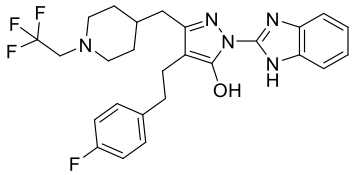
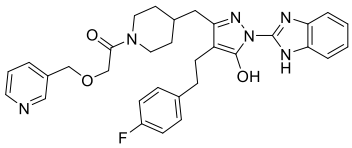
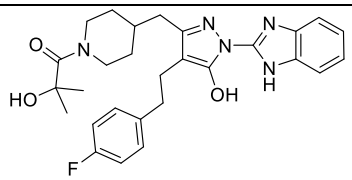
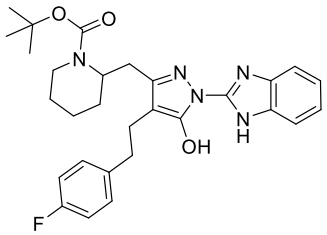
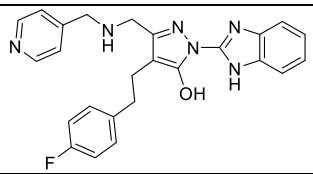
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8017		3.82E-09	6.92E+02	1.00E-03	1:1	31.5	98.4
8026		2.75E-08	1.09E+02	2.56E-03	2-Step	0	94.4
8027		2.08E-07	9.98E+01	5.81E-03	2-Step	14.3	95.6
8028		3.72E-05	4.69E+02	1.20E-02	2-Step	100	84.3
8029		5.44E-07	1.26E+01	7.81E-03	2-Step	39.1	89.8
8030		4.98E-10	6.44E+03	6.53E-03	2-Step	38.8	96.9

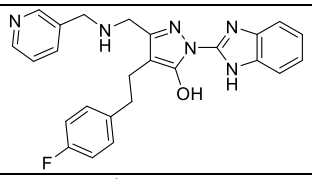
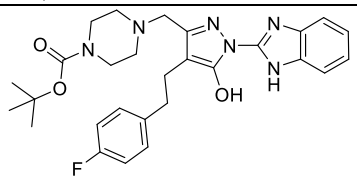
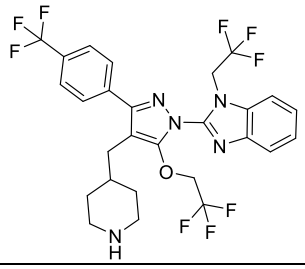
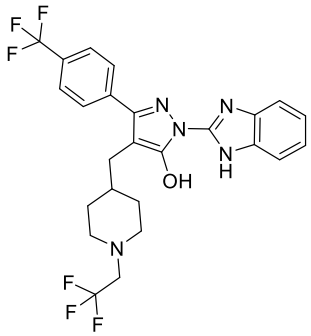
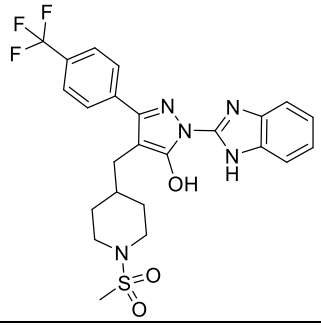
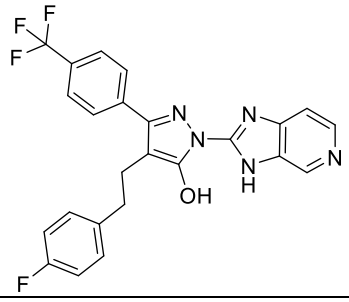
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8031		2.06E-09	1.97E+03	1.02E-02	2-Step	7.5	96.0
8032		1.70E-09	9.06E+03	1.36E-02	2-Step	0	93.0
8046		4.75E-06	6.75E+00	7.81E-03	2-Step	24.4	96.7
8047		2.93E-05	3.47E-03	1.62E-02	2-Step	2.7	99.9
8048		6.17E-10	3.18E+04	1.05E-02	2-Step	0	90.7
8049		3.59E-06	5.05E+00	1.56E-02	2-Step	100	96.4

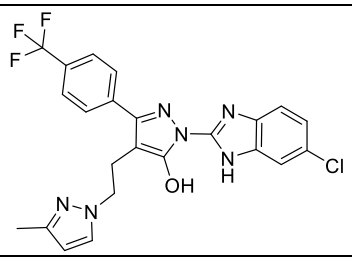
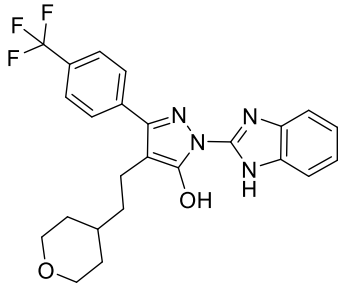
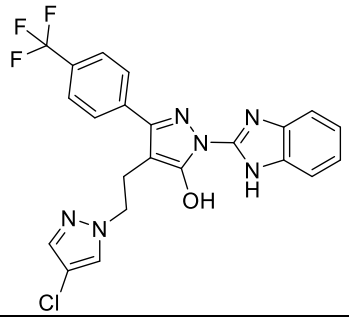
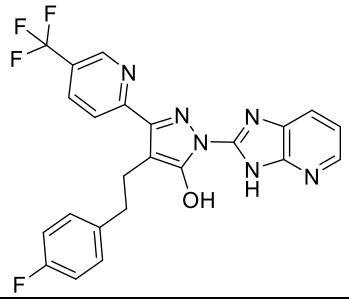
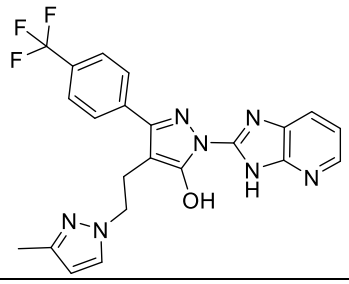
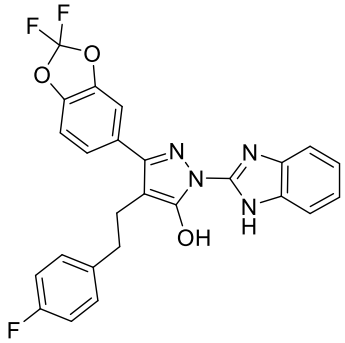
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8050		2.45E-06	1.77E+00	7.19E-04	1:1	10.9	99.0
8052		8.71E-12	4.71E+05	1.60E-02	2-Step	100	87.1
8069		8.17E-07	1.59E+01	1.69E-03	1:1	3.2	100
8071		1.82E-07	2.21E+01	2.48E-03	2-Step	4.4	99.3
8076		7.80E-07	7.31E+00	1.38E-03	1:1	46.5	96.8
8079		3.98E-09	6.59E+03	2.87E-03	2-Step	25.7	98.6

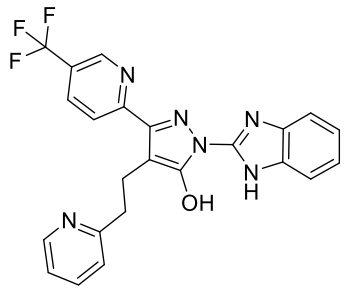
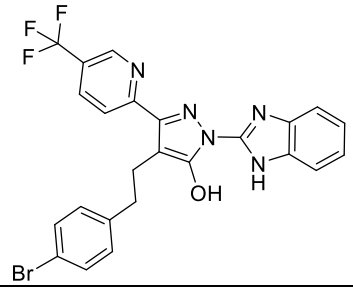
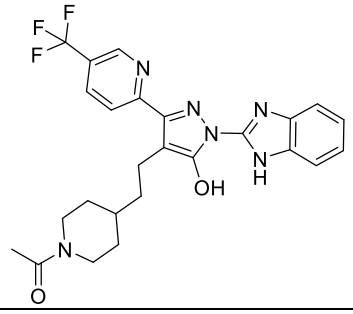
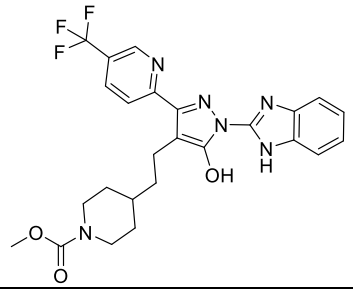
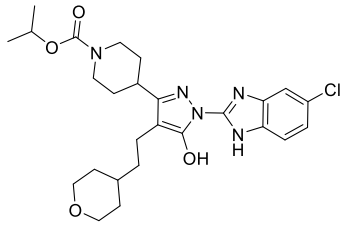
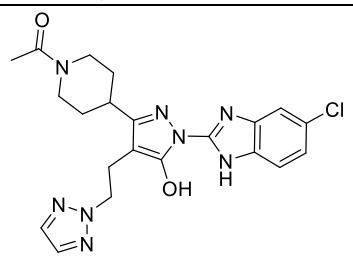
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8104		4.93E-08	4.93E+02	4.76E-03	2-Step	100	79.2
8105		7.31E-07	3.52E+01	9.61E-03	2-Step	0	95.7
8106		6.03E-09	2.07E+03	4.21E-03	1:1	24.6	97.0
8107		1.51E-09	8.59E+03	2.76E-03	2-Step	16	100
8108		1.86E-09	8.05E+03	4.69E-03	1:1	77.5	90.8
8109		3.95E-06	6.92E+00	7.24E-03	2-Step	9.8	100

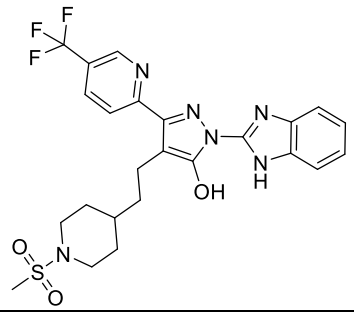
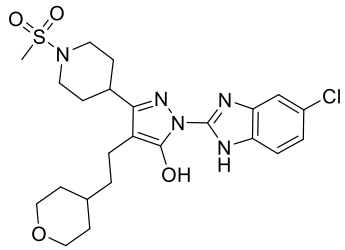
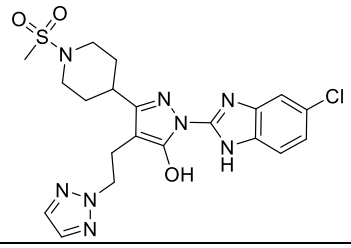
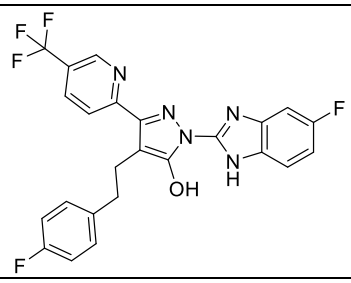
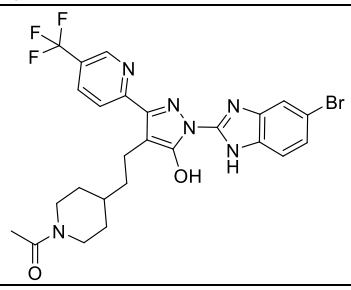
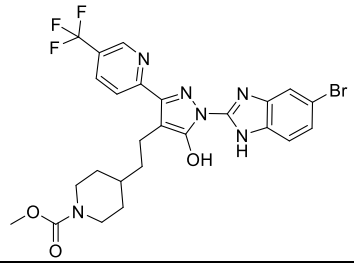
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8118		6.81E-08	9.21E+01	2.95E-03	1:1	54.9	99.8
8124		5.02E-08	3.56E+02	2.36E-03	1:1	36.6	100
8125		8.56E-09	7.34E+02	3.62E-03	1:1	15.4	100
8127		1.81E-06	1.32E+00	2.80E-03	2-Step	92.2	71.4
8128		9.90E-06	1.79E+00	7.48E-04	1:1	20.6	99.1
8130		1.47E-08	1.47E+03	2.87E-03	2-Step	35.7	91.9

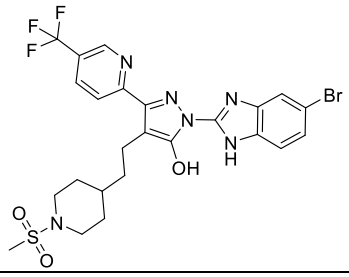
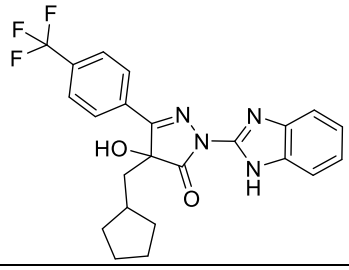
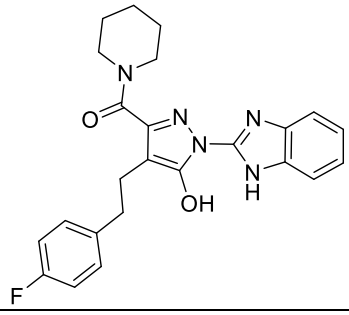
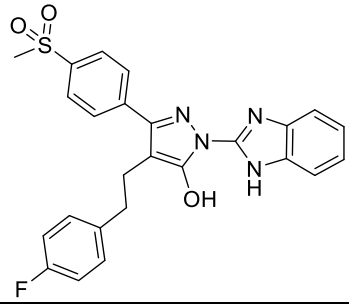
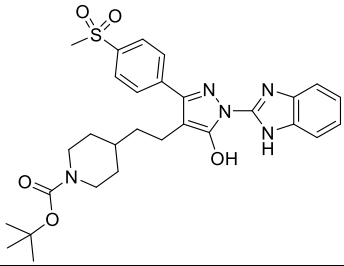
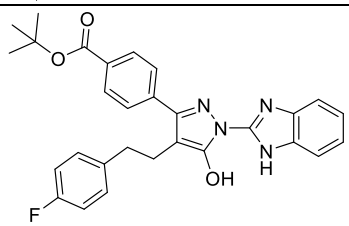
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8132		2.43E-07	5.36E+00	1.02E-03	1:1	0	99.4
8167		1.05E-09	1.65E+04	3.04E-03	1:1	49.2	99.7
8169		2.08E-08	4.68E+02	2.46E-03	1:1	0	99.9
8172		3.54E-07	2.82E+01	2.27E-03	1:1	0	100
8173		7.98E-07	9.47E+00	4.88E-03	1:1	26.5	98.8
8174		9.97E-07	3.50E+00	2.23E-03	1:1	0	97.0
8186		1.80E-13	1.33E+08	4.14E-03	2-Step	0	99.3
8193		3.58E-06	5.97E+00	3.75E-03	2-Step	42.8	100

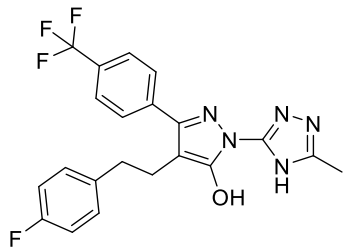
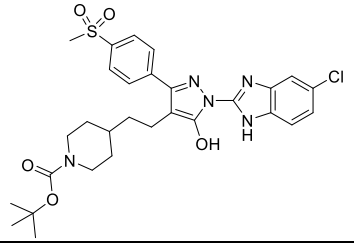
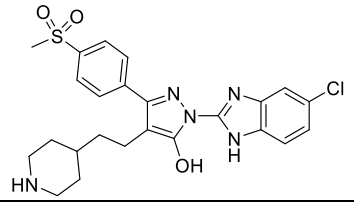
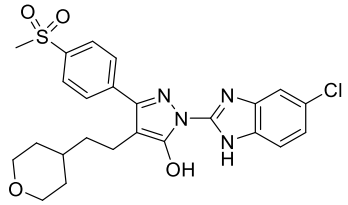
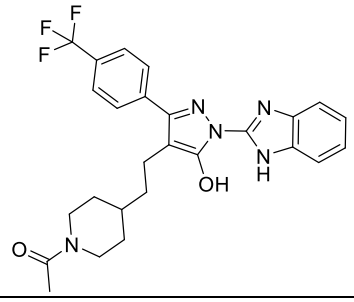
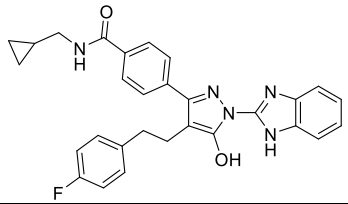
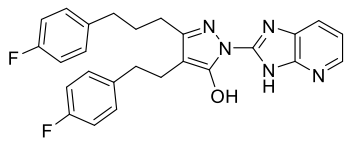
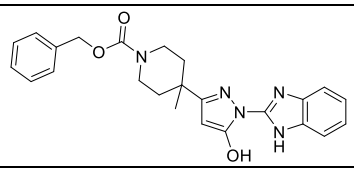
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8194		1.26E-05	3.83E-01	3.57E-03	2-Step	8.3	100
8196		7.22E-06	1.65E+00	2.89E-03	1:1	0	100
8218		1.84E-05	2.22E-01	3.58E-02	1:1	0	99.2
8227		9.10E-08	9.47E+00	1.26E-03	1:1	49.2	93.5
8230		5.97E-07	3.62E+00	1.22E-03	1:1	56.3	89.1
8232		5.76E-10	1.78E+04	1.59E-03	1:1	92.2	99.1

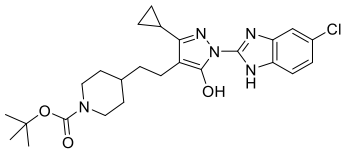
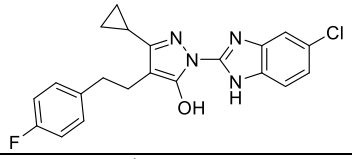
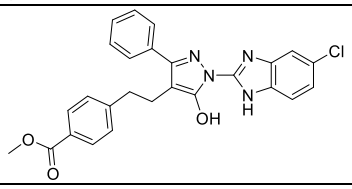
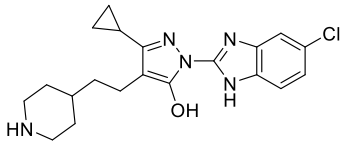
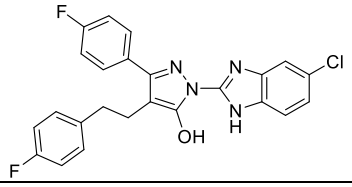
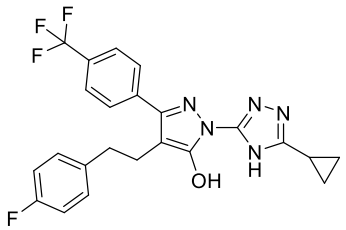
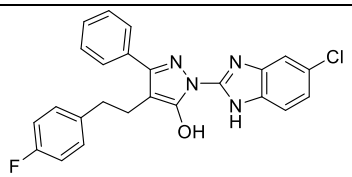
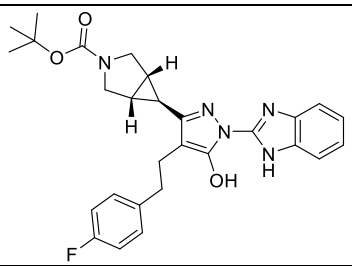
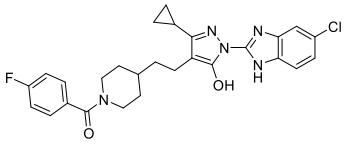
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8282		5.82E-06	1.44E+01	4.48E-03	2-step	0.3	96.6
8284		2.07E-06	1.77E+01	3.41E-03	2-step	44.1	80.8
8343		4.26E-07	1.75E+01	1.02E-03	1:1	0	98.9
8354		3.61E-06	8.82E+00	1.66E-03	1:1	0	98.9
8355		3.96E-07	9.47E+01	3.06E-03	1:1	40.1	98.3
8363		2.11E-14	7.22E+08	4.38E-03	1:1	0	98.2

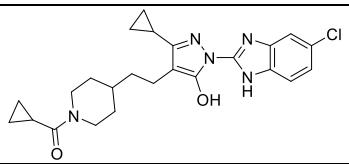
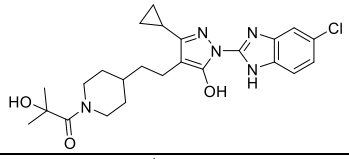
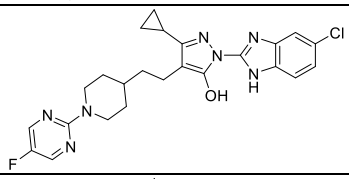
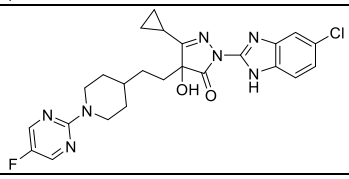
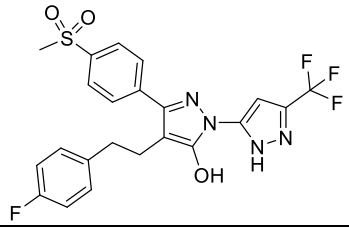
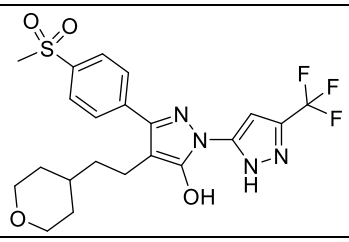
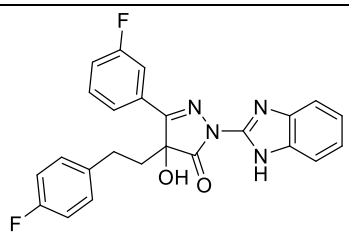
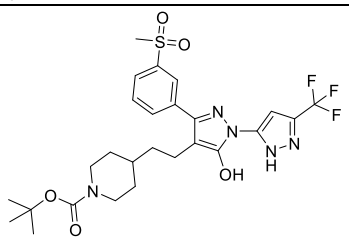
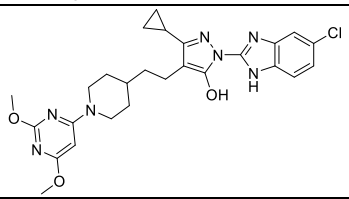
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8364		1.65E-06	3.67E+00	6.42E-04	1:1	5.7	98.5
8400		7.52E-11	4.30E+04	2.11E-03	1:1	59.3	97.6
8401		1.11E-06	3.64E+00	1.32E-03	1:1	63.6	98.3
8402		2.51E-06	3.76E+00	5.47E-03	2-step	25.2	99.1
8404		2.88E-06	4.73E+00	5.85E-03	2-step	100	98.7
8405		3.62E-06	7.61E+00	1.92E-03	1:1	6.6	99.4

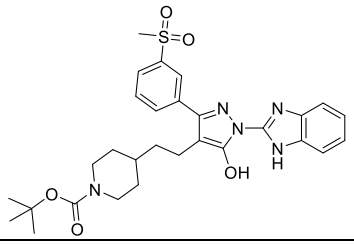
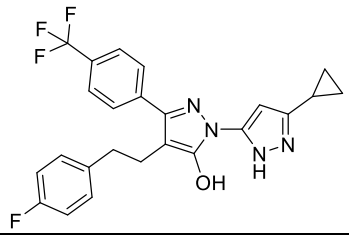
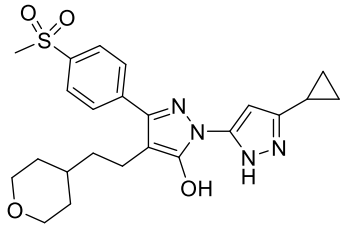
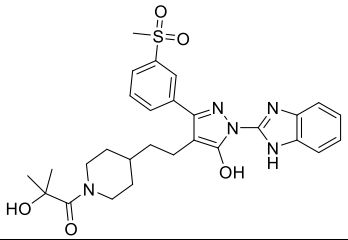
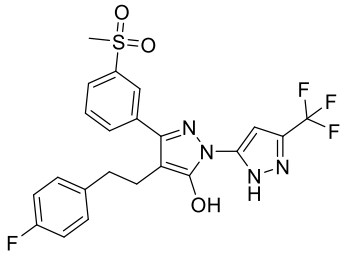
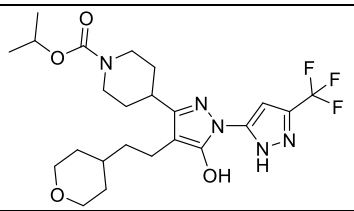
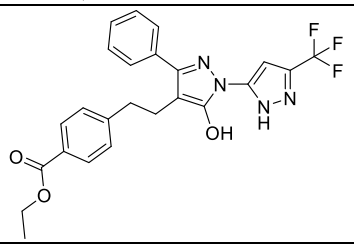
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8419		4.37E-06	3.22E+00	5.13E-03	2-step	22.8	97.1
8420		1.17E-06	3.95E+00	2.34E-03	1:1	9.8	98.6
8440		1.98E-06	4.33E+00	1.81E-03	1:1	0	100
8441		2.02E-09	5.05E+03	1.11E-02	2-step	52.1	81.2
8443		2.63E-08	5.18E+02	8.85E-04	1:1	66.7	74.0
8444		1.40E-09	1.00E+04	2.04E-03	1:1	100	77.8

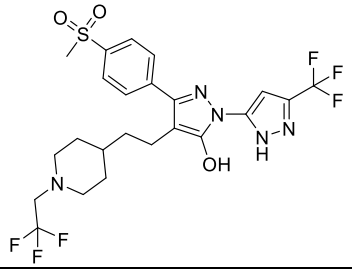
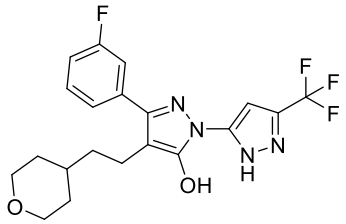
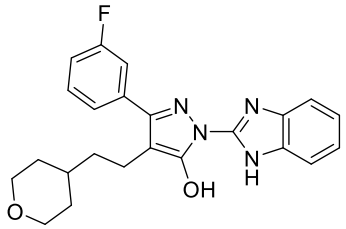
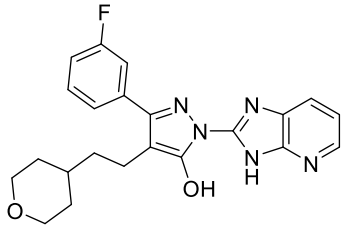
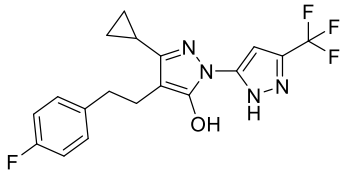
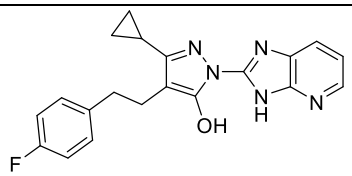
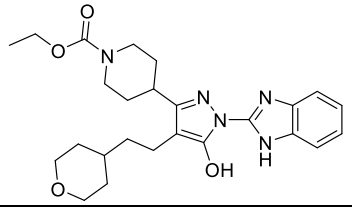
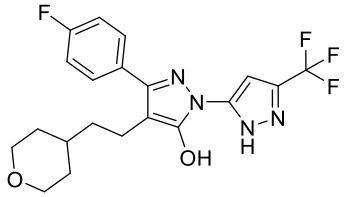
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8445		3.16E-07	4.26E+01	1.62E-03	1:1	62.1	72.9
8515		1.84E-06	1.17E+00	4.45E-03	2-step	2	100
8516		2.67E-06	2.85E+00	7.13E-03	2-step	0	100
8517		3.33E-10	3.26E+04	1.32E-02	2-step	98.1	75.5
8518		3.94E-07	2.62E+01	1.83E-02	2-step	53.4	70.5
8564		6.78E-05	3.95E-02	1.63E-02	2-step	39.2	90.6

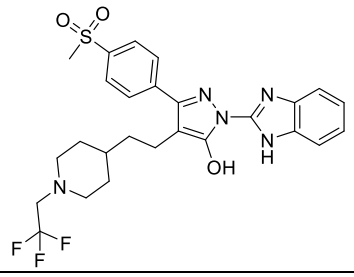
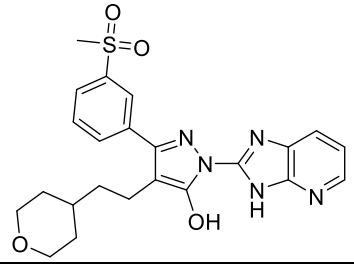
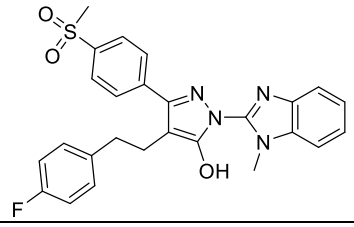
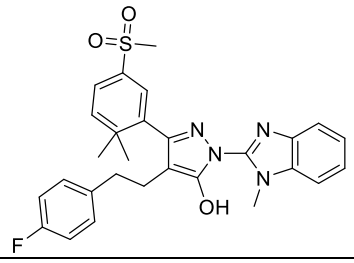
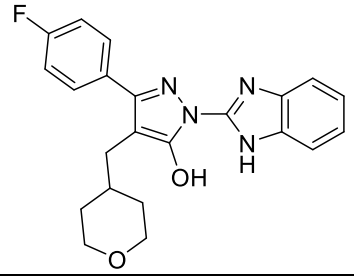
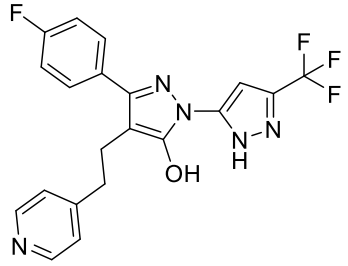
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8566		2.13E-06	7.41E+00	4.77E-03	2-step	39.7	99.7
8567		1.62E-07	5.87E+01	1.00E-02	2-step	53.5	74.5
8611		8.54E-09	3.33E+03	1.75E-02	2-step	0	98.8
8613		2.73E-06	4.96E+00	6.97E-03	2-step	19.2	97.9
8614		4.51E-07	4.44E+01	9.42E-03	2-step	0	98.0
8636		7.40E-07	2.06E+03	2.17E-02	2-step	11.8	95.4
8637		8.90E-07	1.14E+01	6.84E-03	1:1	47.5	98.2
8674		3.17E-06	3.98E+00	2.17E-02	2-step	15.3	99.1

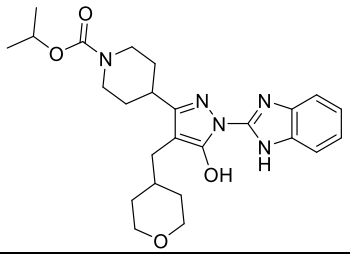
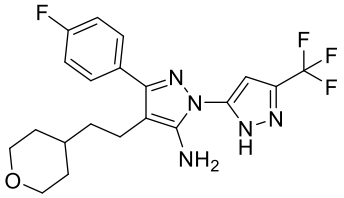
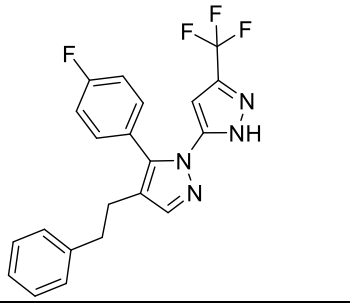
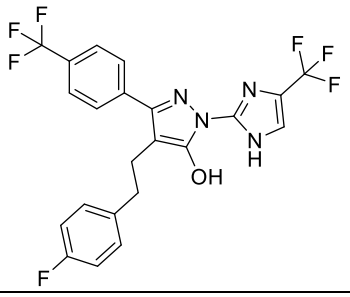
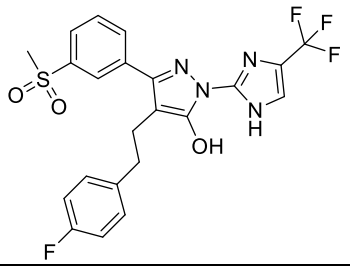
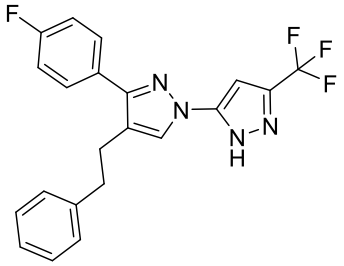
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8675		6.92E-07	1.02E+01	1.32E-02	2-step	0	97.9
8676		1.25E-08	8.01E+02	2.34E-02	2-step	67.1	98.8
8698		1.33E-08	7.82E+02	2.77E-02	2-step	100	75.8
8699		3.15E-12	3.33E+06	3.65E-02	2-step	12.2	95.0
8700		6.93E-08	1.73E+01	4.28E-02	2-step	56	96.5
8701		6.66E-06	5.39E+00	6.08E-02	2-step	4.9	99.5
8702		6.36E-07	5.10E+00	4.14E-02	2-step	25.3	89.6
8765		4.83E-06	3.81E-01	8.59E-03	2-step	4.2	100
8809		2.46E-06	4.16E+00	2.12E-02	2-step	2.5	100

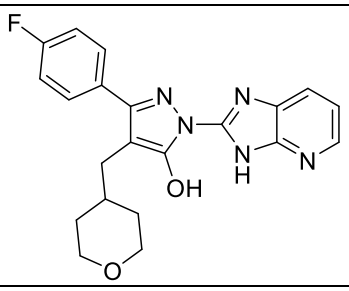
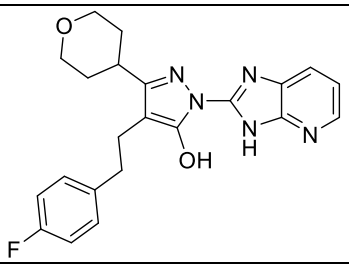
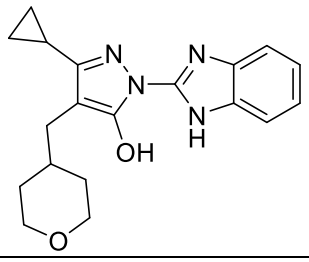
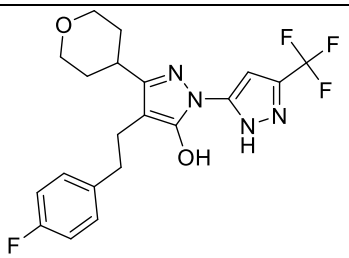
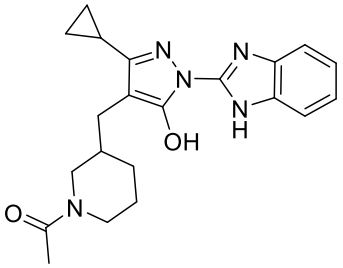
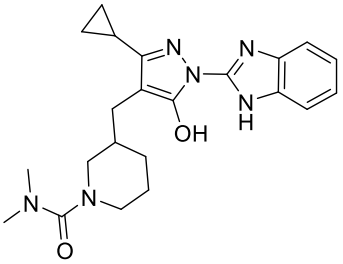
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8810		3.29E-06	5.97E+00	1.96E-02	2-step	0	97.2
8811		1.99E-08	7.54E+02	1.07E-02	2-step	52.3	100
8818		1.53E-11	2.46E+05	2.23E-03	2-step	14.3	100
8819		7.45E-06	1.24E+00	6.05E-03	2-step	0	100
8820		3.51E-05	2.18E+00	1.19E-02	2-step	57.8	100
8821		4.52E-05	5.48E+00	1.24E-02	2-step	25.5	100
8830		2.23E-05	3.88E+00	2.21E-02	2-step	13.5	100
8831		1.52E-05	4.16E+00	1.91E-02	2-step	68.7	100
8833		3.67E-12	1.46E+06	7.18E-03	2-step	13.8	100

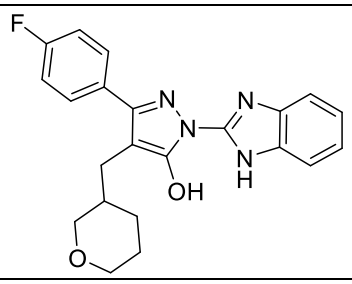
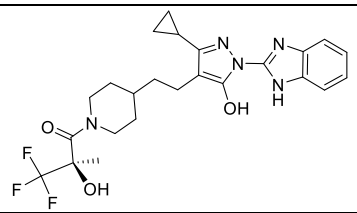
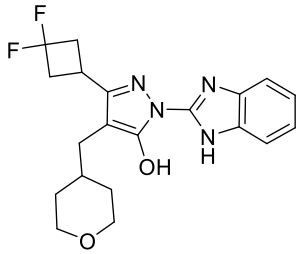
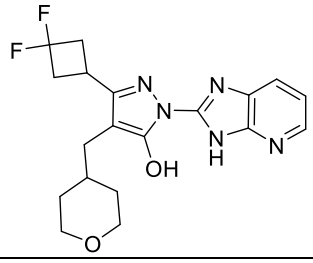
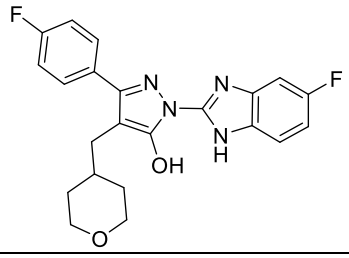
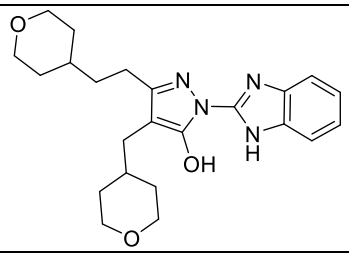
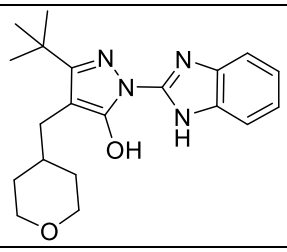
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8835		4.56E-06	1.06E+01	5.18E-03	2-step	95.4	83.7
8864		4.67E-06	5.79E+00	1.06E-02	2-step	0	95.5
8865		8.86E-05	3.74E-01	7.73E-03	1:1	57.3	100
8870		1.20E-06	1.06E+01	5.14E-03	2-step	19.3	97.1
8871		6.49E-06	1.71E+00	1.95E-03	1:1	67.3	99.6
8891		5.14E-05	3.66E-01	6.91E-03	1:1	25.7	100
8900		3.82E-06	8.96E+00	1.39E-02	2-step	13.8	100

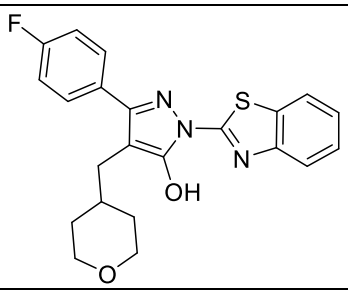
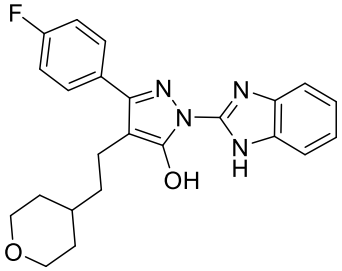
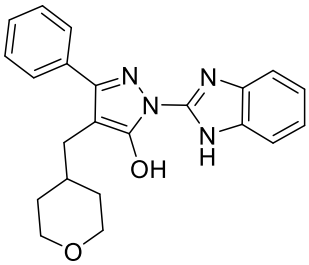
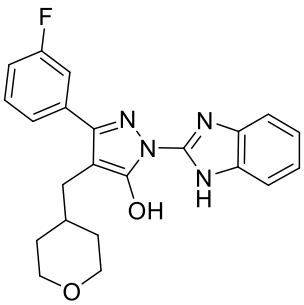
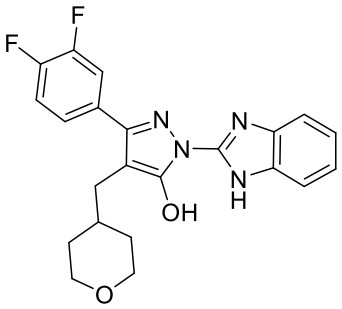
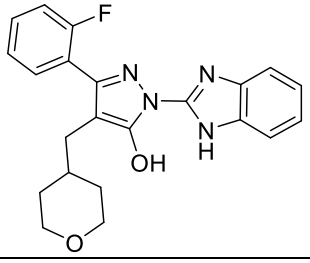
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8914		2.46E-06	2.22E+00	2.05E-03	1:1	51.3	99.7
8959		1.87E-05	2.26E+00	4.94E-03	1:1	0	100
8960		1.07E-06	9.69E+00	4.75E-03	2-step	53.9	77.7
8962		3.15E-06	1.25E+01	5.25E-03	2-step	0	98.7
8963		4.58E-05	7.37E+00	3.07E-02	2-step	0	95.8
8964		4.06E-06	5.81E+00	6.06E-03	2-step	61.4	100
8971		9.09E-06	5.21E+00	9.19E-03	2-step	0	92.2
8973		9.64E-06	3.96E+00	1.31E-03	1:1	0	99.8

Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
8984		2.88E-05	6.80E+00	4.50E-03	1:1	45.4	69.3
8988		7.44E-07	1.13E+01	2.13E-03	1:1	59.3	96.8
9036		2.40E-06	3.63E+00	2.88E-03	1:1	4.1	99.9
9037		4.39E-06	2.32E+00	3.25E-03	1:1	0	95.7
9039		6.26E-06	4.68E+00	8.50E-03	2-step	100	83.0
9068		7.47E-06	1.63E+00	1.44E-03	1:1	12	100

Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
9069		3.29E-06	4.73E+00	3.88E-03	1:1	37.4	95.6
9132		4.75E-06	1.47E+00	2.00E-03	1:1	8.7	100
9215		1.98E-07	2.36E+02	1.15E-02	1:1	3	97.9
9345		7.72E-11	2.23E+05	1.10E-02	2-step	21.9	92.1
9346		3.64E-06	4.87E+00	3.03E-03	1:1	50.6	97.7
9425		9.42E-07	3.64E+00	6.00E-03	1:1	0	100

Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
9539		4.80E-07	2.92E+01	5.15E-03	1:1	33.2	96.7
9540		7.05E-07	1.79E+01	2.97E-03	1:1	0	100
9571		1.37E-06	6.54E+00	3.94E-03	1:1	11.9	100
9577		2.21E-04	2.88E+00	1.67E-02	1:1	17.4	100
9647		4.07E-06	8.13E+00	4.47E-03	1:1	0	99
9649		9.41E-06	3.62E+00	5.13E-03	1:1	61.1	98.8

Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
9651		6.05E-07	7.72E+00	6.67E-04	1:1	67.2	88.8
9653		3.37E-06	4.08E+00	1.38E-03	1:1	5.4	100
9654		2.24E-06	5.08E+00	1.52E-03	1:1	0	98.4
9655		4.49E-06	5.19E+00	1.19E-03	1:1	32.8	99.7
9658		3.48E-10	7.12E+04	5.24E-03	2-step	97.2	85.7
9690		1.09E-05	7.72E+00	8.86E-03	2-step	0	97.9
9746		7.19E-06	2.48E+00	2.62E-03	1:1	74.8	100

Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
9749		7.68E-06	8.01E+00	1.02E-02	2-step	88.7	100
9750		4.42E-06	9.98E+00	1.11E-02	2-step	100	90
9751		2.27E-06	7.61E+00	1.71E-03	1:1	37.8	100
9752		3.52E-06	6.46E+00	2.48E-03	1:1	100	100
9753		3.40E-06	1.04E+01	4.57E-03	2-step	100	95.1
9755		1.17E-05	7.72E+00	1.62E-02	2-step	61.5	99.3

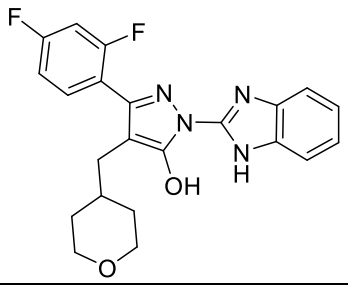
Analog FC-	Structure	K_D, M	T, minutes	RU_{30}/RU_{Nef}	SPR fit	% Infectivity Inhibition	% Cell Viability
9756		3.07E-11	1.02E+06	8.62E-03	2-step	20.2	100

Table S2. Summary of biochemical and biological activities for the top six Nef inhibitor analogs.

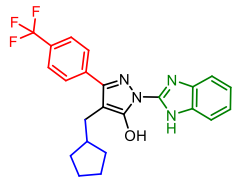
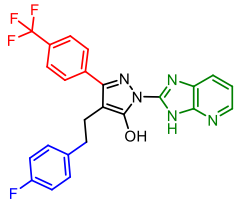
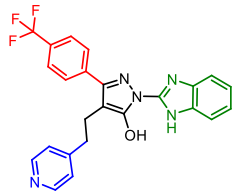
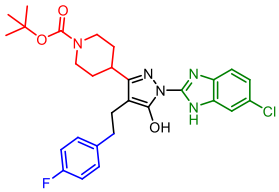
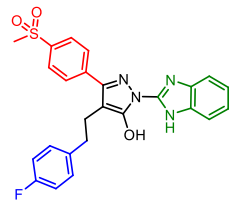
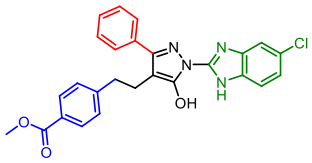
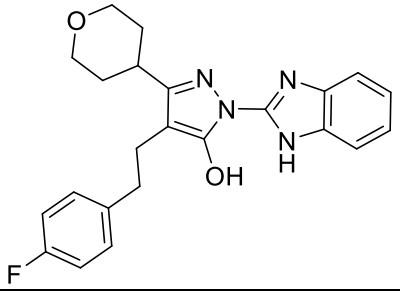
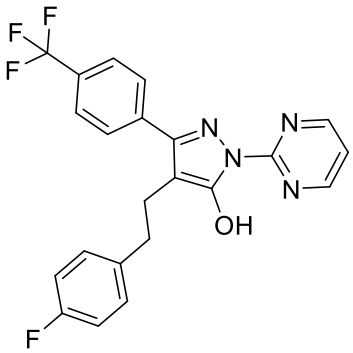
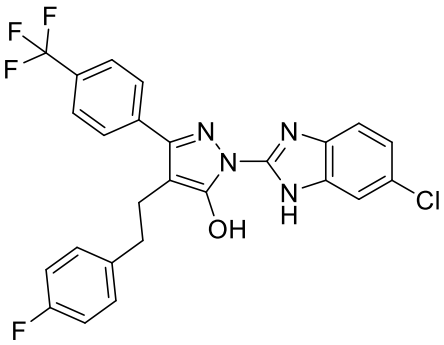
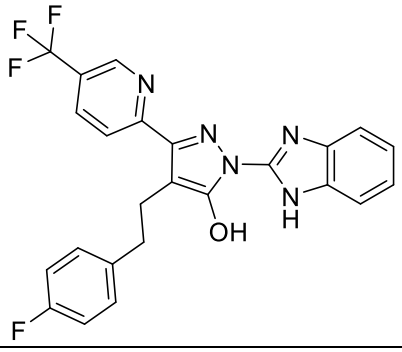
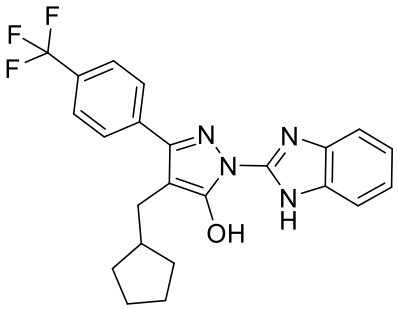
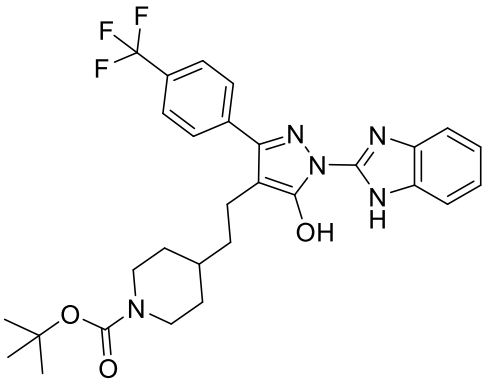
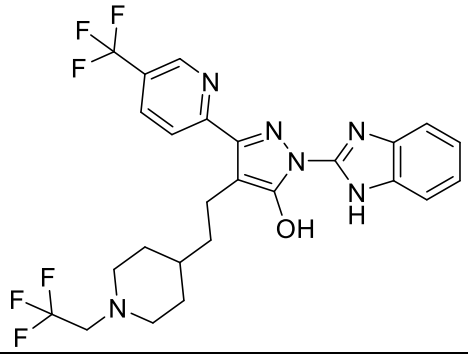
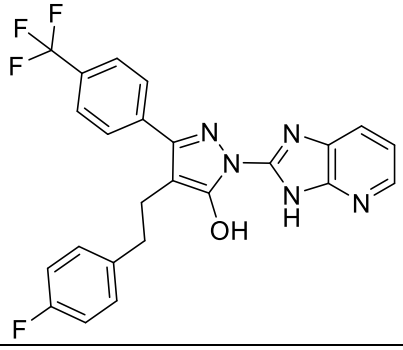
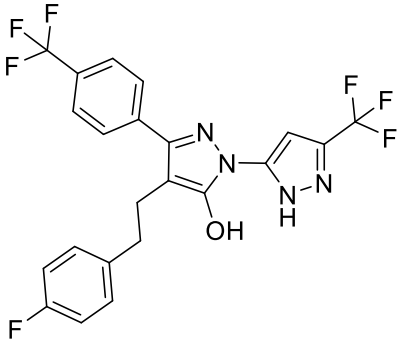
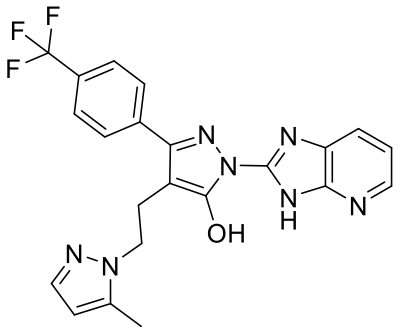
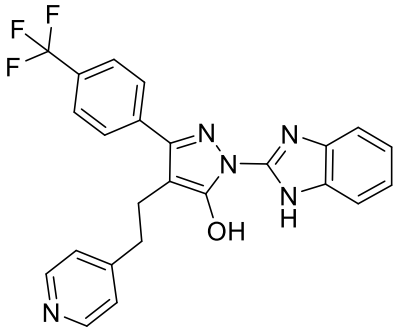
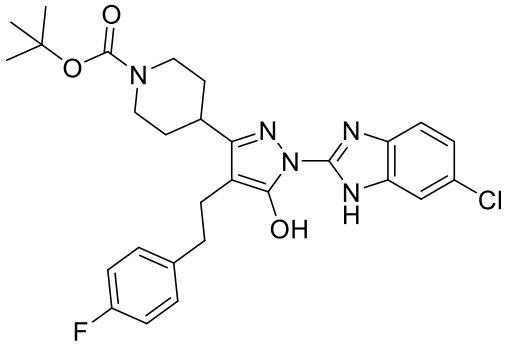
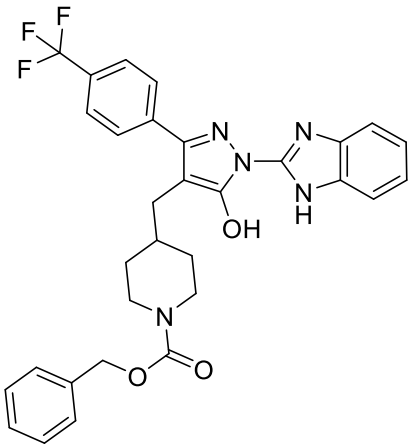
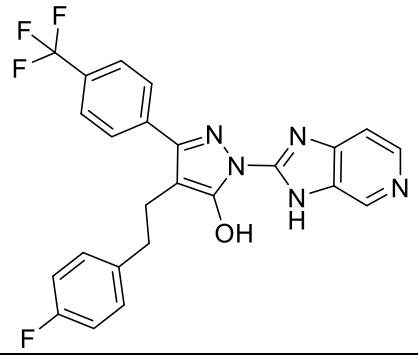
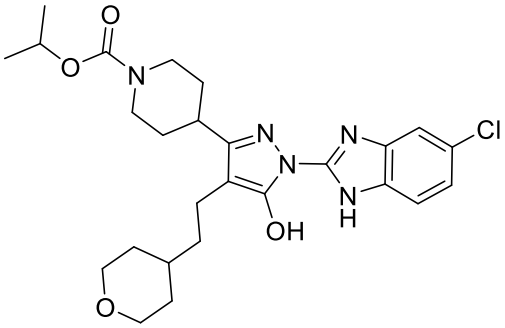
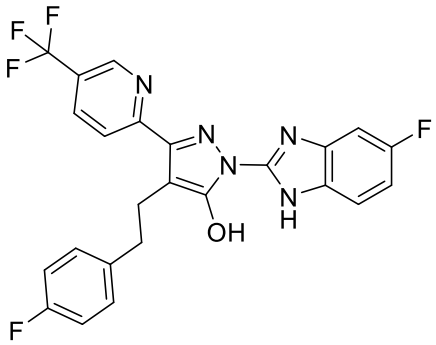
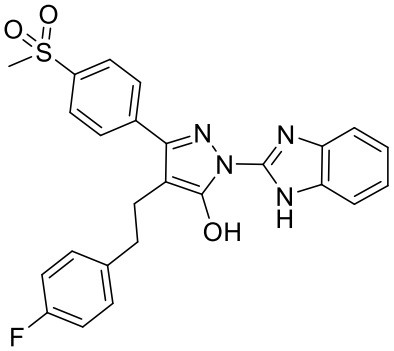
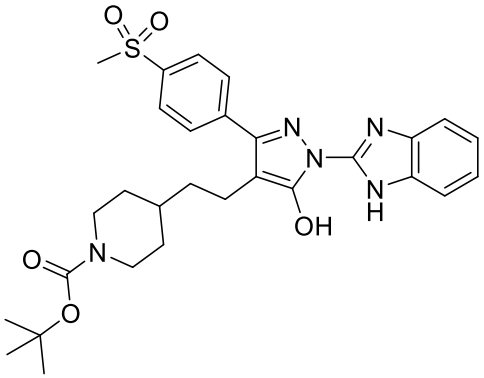
		SPR Data, NL4-3 Nef		TZM-bl reporter cells Analogs @ 1.0 µM		Donor PBMCs infected with HIV-1 NL4-3		CEM-SS Cells
Analog	Structure	K_D, M	Residence Time, min	Infectivity % Inhibition	% Viability	Replication IC_{50}, nM	Viability CC_{50}, nM	% MHC-I Rescue
FC-7902		9.4×10^{-11}	3.8×10^{5}	96.1	85.3	5.01	1,952	18.5
FC-7943		6.0×10^{-11}	6.5×10^{7}	81.2	90.7	2.46	1,806	12.6
FC-7976		2.9×10^{-10}	7.0×10^{4}	100	76.3	1.78	1,563	7.8
FC-8052		8.7×10^{-12}	4.7×10^{5}	100	87.1	0.65	760	20.9
FC-8517		3.3×10^{-10}	3.3×10^{4}	98.1	75.5	9.50	6,442	5.2
FC-8698		1.3×10^{-8}	1.8×10^{2}	100	75.8	31.9	1,153	14.3

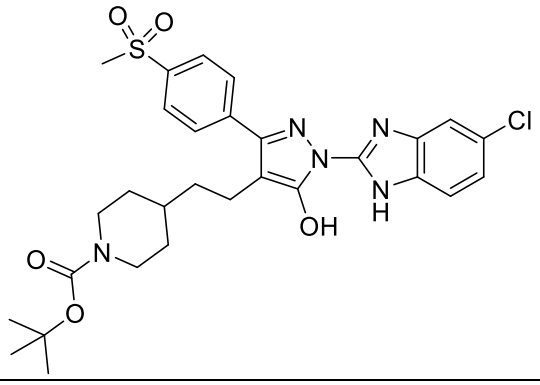
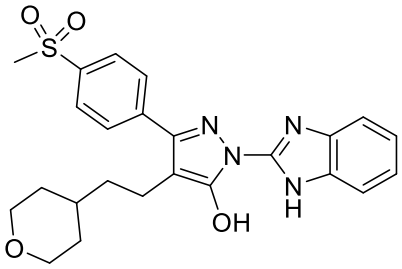
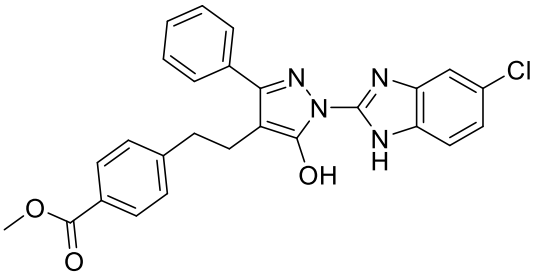
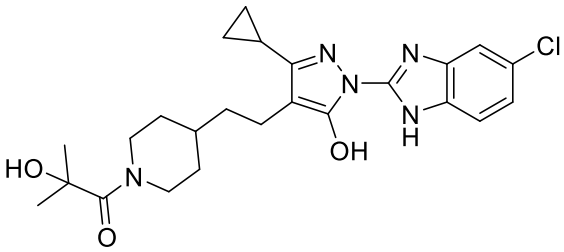
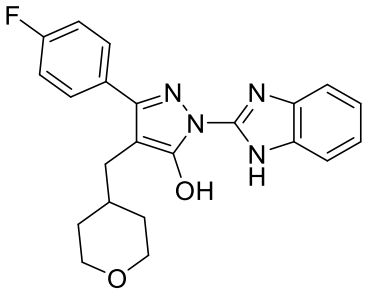
Table S3. Liver microsomal stability of select HIV-1 Nef inhibitors. Mouse liver microsomal stability studies were conducted by Alliance Pharma, Inc., Malvern, PA. Test compounds were prepared at a concentration of 0.5 µM and incubated with mouse liver microsomes (0.5 mg/mL) and an NADPH-regenerating system in potassium phosphate buffer (pH 7.4) at 37 °C. Aliquots were removed at 0, 5, 15, 30, and 45 minutes and quenched with an acetonitrile solution containing an internal standard. Parallel control reactions were run without the cofactor solution. Samples were analyzed by LC-MS/MS, and results recorded as peak area ratios of each analyte to an internal standard. The intrinsic clearance and half-life of each analog were then determined from the first-order elimination rate constant by nonlinear regression. Analog numbers of top-scoring compounds along with their z-scores (based on on-target residence time) are highlighted in red.

Analog FC-	Activity Score (z-score)	Structure	$T_{1/2}$ minutes	Clearance mL/min/g liver
7351	not ranked		3.84	17.3
7867	not ranked		>180	<0.3
7868	**2.31**		**23.0**	**2.89**
7877	1.64		>180	<0.3

Analog FC-	Activity Score (z-score)	Structure	$T_{1/2}$ minutes	Clearance mL/min/g liver
7902	**5.97**		**35.8**	**1.86**
7904	-0.39		49.3	1.35
7942	0.33		51.7	1.29
7943	**3.48**		**154.0**	**0.432**
7946	**2.12**		**52.1**	**1.28**

Analog FC-	Activity Score (z-score)	Structure	$T_{1/2}$ minutes	Clearance mL/min/g liver
7948	0.57		48.7	1.37
7976	**2.53**		**49.3**	**1.35**
8052	**3.77**		**12.0**	**5.55**
8108	0.16		42.5	1.56

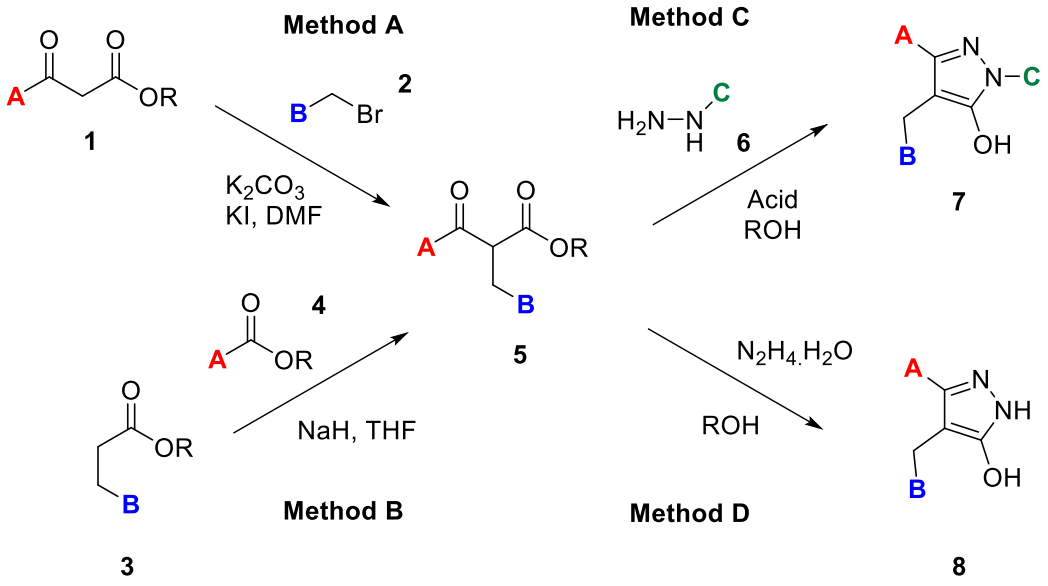
Analog FC-	Activity Score (z-score)	Structure	$T_{1/2}$ minutes	Clearance mL/min/g liver
8232	-0.22		91.9	0.72
8404	-0.33		36.5	1.82
8441	0.49		147.7	0.45
8517	**2.24**		**>180**	**<0.3**
8518	0.14		64.2	1.04

Analog FC-	Activity Score (z-score)	Structure	$T_{1/2}$ minutes	Clearance mL/min/g liver
8567	-0.07		93.5	0.71
8612	not ranked		162.1	0.004
8698	**3.27**		**35.2**	**1.89**
8811	0.25		25.3	2.63
9039	-0.24		79.9	0.832

MATERIALS AND METHODS

Synthesis and characterization of active hydroxypyrazole Nef inhibitor analogs shown in Figures 4 and S2.

Overview of Synthetic Routes.



β-Ketoesters **5** were prepared by two methods. The first method involved alkylation of β-ketoesters **1** with alkyl bromides **2** (Method A). Alternatively, Claisen condensation of enolizable esters **3** with non-enolizable esters **4** gave **5** (Method B). Hydroxypyrazoles of general structure **7** were formed by heating **5** with heteroaryl hydrazines **6**, in the presence of an acid catalyst, in an alcohol solvent (Method C). In most cases p-TsOH was employed as acid catalyst; however, when acid-sensitive functionality, such as a Boc group, was present acetic acid was used. Generally, the alcohol solvent used corresponded to the ester form of **5**. Finally, hydroxypyrazoles of general structure **8**, unsubstituted on nitrogen, were prepared by reaction of β-ketoesters **5** with hydrazine hydrate (Method D).

General Experimental Section

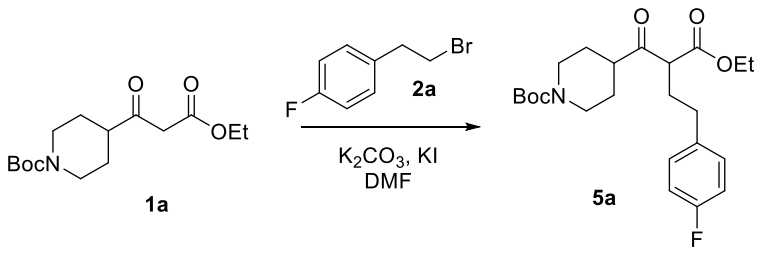
Starting reagents were purchased from commercial suppliers and were used without further purification unless otherwise specified. Normal phase column chromatography was carried out in the indicated solvent system using pre-packed silica gel cartridges on the Isco CombiFlash Companion® or Isco CombiFlash Rf® system. Preparative reversed phase HPLC ("Prep HPLC") was performed on a Phenomenex LUNA 5 µm C18(2) 100 Å 150 x 21.2 mm column or a Waters SunFire® Prep C18 OBD™ 10 µm 150 x 30 mm column with a 12 min mobile phase gradient of 10% acetonitrile/water to 90% acetonitrile/water with 0.1% TFA as buffer using 214 and 254 nm as detection wavelengths. Injection and fraction collection were performed with a Gilson 215 liquid handling apparatus using Unipoint software.

LC-MS data were determined with a Waters Alliance 2695 HPLC/MS using a Phenomenex Luna 3µ C18(2) 100 Å, 75 x 4.6 mm column with a 2996 diode array detector operating from 210−400 nm; the solvent system was 5−95% acetonitrile in water (with 0.1% TFA) over nine minutes using a linear gradient, and retention times (t_R) are in minutes. Mass spectrometry was performed on a Waters ZQ using electrospray in positive ion mode. High resolution mass spectra (HRMS) were obtained on a Bruker Daltonics microTOF II instrument using electrospray ionization in positive mode. Purity of each compound tested in this study was confirmed to be 95% or greater by LC-MS.

Nuclear Magnetic Resonance spectra were recorded on a Varian Mercury 300 spectrometer operating at 299.985 MHz for ^{1}H NMR, at 282.243 MHz for ^{19}F NMR and at 75.439 MHz for ^{13}C NMR. Spectra were taken in the indicated solvent at ambient temperature, and the chemical shifts are reported in parts per million (ppm, δ) relative to the lock of the solvent used. Resonance patterns are recorded with the following notations: br (broad), s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet).

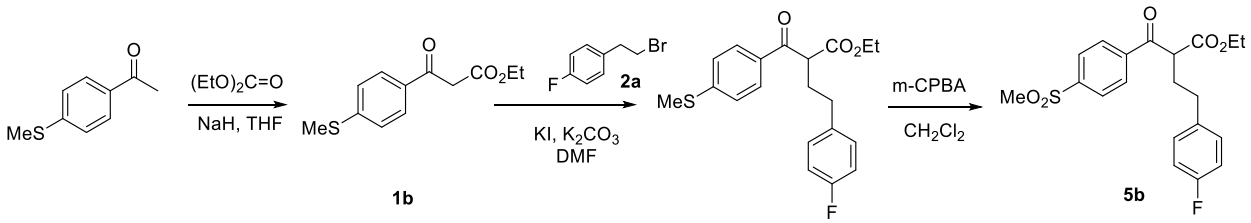
Method A

tert-butyl 4-{3-ethoxy-2-[2-(4-fluorophenyl)ethyl]-3-oxopropanoyl}piperidine-1-carboxylate (5a)



A mixture of β-ketoester **1a** (2.82 g, 9.4 mmol), bromide **2a** (2.20 g, 10.8 mmol), K_2CO_3 (1.43 g, 10.4 mmol), KI (1.72 g, 10.4 mmol) and DMF (20 mL) was stirred at 70 °C for 16 h. The mixture was diluted with EtOAc (80 mL), washed with 5% aq HCl (2 x 15 mL) and brine (15 mL), and dried over Na_2SO_4. Removal of the solvent left a mobile oil (5.41 g). Chromatography on an 80 g silica cartridge, eluted with a 0-40% EtOAc in hexanes gradient, gave a pale yellow oil (3.16 g, 80%) containing ~80% desired **5a** and ~20% O-alkylation product. This material was used without further purification.
LC-MS t_R 5.73 min, m/z 322 [M + Na+]

Ethyl 4-(4-fluorophenyl)-2-(4-methanesulfonylbenzoyl)butanoate (5b)



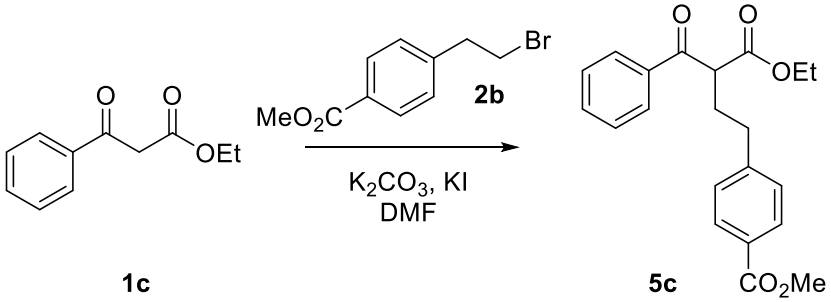
A stirred mixture of 1-[4-(methylsulfanyl)phenyl]ethan-1-one (5.11 g, 30.7 mmol), diethyl carbonate (7.5 mL, 62 mmol) and 60% NaH in oil (2.50 g, 62 mmol) was diluted with dry THF (50 mL) and heated at reflux under N_2 for 5 h. The mixture was cooled, diluted with 5% aq HCl (25 mL), [Caution $H_2\uparrow$]and extracted with EtOAc (2 x 50 mL). The combined EtOAc layer was washed with brine (10 mL), and dried over Na_2SO_4. Removal of the solvent left a dark oil (8.26 g) which was chromatographed on an 80 g silica cartridge, eluted with a 0 – 70% EtOAc in hexanes gradient, to give ethyl 3-[4-(methylsulfanyl)phenyl]-3-oxopropanoate (**1b**, 5.77 g, 79% yield) as a golden oil.
^{1}H NMR (CHLOROFORM-d, major tautomer) δ: 7.79-7.88 (m, 2H), 7.19-7.31 (m, 2H), 4.16-4.23 (m, 2H), 3.93 (s, 2H), 2.51 (s, 3H), 1.24 (t, J=7.1 Hz, 3H).

A mixture of ethyl 3-[4-(methylsulfanyl)phenyl]-3-oxopropanoate (5.77 g, 24.2 mmol), 1-(2-bromoethyl)-4-fluorobenzene (5.16 g, 24.2 mmol), K_2CO_3 (3.68 g, 26.6 mmol), KI (4.42 g, 26.6 mmol) and DMF (30 mL) was heated at 70 °C for 64 h. The mixture was diluted with EtOAc (175 mL), washed with 1% aq HCl (50 mL) and brine (25 mL), and dried over Na_2SO_4. Removal of the solvent left a yellow oil (11.67 g). Chromatography on an 80 g silica cartridge, eluted with a 0-50% EtOAc in hexanes gradient, gave **1b** (7.91 g, 91%) as a pale, yellow oil.
LC-MS t_R 5.72 min, m/z 383, 361.

To a stirred, ice-cold solution of β-ketoester **1b** (7.91 g, 22.0 mmol) in CH_2Cl_2 (200 mL) was added ≤ 77% m-CPBA (11.20 g, ≤50.0 mmol). The mixture was stirred in the ice bath for 3 h and filtered. The filtrate was washed with sat'd aq $NaHCO_3$ (30 mL) and brine (50 mL), and dried over Na_2SO_4. Removal of the solvent left a pasty, white solid (11.90 g) which was chromatographed on a 120 g silica cartridge, eluted with a 0 to 50% EtOAc in hexanes gradient, to afford ethyl 4-(4-fluorophenyl)-2-(4-methanesulfonylbenzoyl)butanoate (7.35g, 85%).
^{1}H NMR (CHLOROFORM-d) δ: 7.97-8.07 (m, 4H), 7.05-7.14 (m, 2H), 6.88-6.99 (m, 2H), 4.25 (t, J=7.0 Hz, 1H), 4.12 (q, J=7.1 Hz, 2H), 3.06 (s, 3H), 2.58-2.70 (m, 2H), 2.21-2.36 (m, 2H), 1.15 (t, J=7.1 Hz, 3H)

LC-MS t_R 5.11 min, m/z 393.

Methyl 4-(3-benzoyl-4-ethoxy-4-oxobutyl)benzoate (5c)

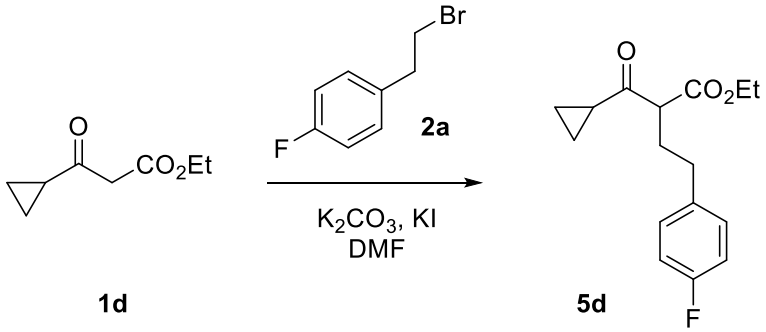


1c **5c**

A mixture ethyl 3-oxo-3-phenylpropanoate (929 mg, 4.8 mmol), methyl 4-(2-bromoethyl)benzoate (1.23 g, 5.0 mmol), K_2CO_3 (735 mg, 5.3 mmol), KI (883 mg, 5.3 mmol) and dry DMF (10 mL) was stirred at 70 ^oC for 18 h. The mixture was diluted with EtOAc (90 mL), washed with 5% aqueous HCl (15 mL) and saturated aqueous $NaHCO_3$ (10 mL) and brine (10 mL), and dried over Na_2SO_4. Removal of the solvent left a yellow oil (1.93 g) which was chromatographed on a 40 g silica cartridge, eluted with a 0-100% EtOAc in hexanes gradient, to afford methyl 4-(3-benzoyl-4-ethoxy-4-oxobutyl)benzoate (**5c**, 920 mg, 54%) as an oil.

^{1}H NMR (CHLOROFORM-d) δ: 7.96 (d, J=8.2 Hz, 2H), 7.87-7.93 (m, 2H), 7.54-7.63 (m, 1H), 7.42-7.50 (m, 2H), 7.25 (d, J = 8.2 Hz, 2H), 4.26 (t, J=7.1 Hz, 1H), 4.09-4.20 (q, 2H), 3.91 (s, 3H), 2.70-2.79 (m, 2H), 2.23-2.43 (m, 2H), 1.00-1.23 (t, 3H).

2-cyclopropanecarbonyl-4-(4-fluorophenyl)butanoate (5d)

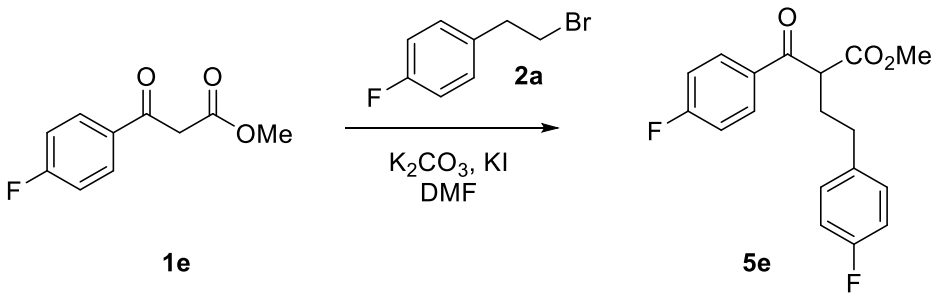


1d **5d**

A mixture of ethyl 3-cyclopropyl-3-oxopropanoate (500 mg, 3.2 mmol), 1-(2-bromoethyl)-4-fluorobenzene (680 mg, 3.3 mmol), K_2CO_3 (490 mg, 3.5 mmol), KI (580 mg, 3.5 mmol) and dry DMF (5 mL) was stirred at 70 ^oC under N_2 for 5 h. The mixture was diluted with EtOAc (90 mL), washed with 5% aq HCl (15 mL) and 1:1 satd aq $NaHCO_3$/brine (15 mL), and dried over Na_2SO_4. Removal of the solvent left an oil (3.46 g) which was chromatographed on a 40 g silica cartridge, eluted with a 0-40% EtOAc in hexanes gradient, to give ethyl 2-cyclopropanecarbonyl-4-(4-fluorophenyl)butanoate (**5d**, 450 mg, 51%) as an oil.

^{1}H NMR (CHLOROFORM-d) δ: 7.08-7.17 (m, 2H), 6.92-7.01 (m, 2H), 4.14-4.26 (m, 2H), 3.55 (t, J=7.3 Hz, 1H), 2.53-2.66 (m, 2H), 2.12-2.25 (m, 2H), 2.03 (tt, J=7.8, 4.5 Hz, 1H), 1.27 (t, J=7.0 Hz, 3H), 1.04-1.11 (m, 2H), 0.87-0.96 (m, 2H).

Methyl 2-(4-fluorobenzoyl)-4-(4-fluorophenyl)butanoate (5e)

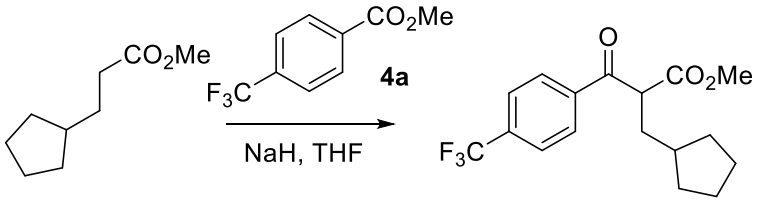


1e **5e**

The title compound was prepared following the procedure described for **5d**, starting with **1e** (500 mg, 2.5 mmol) to afford β-ketoester **5e** (690 mg, 85%) as a colorless oil. LC-MS t_R 5.29 min, m/z 319.

Method B

Methyl 2-(cyclopentylmethyl)-3-oxo-3-[4-(trifluoromethyl)phenyl]propanoate (5f)



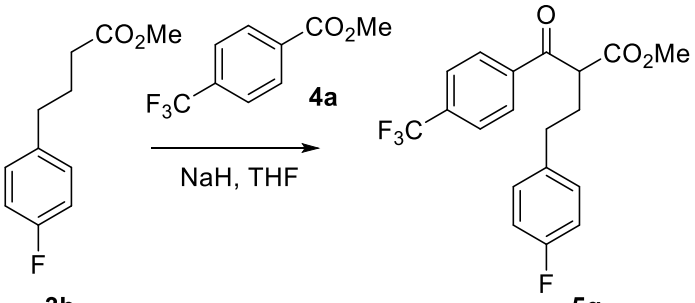
3a **5f**

A mixture of methyl 4-(trifluoromethyl)benzoate (1.77 g, 8.7 mmol), methyl 3-cyclopentylpropanoate (1.13 g, 7.2 mmol) and 60% NaH in oil (0.60 g, 14.9 mmol) was diluted with dry THF (10 mL). MeOH (1 drop) was added and the mixture was stirred at reflux under N_2 for 4 h. The mixture was cooled, diluted with EtOAc (90 mL), washed with 5% aq HCl (20 mL), 1:1 sat'd aq $NaHCO_3$/brine (20 mL) and 9:1 brine/5% aq HCl (20 mL), and dried over Na_2SO_4. Removal of the solvent left an oil (3.17 g) which was chromatographed on a 40 g silica cartridge, eluted with a 0-30% EtOAc in hexanes gradient to give methyl 2-(cyclopentylmethyl)-3-oxo-3-[4-(trifluoromethyl)phenyl]propanoate (**5f**, 2.00 g, 84%) as an oil.

^{1}H NMR (CHLOROFORM-d) δ: 8.09 (d, J=8.1 Hz, 2H), 7.74 (d, J=8.3 Hz, 2H), 4.36 (t, J=7.2 Hz, 1H), 2.04 (dq, J=17.5, 6.9 Hz, 2H), 1.39-1.89 (m, 8H), 0.99-1.34 (m, 3H), 0.85 (br d, J=7.6 Hz, 1H)

^{19}F NMR (CHLOROFORM-d) δ: -63.26.

Methyl 4-(4-fluorophenyl)-2-[4-(trifluoromethyl)benzoyl]butanoate (5g)

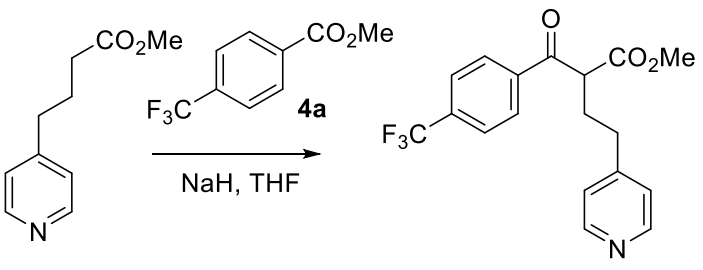


3b **5g**

A mixture of ester **3b** (10.42 g, 53.1 mmol), ester **4a** (16.30 g, 79.7 mmol) and toluene (50 mL) was concentrated under reduced pressure. To the residue was added 60% NaH in oil (5.31 g, 132.8 mmol) and dry THF (100 mL). The mixture was heated at reflux for 3 h, cooled and poured into cold 5% aq HCl (150 mL). [Caution H_2 ↑]. The mixture was extracted with EtOAc (2 x 100 mL). The combined organic layer was washed with brine (40 mL) and dried over Na_2SO_4. Removal of the solvent left a viscous oil (23.89 g). Chromatography on a 120 g silica cartridge, eluted with a 0-10% EtOAc in hexanes gradient, afforded **5g** (15.21 g, 78%) as a colorless oil.

^{1}H NMR $CDCl_3$ δ 7.97-8.00 (d, J=8.2 Hz, 2H), 7.70-7.73 (d, J=8.2 Hz, 2H), 7.08-7.13 (m, 2H), 6.93-6.99 (m, 2H), 4.28 (t, 1H), 3.69 (s, 3H), 2.63-2.69 (m, 2H), 2.30-2.37 (m, 2H).

Methyl 4-(pyridin-4-yl)-2-[4-(trifluoromethyl)benzoyl]butanoate (5h)

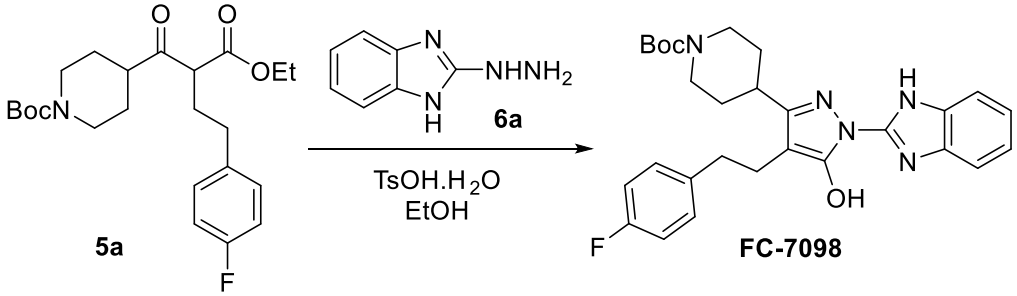


3c **5h**

A mixture of methyl 4-(pyridin-4-yl)butanoate (740 mg, 4.7 mmol), methyl 4-(trifluoromethyl)benzoate (1.16 g, 5.7 mmol) and 60% NaH in oil (380 mg, 9.5 mmol) was diluted with dry THF (8 mL) and stirred at reflux under N_2 for 4 h. The mixture was diluted with EtOAc (90 mL), washed with 1:1 satd aq $NaHCO_3$/water (20 mL), satd aq $NaHCO_3$ (20 mL) and brine (20 mL), and dried over Na_2SO_4. Removal of the solvent left a yellow solid (1.56 g) which was chromatographed on a 24 g silica cartridge, eluted with a 0-100% EtOAc in hexanes gradient to afford methyl 4-(pyridin-4-yl)-2-[4-(trifluoromethyl)benzoyl]butanoate (**5h**, 860 mg, 55%) as a pale yellow oil.
^{1}H NMR (CHLOROFORM-d) δ: 8.46-8.55 (m, 2H), 8.02 (d, J=7.9 Hz, 2H), 7.73 (d, J=8.5 Hz, 2H), 7.06-7.14 (m, 2H), 4.28 (t, J=7.1 Hz, 1H), 3.68 (s, 3H), 2.62-2.73 (m, 2H), 2.29-2.42 (m, 2H)

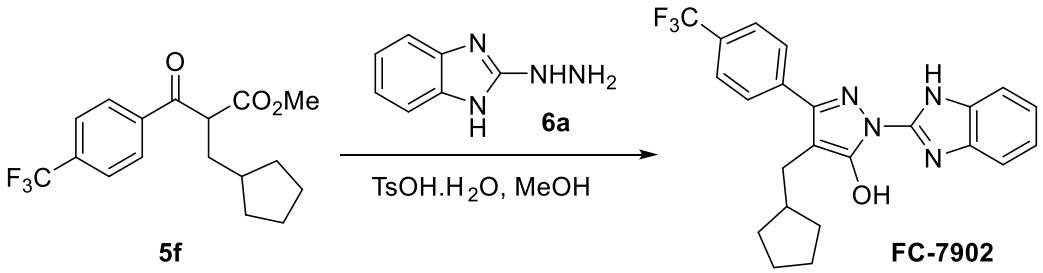
Method C

tert-Butyl 4-[1-(1H-1,3-benzodiazol-2-yl)-4-[2-(4-fluorophenyl)ethyl]-5-hydroxy-1H-pyrazol-3-yl]piperidine-1-carboxylate (FC-7098)



A mixture of ~80% pure β-ketoester **5a** (950 mg, ~1.8 mmol), hydrazine **6a** (840 mg, 5.7 mmol), HOAc (1.5 mL) and EtOH (1.5 mL) was heated in the microwave at 130 °C for 4 h. The mixture was diluted with EtOAc (100 mL), water (10 mL), sat'd aq $NaHCO_3$ (2 x 10 mL) and brine (10 mL), and dried over Na_2SO_4. Removal of the solvent left a rust colored solid (1.47 g) which was chromatographed on an 80 g silica cartridge, eluted with a 0-50% EtOAc in hexanes gradient, to give an oil (0.40 g). Prep HPLC gave tert-butyl 4-[1-(1H-1,3-benzodiazol-2-yl)-4-[2-(4-fluorophenyl)ethyl]-5-hydroxy-1H-pyrazol-3-yl]piperidine-1-carboxylate TFA salt (220 mg, 20%) as a white solid.
^{1}H NMR (CD_3OD) δ: 7.54-7.75 (m, 2H), 7.36-7.51 (m, 2H), 7.19 (dd, J=8.5, 5.5 Hz, 2H), 6.88-7.09 (m, 2H), 4.10 (br d, J=13.3 Hz, 2H), 2.61-2.94 (m, 6H), 2.43-2.55 (m, 1H), 1.52-1.81 (m, 4H), 1.46 (s, 9H).
^{19}F NMR (CD_3OD) δ: -77.20, -119.43.
LC-MS t_R 4.93 min, 506, 450, 406.
HRMS calc'd for $C_{28}H_{33}FN_5O_3$ 506.2562, found 506.2553.

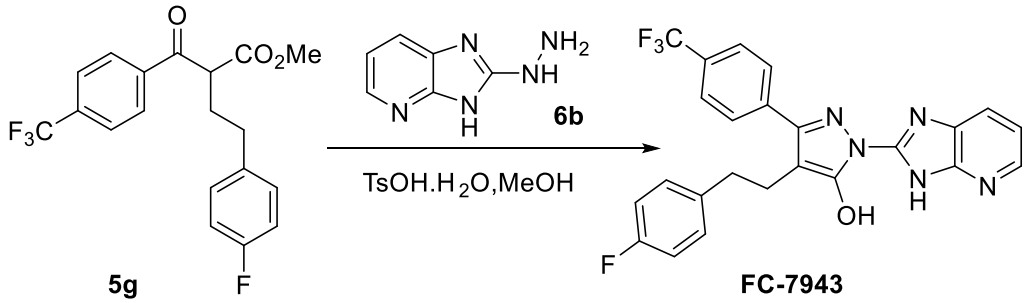
1-(1H-1,3-benzodiazol-2-yl)-4-(cyclopentylmethyl)-3-[4-(trifluoromethyl)phenyl]-1H-pyrazol-5-ol (FC-7902)



A mixture of β-ketoester **5f** (225 mg, 0.69 mmol), hydrazine **6a** (155 mg, 1.03 mmol), $TsOH.H_2O$ (cat. qty.) and MeOH (4 mL) was heated in the microwave at 130 °C for 3 h. The mixture was diluted with EtOAc (100 mL), washed with 5% aq HCl (10 mL), satd aq $NaHCO_3$ (10 mL) and brine (10 mL), and dried over Na_2SO_4. Removal of the solvent left an oil (270 mg) which was purified by prep HPLC to give the title compound as its TFA salt (120 mg, 32%) as a white solid.
^{1}H NMR (METHANOL-d_4) δ: 7.90-8.01 (m, 2H), 7.80 (d, J=8.0 Hz, 2H), 7.59-7.70 (m, 2H), 7.32-7.45 (m, 2H), 2.64 (d, J=7.5 Hz, 2H), 1.92-2.14 (m, 1H), 1.37-1.74 (m, 6H), 1.06-1.24 (m, 2H)
^{19}F NMR (METHANOL-d_4) δ: -77.2, 64.18.
LC-MS t_R 5.68 min, m/z 427.

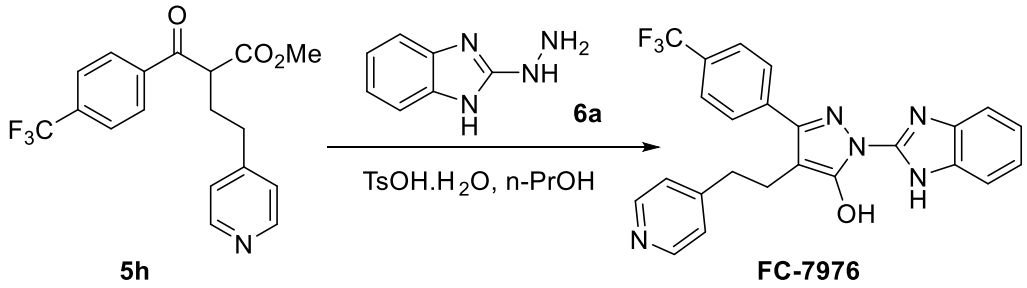
HRMS calc'd for $C_{23}H_{22}F_3N_4O$ 427.1740, found 427.1730.

4-[2-(4-fluorophenyl)ethyl]-1-{3H-imidazo[4,5-b]pyridin-2-yl}-3-[4-(trifluoromethyl)phenyl]-1H-pyrazol-5-ol (FC-7943)



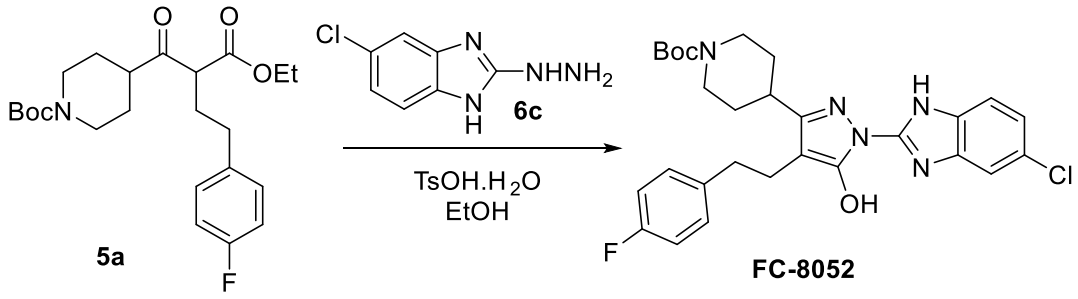
Hydrazine **6b** was prepared following the procedure in DE 3,340,932. A mixture of β-ketoester **5g** (275 mg, 0.75 mmol), hydrazine **6b** (140 mg, 0.94 mmol), $TsOH.H_2O$ (cat. qty.) and MeOH (4 mL) was heated in the microwave at 130 °C for 3 h. The mixture was diluted with EtOAc (100 mL), washed with satd aq $NaHCO_3$ (15 mL) and brine (15 mL), and dried over Na_2SO_4. Removal of the solvent left an oil (360 mg) which was purified by prep HPLC to afford the title compound TFA salt (30 mg, 5%) as a pale yellow solid.
^{1}H NMR (DMSO-d_6) δ: 8.38 (dd, J=5.0, 1.5 Hz, 1H), 8.01 (dd, J=8.0, 1.5 Hz, 1H), 7.83-7.93 (m, 4H), 7.26-7.39 (m, 1H), 7.14-7.24 (m, 2H), 7.01-7.10 (m, 2H), 2.79-2.88 (m, 4H).
^{19}F NMR (CD_3OD) d: -64.29, -77.05, -119.66.
^{13}C NMR (DMSO-d_6) δ: 161.1 (d, J = 241.4 Hz), 153.5, 149.5, 148.1, 137.2, 134.9, 134.7, 130.6, 130.4, 128.9 (q, J = 33 Hz), 127.5, 125.9, 124.4 (q, J = 272.3 Hz), 118.4, 115.3, 115.2, 115.0, 103.8, 34.0, 24.2. Peaks due to TFA not reported.
LC-MS t_R 4.47 min, m/z 468.
HRMS calc'd for $C_{24}H_{18}F_4N_5O$ 468.1442 , found 468.1442.

1-(1H-1,3-benzodiazol-2-yl)-4-[2-(pyridin-4-yl)ethyl]-3-[4-(trifluoromethyl)phenyl]-1H-pyrazol-5-ol (FC-7976)



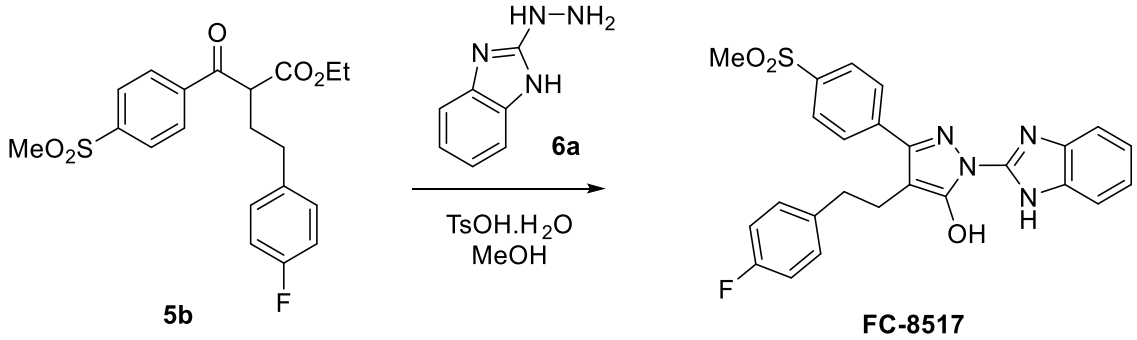
A mixture of β-ketoester **5h** (720 mg, 2.05 mmol), hydrazine **6a** (380 mg, 2.56 mmol), $TsOH.H_2O$ (~200 mg, ~1 mmol) and n-PrOH (12 mL) was heated in the microwave at 130 °C for 3 h. The mixture was diluted with EtOAc (100 mL), washed with 1:1 water/sat'd aq $NaHCO_3$ (15 mL) and brine (15 mL), and dried over Na_2SO_4. Removal of the solvent left an orange oil (1.27 g). Chromatography on a 40 g silica cartridge, eluted with a 0-20% MeOH in CH_2Cl_2 gradient gave an orange solid (610 mg) which was further purified by prep HPLC to afford FC-7976 bis TFA salt (400 mg, 29%) as an off-white solid.
NMR CD_3OD δ: 8.61 (d, J=5.9 Hz, 2H), 7.79-7.88 (m, 6H), 7.73-7.76 (m, 2H), 7.52-7.55 (m, 2H), 3.14-3.17 (m, 4H);
^{19}F NMR (METHANOL-d_4) δ: -64.13, -77.29.
^{13}C NMR (DMSO-d6) δ: 162.0, 157.8, 153.2, 143.7, 141.7, 136.9, 133.5, 129.4 (q, J = 30.9 Hz), 128.7, 127.3, 125.9, 124.6 (J = 271.5 Hz), 123.8, 114.5, 97.7, 35.4, 22.7. Peaks due to TFA not reported.
LC-MS t_R 3.66 min, m/z 450.
HRMS calc'd for $C_{24}H_{19}F_3N_5O$ 450.1536, found 450.1546.

tert-butyl 4-[1-(5-chloro-1H-1,3-benzodiazol-2-yl)-4-[2-(4-fluorophenyl)ethyl]-5-hydroxy-1H-pyrazol-3-yl]piperidine-1-carboxylate (FC-8052)



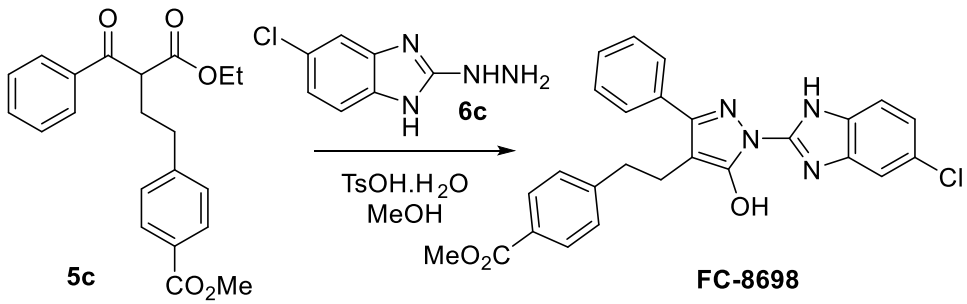
A mixture of ~80% β-ketoester **5a** (1.06 g, ~2.0 mmol), hydrazine **6c** (580 mg, 3.2 mmol), HOAc (1.5 mL) and EtOH (1.5 mL) was heated in the microwave at 130 °C for 4 h. The mixture was diluted with EtOAc (100 mL), washed with water (10 mL), sat'd aq $NaHCO_3$ (2 x 10 mL) and brine (10 mL), and dried over Na_2SO_4. Removal of the solvent left a dark red-brown solid (1.47 g) which was chromatographed on an 80 g silica cartridge, eluted with a 0-50% EtOAc in hexanes gradient, to give an oil (300 mg). Prep HPLC gave the title compound TFA salt (180 mg, 16%) as a white solid.
^{1}H NMR (METHANOL-d_4) δ: 7.52-7.63 (m, 2H), 7.31 (dd, J=8.6, 2.0 Hz, 1H), 7.14-7.23 (m, 2H), 6.93-7.05 (m, 2H), 4.10 (br d, J=13.3 Hz, 2H), 2.62-2.90 (m, 6H), 2.42-2.58 (m, 1H), 1.70-1.48 (br dd, J=17.5, 4.3 Hz, 4H), 1.46 (s, 9H).
^{19}F NMR (METHANOL-d_4) δ: -77.45, -119.47.
LC-MS t_R 5.72 min, m/z 542, 540, 442, 440.
HRMS calc'd for $C_{28}H_{32}ClFN_5O_3$ 540.2172, found 540.217

1-(1H-1,3-benzodiazol-2-yl)-4-[2-(4-fluorophenyl)ethyl]-3-(4-methanesulfonylphenyl)-1H-pyrazol-5-ol (FC-8517)



A mixture of β-ketoester **5b** (184 mg, 0.47 mmol), hydrazine **6a** (140 mg, 0.94 mmol), $TsOH.H_2O$ (cat. qty.) and EtOH (4 mL) was heated in the microwave at 130 °C for 4 h. Preparative HPLC gave the title compound (194 mg, 70%) as its mono-TFA salt.
^{1}H NMR (CD_3OD) δ: 8.02-8.05 (m, 2H), 7.83-7.86 (m, 2H), 7.73-7.76 (m, 2H), 7.52-7.56 (m, 2H), 7.00-7.05 (m, 2H), 6.82-6.89 (m, 2H), 3.20 (s, 3H), 2.95-3.01 (m, 2H), 2.76-2.79 (m, 2H);
^{19}F NMR (METHANOL-d_4) δ: -77.40, -119.50.
^{13}C NMR (DMSO-d_6) δ: 161.1 (d, J = 241 Hz), 158.3, 156.7, 152.8, 143.8, 141.1, 137.7, 133.8, 130.5, 128.7, 127.7, 123.9, 115.3, 114.6, 100.4, 44.0, 34.6, 24.6. Peaks due to TFA not reported.
LC-MS t_R 4.40 min, m/z 440.
HRMS calc'd for $C_{25}H_{22}FN_4O_3S$ 477.1391, found 477.1393.

Methyl 4-{2-[1-(5-chloro-1H-1,3-benzodiazol-2-yl)-5-hydroxy-3-phenyl-1H-pyrazol-4-yl]ethyl}benzoate (FC-8698)



A mixture of β-ketoester **5c** (483 mg, 1.4 mmol), hydrazine **6c** (250 mg, 1.4 mmol), $TsOH.H_2O$ (cat. qty.) and MeOH (4 mL) was heated at 130 °C for 3 h. The mixture was diluted with EtOAc (100 mL), washed with 5% aq HCl (15 mL) and 9:1 brine/satd aq $NaHCO_3$ (10 mL), and dried over Na_2SO_4. Removal of the solvent left a brown solid (690 mg) which was purified by prep HPLC to give the title compound TFA salt (200 mg, 25%) as an off-white solid.

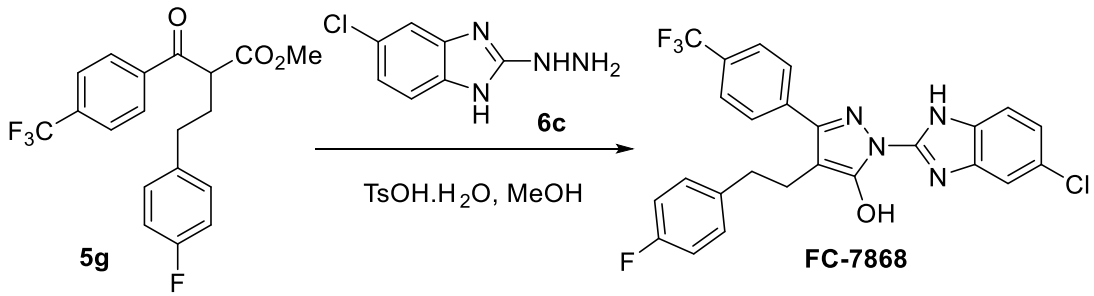
^{1}H NMR (METHANOL-d_4) δ: 7.81 (d, J=7.8 Hz, 2H), 7.40-7.63 (m, 7H), 7.29 (dd, J=8.6, 2.0 Hz, 1H), 7.18 (d, J=7.9 Hz, 2H), 3.86 (s, 3H), 3.30 (br s, 2H), 2.89 (br s, 2H).

^{13}C NMR (DMSO-d_6) δ: 166.6, 159.6, 152.6, 147.5, 145.5, 138.2, 135.5, 130.9, 130.0, 129.7, 129.6, 129.0, 128.3, 127.8, 126.6, 122.5, 116.1, 114.9, 102.7, 52.4, 34.7, 24.1.

LC-MS t_R 5.42 min, m/z 475, 473.

HRMS calc'd for $C_{26}H_{22}ClN_4O_3$ 473.1375, found 473.1375.

1-(6-chloro-1H-1,3-benzodiazol-2-yl)-4-[2-(4-fluorophenyl)ethyl]-3-[4-(trifluoromethyl)phenyl]-1H-pyrazol-5-ol (FC-7868)



A mixture of β-ketoester **5g** (318 mg, 0.86 mmol), hydrazine **6c** (225 mg, 1.23 mmol), $TsOH.H_2O$ (cat. qty.) and MeOH (4 mL) was heated in the microwave at 130 °C for 3 h. The mixture was diluted with EtOAc (100 mL), washed with 5% aq HCl (10 mL), satd aq $NaHCO_3$ (10 mL) and brine (10 mL), and dried over Na_2SO_4. Removal of the solvent left an oil (500 mg). Prep HPLC gave 1-(6-chloro-1H-1,3-benzodiazol-2-yl)-4-[2-(4-fluorophenyl)ethyl]-3-[4-(trifluoromethyl)phenyl]-1H-pyrazol-5-ol TFA salt (250 mg, 33%) as a white solid.

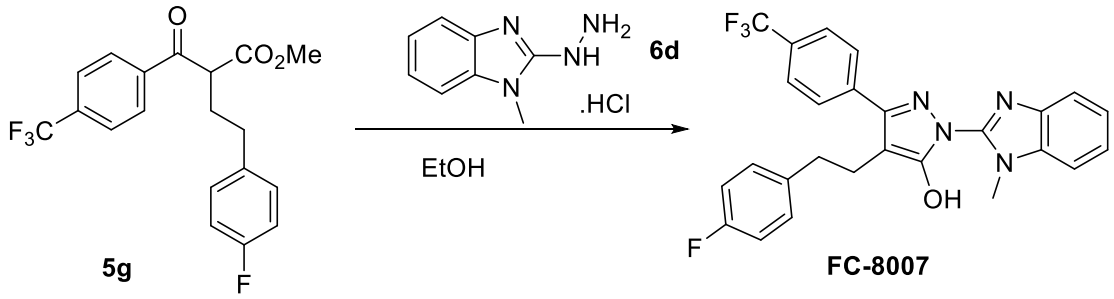
^{1}H NMR (METHANOL-d_4) δ: 8.35 (dd, J=5.3, 1.4 Hz, 1H), 8.05-8.12 (m, 1H), 7.65-7.80 (m, 4H), 7.38 (dd, J=8.0, 5.3 Hz, 1H), 7.06 (dd, J=8.6, 5.4 Hz, 2H), 6.79-6.91 (m, 2H), 2.78-2.94 (m, 5H)

^{19}F NMR (METHANOL-d_4) δ: -64.22, -77.52, 119.56.

LC-MS t_R 6.15 min, m/z 503, 501.

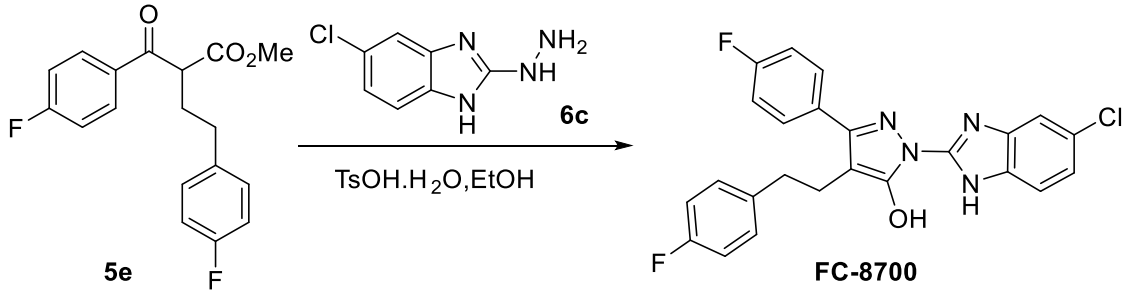
HRMS calc'd for $C_{25}H_{18}ClF_4N_4O$ 501.1100, found 501.1083.

4-[2-(4-fluorophenyl)ethyl]-1-(1-methyl-1H-1,3-benzodiazol-2-yl)-3-[4-(trifluoromethyl)phenyl]-1H-pyrazol-5-ol (FC-8007)



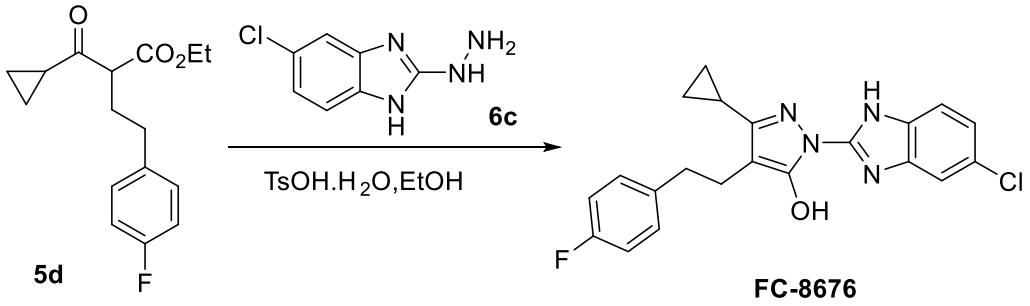
A mixture of β-ketoester **5g** (331 mg, 0.90 mmol), hydrazine **6d** HCl salt (119 mg, 0.60 mmol) and MeOH (2 mL) was stirred at 40 °C for 3 d. The mixture was diluted with EtOAc (100 mL), washed with 5% aq HCl (10 mL), sat'd aq $NaHCO_3$ (10 mL) and brine (10 mL), and dried over Na_2SO_4. Removal of the solvent left an oil (360 mg) which was purified by prep HPLC to give the title compound TFA salt (160 mg, 55%) as a white solid.
^{1}H NMR (CD_3OD) δ: 7.73 (d, J=3.3 Hz, 6H), 7.39-7.53 (m, 2H), 7.05 (dd, J=8.5, 5.5 Hz, 2H), 6.81-6.92 (m, 2H), 4.04 (s, 3H), 2.86-2.97 (m, 2H), 2.82 (d, J=7.0 Hz, 2H)
^{19}F NMR (METHANOL-d_4) δ: -64.24, -77.57, -119.62.
LC-MS t_R 6.63 min, m/z 481.
HRMS calc'd for $C_{26}H_{21}F_4N_4O$ 481.1646, found 481.1646.

1-(5-chloro-1H-1,3-benzodiazol-2-yl)-3-(4-fluorophenyl)-4-[2-(4-fluorophenyl)ethyl]-1H-pyrazol-5-ol (FC-8700)



A mixture of β-ketoester **5e** (79 mg, 0.25 mmol), hydrazine **6c** (45 mg, 0.25 mmol), $TsOH.H_2O$ (cat. qty.) and EtOH (2 mL) was heated in the microwave at 130 °C for 3 h. Prep HPLC afforded FC-8700 as its TFA salt (28 mg) as a white solid.
^{1}H NMR (METHANOL-d_4) δ: 7.50-7.64 (m, 4H), 7.30 (dd, J=8.7, 1.9 Hz, 1H), 7.19 (t, J=8.6 Hz, 2H), 7.01-7.11 (m, 2H), 6.82-6.93 (m, 2H), 2.72-2.88 (m, 4H).
^{19}F NMR (METHANOL-d_4) δ: -77.46, -113.07, -119.58.
LC-MS t_R 5.95 min, m/z 451.
HRMS calc'd for $C_{24}H_{18}ClF_2N_4O$ 451.1132, found 451.1139.

1-(5-chloro-1H-1,3-benzodiazol-2-yl)-3-cyclopropyl-4-[2-(4-fluorophenyl)ethyl]-1H-pyrazol-5-ol (FC-8676)



A mixture of β-ketoester **5d** (100 mg, 0.36 mmol), hydrazine **6c** (66 mg, 0.36 mmol), $TsOH.H_2O$ (cat. qty.) and EtOH (3 mL) was heated in the microwave at 130 °C for 3 h. The mixture was diluted with EtOAc (100 mL), washed with 5% aq HCl (10 mL), sat'd aq $NaHCO_3$ (10 mL) and brine (10 mL), and dried over Na_2SO_4. Removal of the solvent left an oil (180 mg) which was purified by prep HPLC to provide the title compound TFA salt (100 mg, 55%) as a white solid.

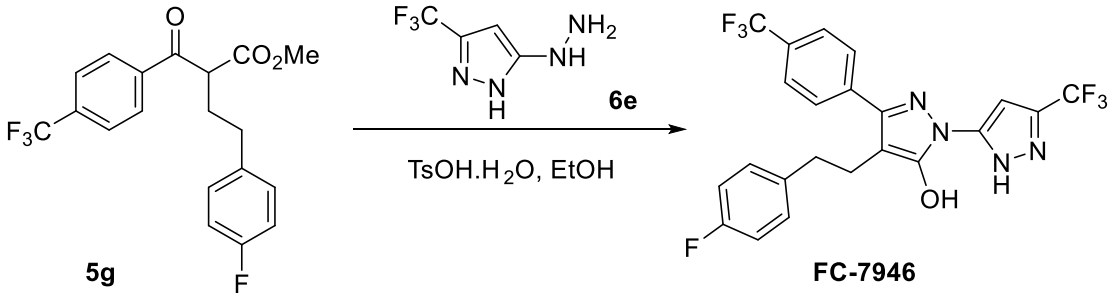
^{1}H NMR (CD_3OD) δ: 7.61 (d, J=1.9 Hz, 1H), 7.56 (d, J=8.6 Hz, 1H), 7.34 (dd, J=8.6, 1.9 Hz, 1H), 7.20 (dd, J=8.5, 5.5 Hz, 2H), 6.92-7.02 (m, 2H), 2.83-2.92 (m, 2H), 2.75 (s, 2H), 1.67-1.78 (m, 1H), 0.87-1.00 (m, 4H)

^{19}F NMR (METHANOL-d_4) δ: -77.52, -119.70.

LC-MS t_R 5.07 min, 399, 397.

HRMS calc'd for $C_{21}H_{19}ClFN_4O$ 397.1226, found 397.1222

4-[2-(4-fluorophenyl)ethyl]-5'-(trifluoromethyl)-3-[4-(trifluoromethyl)phenyl]-2'H-[1,3'-bipyrazol]-5-ol (FC-7946)



A mixture of β-ketoester **5g** (350 mg, 0.92 mmol), hydrazine **6e** (148 mg, 0.89 mmol), $TsOH.H_2O$ (cat. qty.) and EtOH (4 mL) was heated in the microwave at 130 °C for 1 h. Prep HPLC afforded the title compound (350 mg, % yield) as an off-white solid.

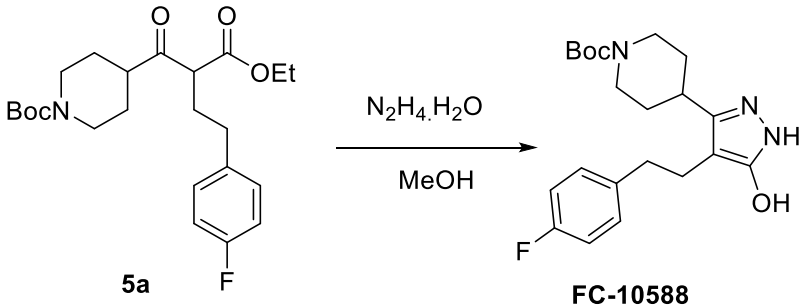
^{1}H NMR (METHANOL-d_4) δ: 7.60-7.73 (m,4H), 6.97-7.06 (m,2H), 6.80-6.90 (m,3H), 2.81-2.90 (m, 2H), 2.77 (d, J=6.6 Hz, 2H).

LC-MS t_R 5.88 min, m/z 485.

HRMS calc'd for $C_{22}H_{16}F_7N_4O$ 485.1207, found 485.1201.

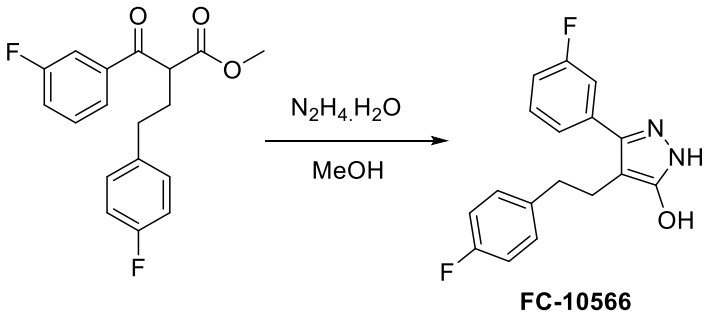
Method D

tert-butyl 4-{4-[2-(4-fluorophenyl)ethyl]-5-hydroxy-1H-pyrazol-3-yl}piperidine-1-carboxylate (FC-10558)



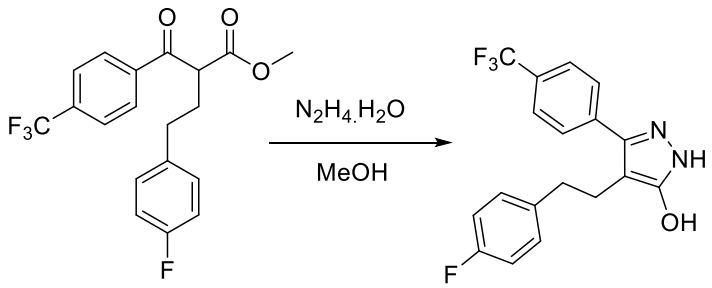
To a stirred solution of ~80% pure β-ketoester **5a** (93 mg, 0.22 mmol), prepared by Method A, in MeOH (2 mL) was treated with hydrazine hydrate (0.015 mL, 0.31 mmol). The mixture was stirred at 80 °C for 15 h. Prep HPLC gave the title compound (57 mg, 66%) as a white solid.
^{1}H NMR (METHANOL-d_4) δ: 7.09-7.19 (m, 2H), 6.99 (br dd, J=9.1, 4.1 Hz, 2H), 4.09 (br d, J=13.3 Hz, 2H), 2.79 (d, J=6.2 Hz, 2H), 2.58-2.76 (m, 4H), 2.50 (br t, J=7.7 Hz, 1H), 1.43-1.47 (s, 9H), 1.34-1.43 (m, 4H).
^{19}F NMR (METHANOL-d_4) δ: -119.30.
LC-MS t_R 4.27 min, m/z 390, 334, 290.
HRMS calc'd for $C_{21}H_{29}FN_3O_3$ 390.2187, found 390.2175

3-(3-fluorophenyl)-4-[2-(4-fluorophenyl)ethyl]-1H-pyrazol-5-ol (FC-10566)



To a stirred solution of methyl 2-(3-fluorobenzoyl)-4-(4-fluorophenyl)butanoate (105 mg, 0.33 mmol), prepared by Method B, in MeOH (2 mL) was added hydrazine hydrate (0.018 mL, 0.37 mmol). The mixture was stirred at 80 °C for 2 d. The mixture was concentrated and the residue was chromatographed on a 12 g silica cartridge, eluted with a 0-10% MeOH in CH_2Cl_2 gradient to give a white solid (53 mg) which was further purified by prep HPLC to give the title compound (45 mg, 45%) as a white solid.
^{1}H NMR (METHANOL-d_4) δ: 7.45 (td, J=8.1, 5.9 Hz, 1H), 7.09-7.23 (m, 2H), 6.95-7.06 (m, 3H), 6.81-6.93 (m, 2H), 2.71-2.87 (m, 4H).
^{19}F NMR (METHANOL-d_4) δ: -114.04, -119.63.
LC-MS t_R 4.30 min, m/z 301.
HRMS calc'd for $C_{17}H_{15}F_2N_2O$ 301.1147, found 301.1145.

4-[2-(4-fluorophenyl)ethyl]-3-[4-(trifluoromethyl)phenyl]-1H-pyrazol-5-ol (FC-10567)



5g **FC-10567**

A solution of β-ketoester **5g** (96 mg, 0.26 mmol) in MeOH (2 mL) was treated with hydrazine hydrate (0.02 mL, 0.42 mmol). The mixture was stirred at 40 °C for 3 d and purified by preparative HPLC to give the title compound (62 mg, 68%) as a white solid.

^{1}H NMR (METHANOL-d_4) δ: 7.72 (d, J=8.1 Hz, 2H), 7.43-7.50 (m, 2H), 6.93-7.02 (m, 2H), 6.77-6.87 (m, 2H), 2.72-2.88 (m, 4H).

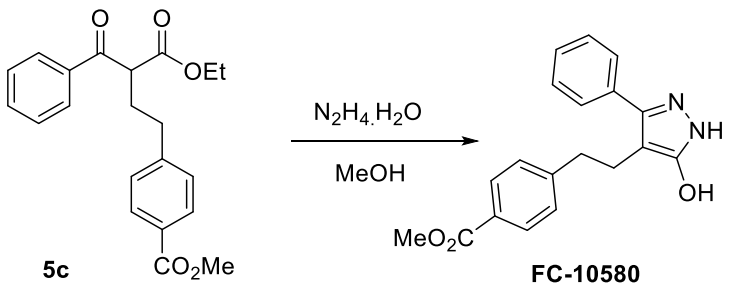
^{19}F NMR (METHANOL-d_4) δ: -64.34, -77.48, -119.64 (t, J=8.9 Hz, 1F).

^{13}C NMR (DMSO-d_6) δ: 161.1 (d, J = 241 Hz), 159.5, 140.5, 137.8, 134.7, 130.4, 128.8 (q, J = 33 Hz), 128.0, 126.0, 124.5 (q, J = 272 Hz) 115.1, 101.8, 34.8, 24.4. Peaks due to TFA not reported.

LC-MS t_R 4.73 min, m/z 351.

HRMS calc'd for $C_{18}H_{15}F_4N_2O$ 351.1115, found 351.1114.

Methyl 4-[2-(5-hydroxy-3-phenyl-1H-pyrazol-4-yl)ethyl]benzoate (FC-10580)



5c **FC-10580**

To a stirred solution of β-ketoester **5c** (112 mg, 0.32 mmol) in MeOH (3 mL) was added hydrazine hydrate (0.015 mL, 0.32 mmol). The mixture was stirred at 40 °C for 6 d and purified by prep HPLC to give the title compound (25 mg, 25%) as a white solid.

^{1}H NMR (METHANOL-d_4) δ: 7.80 (d, J=8.4 Hz, 2H), 7.38-7.47 (m, 3H), 7.27-7.34 (m, 2H), 7.12 (d, J=8.2 Hz, 2H), 3.87 (s, 3H), 2.86 (s, 4H).

^{13}C NMR (DMSO-d_6) δ: 166.6, 160.4, 148.0, 140.6, 131.2, 129.7, 129.6, 129.2, 129.0, 127.7, 127.3, 100.5, 52.4, 35.8, 24.2.

LC-MS t_R 3.98 min, m/z 323.

HRMS calc'd for $C_{19}H_{19}N_2O_3$ 323.139, found 323.1378.

Surface plasmon resonance (SPR). Recombinant, full-length HIV-1 Nef protein (NL4-3 allele) was expressed in soluble form in *E. coli* and purified as described elsewhere.[1] SPR analysis was performed on a Reichert 4SPR instrument (Reichert Technologies) using carboxymethyl dextran hydrogel biosensor chips (Reichert #13206066). Recombinant purified Nef was covalently attached to the chip surface via standard amine coupling chemistry[2]. Compounds were prepared in phosphate-buffered saline (PBS) plus 1% DMSO and flowed past the immobilized Nef protein and a reference channel at a flow rate of 50 µL/min for 90 s over a range of concentrations. The binding reaction was followed by dissociation for 180 s, followed by surface regeneration using 5 mM NaOH at a flow rate of 50 µL/min for 30 sec. All sensorgrams were recorded in triplicate at each concentration, corrected for buffer effects, and fitted with either the 1:1 Langmuir binding model or the two-state induced-fit model using the TraceDrawer software (Reichert). The integrity of the Nef protein on the chip was verified at the beginning and end of each analysis by confirming interaction with the recombinant SH3 domain of the Src-family kinase, Hck.

HIV-1 infectivity assays. HIV-1 stocks were produced in 293T cells transfected with a proviral clone of HIV-1_{NL4-3} using the XtremeGENE 9 transfection reagent (Sigma Aldrich). Viral supernatants were collected 72 h post-transfection and amplified in the T cell line MT2 as described elsewhere [3]. Viral titers were quantified by HIV-1 p24 AlphaLISA assay (PerkinElmer, #AL291F) according to the manufacturer's protocol. HIV-1 infectivity was measured using the TZM-bl reporter cell line[4,5] (NIH AIDS Research and Reference Reagent Program) in which the HIV-1 LTR is linked to the expression of firefly luciferase. TZM-bl cells (2.5 x 10^4/well in 96-well plates) were allowed to adhere overnight prior to infection. Cells and HIV-1_{NL4-3} (9000 pg p24/ml) were incubated separately with compounds for 3 h and then combined to a final volume of 200 µl in each well. Following incubation for 48 h at 37 °C, the cells were lysed in 50 µl luciferase cell culture lysis reagent (Promega). Lysates (40 µl) were then transferred to white 96-well plates followed by 50 µl injections of luciferase reagent per well (Promega). Luminescence was then recorded with a delay time of 2 s and an integration period of 10 s. The cytotoxicity of each compound was independently evaluated in in uninfected TZM-bl cells using the CellTiter-Blue cell viability assay (Promega). Each experiment included at three wells infected with wild type or ΔNef HIV-1 in the absence of compounds, and the difference in the luciferase activity between them defined the assay window in terms of Nef-dependent enhancement of HIV-1 replication. All compounds were assayed in triplicate at a final concentration of 1.0 µM and suppression of infectivity was calculated relative to the wild- type and ΔNef controls.

Isolation of donor peripheral blood mononuclear cells and replication assays. Donor PBMCs were isolated from buffy coats using Ficoll-Paque PLUS (GE Healthcare) and activated with 1 µg/mL PHA (Sigma, #L1668) and 50 U/mL IL-2 (BD Pharminogen, #CB-40043B) for 3 days. PBMCs were infected with HIV-1 for 4 days in the presence or absence of test compounds using DMSO as the carrier solvent (0.1% final concentration). Viral replication was measured by AlphaLISA assay (PerkinElmer) for HIV-1 p24 in the culture supernatants.

Cell-based assays for Nef-mediated activation of Itk and Hck. The coding regions for full-length Itk and Hck were amplified by PCR and fused in-frame with a V5 epitope tag followed by the C-terminal coding fragment of the Venus protein (Venus residues Ala154 to Lys238) at their C termini as described[6]. A complementary HIV-1 Nef (B clade; SF2 allele) expression constructs fused the N-terminal coding fragment of the Venus protein (residues Val-2 to Asp-173) to the Nef C-terminus as described[6]. The resulting PCR products were subcloned into the mammalian expression vector, pCDNA3.1(-) (ThermoFisher/Invitrogen).

Human embryonic kidney 293T cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products). Primary antibodies for this assay were obtained from ThermoFisher (V5 tag mouse monoclonal, #R960-25), Sigma (V5 tag rabbit polyclonal, #AB3792), Santa Cruz (pTyr antibody pY99, #sc-7020), Millipore (mouse anti-pSrc-pY416 clone 9A6, #05-677) and the NIH AIDS Reagent Program (HIV-1 Nef Monoclonal 6.2, #1539). Secondary antibodies were obtained from Southern Biotech (goat anti-rabbit IgG mouse, #4050-07; goat anti-mouse IgG, #1031-07; goat anti-mouse IgG, #1031-04), and ThermoFisher/Molecular Probes (Pacific Blue goat anti-mouse IgG, #P31582; Pacific Blue goat anti-rabbit IgG, #P10994).

For the assay, 293T cells were plated in 35 mm microwell dishes (MatTek, # P35G-1.5-14-C) and cultured overnight. Cells were then transfected with expression vectors using X-tremeGENE 9 DNA transfection reagent. For inhibitor studies, cells were treated with Nef inhibitors or the DMSO carrier solvent (0.1% final concentration) four hours post-transfection. Forty hours later, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 2% BSA in PBS overnight. Cells were immunostained with anti-V5, anti-pTyr, or anti-pY416 (all antibodies

diluted 1:1000 in PBS with 2% BSA) for 1 h at room temperature. Cells were washed and stained with secondary antibodies conjugated to Texas Red or Pacific Blue at dilutions of 1:500 and 1:1000, respectively. Immunostained images, along with the BiFC (Nef-kinase interaction signal) were acquired using confocal microscopy (Fluoview FV1000, Olympus) with a 60 X objective using x-y scan mode. Single-cell image analysis was performed with the Java-based image processing program, ImageJ. Immunofluorescence intensities with each antibody were measured for at least 100 cells, and single-cell data were calculated as the fluorescence ratio of kinase activity (pTyr for Itk or Hck pY416) to kinase protein (Itk or Hck) signals.

MHC-I downregulation assay. Nef-dependent downregulation of cell-surface MHC-I was assayed using the CEM-SS T cell line, which has been engineered to over-express an HLA-A*02 allele.[7] CEM-SS cells (1.5×10^6) were transfected by electroporation with the pSelect-GFPzeo expression plasmid which expresses GFP as a gating marker as well as HIV-1 Nef (B-clade, SF2 allele). Immediately after transfection, cells were treated with test compounds using DMSO as a carrier solvent (0.1% final concentration). Forty-eight hours later, cells were harvested and immunostained for cell-surface HLA-A*02 for 1 hour at room temperature and analyzed by flow cytometry.

References

1. Moroco, J. A.; Alvarado, J. J.; Staudt, R. P.; Wales, T. E.; Smithgall, T. E.; Engen, J. R. (2018) Remodeling of HIV-1 Nef Structure by Src-Family Kinase Binding. *J. Mol. Biol. 430*, 310-321.

2. Grover, P.; Shi, H.; Baumgartner, M.; Camacho, C. J.; Smithgall, T. E. (2015) Fluorescence Polarization Screening Assays for Small Molecule Allosteric Modulators of ABL Kinase Function. *PLoS One* **,** *10*, e0133590.

3. Narute, P. S.; Smithgall, T. E. (2012) Nef alleles from all major HIV-1 clades activate Src-family kinases and enhance HIV-1 replication in an inhibitor-sensitive manner. *PLoS. One. 7*, e32561.

4. Platt, E. J.; Wehrly, K.; Kuhmann, S. E.; Chesebro, B.; Kabat, D. (1998) Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J. Virol. 72*, 2855-2864.

5. Derdeyn, C. A.; Decker, J. M.; Sfakianos, J. N.; Wu, X.; O'Brien, W. A.; Ratner, L.; Kappes, J. C.; Shaw, G. M.; Hunter, E. (2000) Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. *J. Virol. 74*, 8358-8367.

6. Poe, J. A.; Smithgall, T. E. (2009) HIV-1 Nef dimerization is required for Nef-mediated receptor downregulation and viral replication. *J. Mol. Biol. 394*, 329-342.

7. Anmole, G.; Kuang, X. T.; Toyoda, M.; Martin, E.; Shahid, A.; Le, A. Q.; Markle, T.; Baraki, B.; Jones, R. B.; Ostrowski, M. A.; Ueno, T.; Brumme, Z. L.; Brockman, M. A. (2015) A robust and scalable TCR-based reporter cell assay to measure HIV-1 Nef-mediated T cell immune evasion. *J Immunol. Methods* **,** *426*, 104-113.