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Supplemental Information

A Strain-Specific Inhibitor

of Receptor-Bound HIV-1

Targets a Pocket near the Fusion Peptide

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Fig. S1. Cryo-EM maps and models of detergent-bound HIV Env in complex with receptor. Related to figure 1. (A) Fourier shell correlation (*top*) and distribution plot of particle orientations (*bottom*) and (B) local resolution estimates (colored by Å) of C1 reconstruction of B41-CD4-17b. (C) Fourier shell correlation (*top*) and distribution plot of particle orientations (*bottom*) and (D) local resolution estimates (colored by Å) of C3 reconstruction of B41-CD4-17b. (E) Density and modeled atoms for DDM in three protomers of the asymmetric reconstruction of B41-CD4-17b.



Fig. S2. Selection of small molecule candidates by *in silico* screening and differential scanning fluorimetry. Related to figure 2. Plot of scores obtained in the C1 asymmetric structure (y-axis) against scores obtained in the C3 symmetric structure (x-axis). Each of the three panels corresponds to a different FP conformation as represented in Fig. 1A: (A) gp41^B, (B) gp41^E and (C) gp41^K. (D) Top 5 candidates based on a selection criteria of a ΔT_m value equal or greater than $\pm 1.0^{\circ}$ C against at least 2 different Env genotypes, in the presence or absence of sCD4. (E) Negative-stain 2D class averages of CZA97 SOSIP+sCD4 complexed with the top 5 candidates in (D) reveals that changes in stability are not due to trimer dissociation/denaturation. (F) Fourier shell correlation (*top*) and distribution plot of particle orientations (*bottom*) and (G) local resolution estimates (colored by Å) of C3 reconstruction of B41-CD4-17b frozen with LMNG. (H) Comparison of fusion peptides from B41-CD4-17b frozen with either DDM (asymmetric model) or LMNG. (I) Comparison of EM map (contoured at 1.5 σ) near the ligand binding pocket of B41-CD4-17b frozen with either DDM (green) or LMNG (blue).



Fig. S3. Targeting the DDM-pocket of gp41 with small molecules. Related to figure 3. (A) Change in T_m of B41 SOSIP in the presence of various detergents. (B) Comparison of DDM and LMNG structures. (C) Global Fourier shell correlation (FSC), (D) side view of the final 3D reconstruction colored by local resolution estimates (in Å), and (E) distribution plot of particle orientations of B41+CD4+17b+GO35. (F) Select 2D class averages of B41+CD4+17b+GO35.



Fig. S4. Cytotoxicity and neutralization assay profiles of small molecules. Related to figure 4. (A) Cytotoxicity measurements of GO35 and GO52. Assays performed as duplicates. Mean values plotted with standard deviation represented by vertical bars. (B) Neutralization activity of 80 small molecules against BG505 N332 HIV-1 and A-MLV viruses. All molecules tested at 30 μ M final concentration. Assay performed as duplicates (N=2) and mean value is plotted. BMS-626529 and T20 (enfuvirtide) are known HIV-1 fusion inhibitors included as controls.



Fig. S5. Cryo-EM reconstructions of B41+CD4+17b+GO52. Related to figure 5. (A) Select 2D class averages of B41+CD4+17b+GO52. Global Fourier shell correlations (FSC; *left*), local resolution estimates (colored by resolution in Å; *middle*) and distribution plot of particle orientations (*right*), of the (**B**) C3 symmetric and (**C**) asymmetric reconstructions of B41+CD4+17b+GO52. (**D**) Superimposition of the 3 asymmetric gp41 chains of (**C**) with a focus on the FP and FPPR regions. (**E**) Lysine (dark blue) and arginine (light blue) residues within 10 Å of gp41 HR1 region 548-562. (**F**) Comparison of P43 (gp120) interactions with the FP in the three asymmetric protomers of (**C**).



B41+CD4+17b DDM (C3) EMD-20151 (3.3 Å) PDB 6opo (chain B)



B41+CD4+17b DDM (C1) EMD-20152 (3.6 Å) PDB 6opp (chain B)



B41+CD4+17b DDM (C1) EMD-20152 (3.6 Å) PDB 6opp (chain E)



B41+CD4+17b DDM (C1) EMD-20152 (3.6 Å) PDB 6opp (chain K)



BG505+CD4+E51 Class1 (C1) EMD-20605 (3.3 Å) PDB 6u0l (chain X)



BG505+CD4+E51 Class1 (C1) EMD-20605 (3.3 Å) PDB 6u0l (chain Y)



BG505+CD4+E51 Class1 (C1) EMD-20605 (3.3 Å) PDB 6u0l (chain Z)



BG505+CD4+E51 Class2 (C1) EMD-20608 (3.5 Å) PDB 6u0n (chain X)



BG505+CD4+E51 Class2 (C1) EMD-20608 (3.5 Å) PDB 6u0n (chain Y)



BG505+CD4+E51 Class2 (C1) EMD-20608 (3.5 Å) PDB 6u0n (chain Z)

Fig. S6. The B41 binding FPPR is more ordered than in BG505. **Related to figure 6.** Comparison of CD4- and DDM-bound C3-symmetric and asymmetric B41 cryo-EM maps with asymmetric reconstructions of CD4-bound BG505, with special focus on the FPPR (denoted by a white arrow).



Fig. S7. A schematic of iterative drug design using a combination of cryo-EM, virtual screening, and assays. Related to figures 1, 2, 3, 4, and 5. Created with BioRender.com.

Table S1. Difference in thermal stability of CD4-bound SOSIP trimers in the presence of small molecules relative to no small molecule controls as measured by differential scanning fluorimetry. Related to figures 2 and 3. Absent values marked with a star denote that the small molecule had a high level of intrinsic fluorescence that interfered with the method.

Small	ZINC ID	AMC011	BG505+	B41+	CZA97+	DU422+	JRFL+	Small	ZINC ID	AMC	BG505+	B41	CZ	DU 42	JR FI
ule		+CD4	CD4	CD4	CD4	CD4	CD4	ule		+CD4	CD4	CD4	A9/ +C	42	FL +
													D4	CD 4	CD 4
GO1	ZINC0018 5349	-0.1	0.1	-0.4	-0.1	0.6	0	GO31	ZINC6745 9760	-0.4	-0.4	-0.3	-0.1	0.3	0
GO2	ZINC0195 0659	-0.1	-0.1	-0.3	-0.5	0.6	-0.2	GO32	ZINC7730 0595	*	*	*	*	*	*
GO3	ZINC0023 5832	-0.1	0	-0.3	-0.3	0.7	-0.3	GO33	ZINC6749 1033	0.1	-0.1	-0.1	0.1	0	0
GO4	ZINC0029 1882	-0.1	0.2	-0.3	-0.3	1	-0.2	GO34	ZINC9724 4092	-0.5	-0.4	-1.1	-1	1.2	- 0.8
GO5	ZINC0023 3220	-0.1	-0.1	-0.3	-0.3	1.1	-0.1	GO35	ZINC9613 2694	-0.6	-1.1	-3.1	-1.5	- 1.1	- 1.9
GO6	ZINC0190 7589	0.1	0.1	-0.3	0	1.1	-0.2	GO36	ZINC9132 5610	-0.4	-0.4	-0.7	-0.8	0.3	- 0.8
G07	ZINC0399 8067	0	0.1	-0.3	-0.2	0.6	-0.2	GO37	ZINC6767 5494	0	0.2	-0.4	-0.6	0.8	- 0.2
GO8	ZINC0025 8817	0	0.1	-0.5	0	0.4	-0.2	GO38	ZINC2078 2685	0	-0.5	-0.7	-0.6	- 0.4	- 0.5
GO9	ZINC0003 5601	0.2	0	0	-0.1	0	-0.2	GO39	ZINC6539 4371	-0.2	-0.1	-0.2	0	- 0.2	- 0.1
GO10	ZINC1236 2428	0.1	0.1	-0.2	-0.1	0.4	-0.1	GO40	ZINC6539 4381	*	*	*	*	*	*
G011	ZINC0012 3118	0.2	0	-0.4	-0.1	0.5	0	GO41	ZINC5515 3733	0.2	0	-0.9	-0.6	0.8	- 0.4
GO12	ZINC1824 7429	0.1	0.1	-0.2	-0.2	0.5	0.2	GO42	ZINC7174 5481	-0.6	-0.7	-1.1	-0.4	- 0.6	- 0.7
GO13	ZINC0007 4920	0.1	-0.1	-0.2	-0.1	0.5	0.1	GO43	ZINC9536 6696	0.1	0.2	-0.4	-0.4	1.4	0.2
GO14	ZINC0056 9594	-0.1	0.1	-0.2	-0.4	1.3	-0.2	GO44	ZINC7738 6563	*	*	*	*	*	*
GO15	ZINC0046 1274	-0.3	-0.1	-0.2	-0.1	-0.3	-0.1	GO45	ZINC6780 3542	0	0.1	-0.3	-0.4	1.1	- 0.2
GO16	ZINC0046 2885	0.1	0.2	-0.2	-0.1	0.4	0	GO46	ZINC7738 9895	-0.3	-0.4	-1	-0.6	0.3	- 0.7
GO17	ZINC0046 3378	*	*	*	*	*	*	GO47	ZINC9614 9848	*	*	*	*	*	*
GO18	ZINC0046 9391	-0.2	-0.1	0.1	0.1	0.2	-0.1	GO48	ZINC5525 2643	-0.2	0.1	-0.5	-0.5	0.1	- 0.2
GO19	ZINC0426 3843	0.8	-0.2	-0.2	0.1	1	-0.2	GO49	ZINC6542 2611	-0.1	0	-0.1	-0.2	- 0.3	- 0.1
GO20	ZINC0505 9895	-1.3	-0.9	-0.7	-1.2	-1.3	-0.8	GO50	ZINC1497 4795	-0.1	-0.1	-0.7	-0.6	0.1	0.2
GO21	ZINC0062 1192	0.2	0	-0.1	0	0.8	-0.4	GO51	ZINC6545 5118	0.1	0.4	-0.6	0	0.2	- 0.1
GO22	ZINC0506 1043	0	-0.2	-0.5	-0.2	1.1	0	GO52	ZINC9768 1845	-0.3	0	-0.4	-0.7	1.3	- 0.2
GO23	ZINC0424 7959	0.1	0.2	-0.1	-4.3	2.4	0	GO53	ZINC9181 9525	-0.1	-0.2	-0.8	-0.6	0.8	- 0.5
GO24	ZINC0054 1458	0.2	0.2	-0.3	-0.1	1.1	0.2	GO54	ZINC6784 3155	-0.4	-0.3	-0.6	-0.2	- 0.2	- 0.3
GO25	ZINC0142 8810	0.2	0.2	-0.5	-0.1	0.8	0.1	GO55	ZINC9776 6602	-0.2	-0.2	-0.1	-0.5	0	- 0.3
GO26	ZINC1046 1636	0.2	0.3	-0.1	-0.4	2	0.2	GO56	ZINC7217 1577	-0.4	-0.3	-0.5	-0.4	- 0.2	- 1.8
GO27	ZINC0676 7131	0.3	0.2	-0.2	-0.2	2.1	-0.1	GO57	ZINC6797 5161	-0.7	-1	-1	-1.2	-2	- 1.6
GO28	ZINC3635 1253	*	*	*	*	*	*	GO58	ZINC6797 5538	*	*	*	*	*	*
GO29	ZINC3635 8921	0.2	0.1	-0.1	-0.1	1.9	0	GO59	ZINC7217 3974	-0.1	-0.4	-0.6	-0.7	- 0.2	- 0.4
GO30	ZINC7728 6437	*	*	*	*	*	*								