Toremifene interacts with and destabilizes the Ebola virus glycoprotein

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Ebola viruses (EBOVs) are responsible for repeated outbreaks of fatal infections, including the recent deadly epidemic in West Africa. There are currently no approved therapeutic drugs or vaccines for the disease. EBOV has a membrane envelope decorated by trimers of a glycoprotein (GP, cleaved by furin to form GP1 and GP2 subunits), which is solely responsible for host cell attachment, endosomal entry and membrane fusion1–7. GP is thus a primary target for the development of antiviral drugs. Here we report the first, to our knowledge, unliganded structure of EBOV GP, and high-resolution complexes of GP with the anticancer drug toremifene and the painkiller ibuprofen. The high-resolution apo structure gives a more complete and accurate picture of the molecule, and allows conformational changes introduced by antibody and receptor binding to be deciphered8–10. Unexpectedly, both toremifene and ibuprofen bind in a cavity between the attachment (GP1) and fusion (GP2) subunits at the entrance to a large tunnel that links with equivalent tunnels from the other monomers of the trimer at the three-fold axis. Protein–drug interactions with both GP1 and GP2 are predominately hydrophobic. Residues lining the binding site are highly conserved among filoviruses except Marburg virus (MARV), suggesting that MARV may not bind these drugs. Thermal shift assays show up to a 14 °C decrease in the protein melting temperature after toremifene binding, while ibuprofen has only a marginal effect and is a less potent inhibitor. These results suggest that inhibitor binding destabilizes GP and triggers premature release of GP2, thereby preventing fusion between the viral and endosome membranes. Thus, these complex structures reveal the mechanism of inhibition and may guide the development of more powerful anti-EBOV drugs.

The recent outbreak of EBOV in West Africa, the worst of more than 30 in the past 40 years, comprised more than 28,000 cases and over 11,000 deaths11. In the urgent need to find therapeutics, many small compounds and existing Food and Drug Administration (FDA)-approved drugs have been screened in vitro or in silico (for example, ibuprofen was suggested by docking experiments12) to find lead compounds for drug development or repurpose drugs for the treatment of EBOV disease9–11. Among these, a set of selective estrogen receptor modulators (SERMs) stand out as potential inhibitors from in vitro and in vivo studies13; however, their mechanism of action remains largely unknown. Using recombinant EBOV GP we tested whether nine such compounds could directly bind by a thermal-shift assay (Methods). The results show that toremifene in particular markedly decreases the melting temperature (Tm) of EBOV GP, by up to 14 °C at 100 μM (Fig. 1). This contrasts with the action of inhibitors on most protein targets, which tend to increase stability14, although destabilization has been reported before15. Benztrapine16, the G-protein–coupled receptor (GPCR) antagonist, also decreases the Tm of GP by 4 °C, while other compounds, including ibuprofen, showed Tm shifts of less than 2 °C (Fig. 1, Extended Data Fig. 1). The destabilization effects of toremifene and ibuprofen are both pH and concentration dependent (Fig. 1). The binding constants (Kd values) determined by this assay are 16 μM for toremifene and 6 mM for ibuprofen (Extended

Figure 1 | Summary of thermal-shift assays. a, The effects of toremifene and ibuprofen on the melting temperature of EBOV GP at different pHs. The raw fluorescence traces are shown in Extended Data Fig. 1. The protein melting temperature at pH 5.2 at which the crystals were grown is taken as the reference point. b, The melting temperatures of EBOV GP at different concentrations of toremifene or ibuprofen, at pH 5.2. Data are mean ± s.d. (n = 3).

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Data Fig. 1). In a mouse model, toremifene appears to be even more potent (half-maximum inhibitory concentration (IC50) ~1 μM).

The crystal structure of unliganded EBOV GP was determined at 2.2 Å resolution, with good R-factors and stereochemistry (Extended Data Table 1). Three copies each of the GP1 and GP2 subunits (Fig. 2a) form the biological trimer around the crystallographic three-fold axis (Fig. 2b, c). This structure, although crystallized at pH 5.2, represents the pre-fusion state of the molecule, with the GP1 receptor-binding site blocked by a glycan cap (Fig. 2e). GP1 is predominantly composed of β-strands, forming a large semi-circular groove at the centre of the subunit that clamps the α3 helix and β19–β20 strands of GP2 (Fig. 2d). The glycan cap is removed in the late endosome by cathepsin B/L to expose the receptor Niemann–Pick disease type C1 (NPC1)-binding site9,20,21. GP2 catalyses membrane fusion and contains N-terminal (α11, and β1) heptad repeats linked by a CX6CC motif (residues 601–609, Fig. 2a). The C-terminal heptad repeat, disordered in all previously published GP structures8–10, contributes to the trimer interface in our structure (Fig. 2b, c) and contains N618, which is glycosylated as predicted. The well-ordered CX6CC motif forms intrasubunit (C601–C608), and intersubunit (C53–C609) disulfide bonds (Fig. 3). Mutation of any of these cysteine residues renders the virus incapable of entering host cells22. In the fusion process, GP2 undergoes conformational changes in which α5 refolds onto a helix coalesced from α3 and α4 to form a six-helix bundle23 (Extended Data Fig. 2). In our pre-fusion structure, the hydrophobic GP2 fusion loop (residues 511–554) (Fig. 2a) projects into a neighbouring monomer and is stabilized in a shallow depression surrounded by loops β34–β35 and β10–β11, and α3. Apart from residues 521–526, which have very loose interactions with the rest of the protein, the fusion loop in this pH 5.2 apo GP is very similar to that in the KZ52 Fab complex crystallized at pH 8.3 (Extended Data Fig. 3). This is in contrast to the large conformational changes reported for the isolated fusion loop at different pHs (ref. 24), suggesting that GP1 maintains GP2 in the pre-fusion state until their separation triggered by receptor binding.

In total, 319 out of 394 Cα atoms in our apo GP structure match with the GP–Fab complex8, with a root mean squared deviation (r.m.s.d.) value of 1.1 Å (Fig. 3a–d), however, there are marked differences, beyond the C-terminal heptad repeat and CX6CC motif. The β1–β2 hairpin interacts directly with the KZ52 Fab and is pushed 6 Å inwards in the Fab complex (Fig. 3c). The glycan cap is better ordered in the apo structure, revealing an extra strand, β18′, inserted between β17 and β18, overlapping β18 in the Fab complex but running in the opposite direction (Fig. 3b). Several cross-reactive neutralizing monoclonal antibodies from EBOV survivors bind to the cap25, suggesting that this conserved epitope is important for antibody-mediated clearance of the virus. Our structure defines this conformational epitope.

In the apo GP structure (excluding the glycan cap), 230 Cα atoms overlay with the GP in the NPC1 receptor complex8, with an r.m.s.d. of 0.9 Å (Fig. 3e). NPC1 binding draws helix α1 approximately 2 Å towards the receptor, causing the preceding 310-helix α1′ to unwind, disrupting interactions with α3 of GP2, as described previously9 (Fig. 3f). These structural changes also break hydrogen bonds from α1′ to the amide groups of residues K510 and N512, disordering the N-terminal residues 502–507 of GP2. The structural differences continue to the other side of α3. In the NPC1-bound structure, the α3 helix starts two residues earlier and the β1–β2 hairpin bends inwards, adopting a conformation similar to that in the KZ52 Fab complex (Fig. 3c, g). In addition, there is a large tunnel between neighbouring monomers (Fig. 4, Extended Data Fig. 4), whose hydrophobic entrance is formed by surrounding residues from the β1–β2 hairpin, β3, β6 and β13 of GP1, and α3, β19 and β20 of GP2. Residues 192–195 (with sequence DFFS, and named DFF lid thereafter) form a tight turn with F193 and F194 plugging the entrance of the tunnel and making tight
obtained by crystal soaking and refined to 2.7 Å resolution (Extended Data Fig. 6). Perhaps preventing premature release of GP2 (Extended Data Fig. 6).

The N563 glycan (which is resistant to enzymatic deglycosylation), interactions. Both α1′ and α1 are shielded by residues 287–293 and the N563 glycan (which is resistant to enzymatic deglycosylation), perhaps preventing premature release of GP2 (Extended Data Fig. 6).

Structures of GP–toremifene and GP–ibuprofen complexes were obtained by crystal soaking and refined to 2.7 Å resolution (Extended Data Table 1). Both inhibitors have well-defined electron density and bind at the same site at the entrance of the large tunnel by expelling the DFF lid (Fig. 4 and Extended Data Figs 4, 5, 7). In addition to the tunnel entrance residues already mentioned, the tunnel is lined by residues from the N-terminal loop, the β1–β2 hairpin, β2–β3 loop of GP1, and α3 and α4 of GP2 from a neighbouring monomer, and interconnected with the other two tunnels in the trimer at the three-fold axis (Fig. 4b and Extended Data Fig. 4). Y517 makes dominant interactions with toremifene by contacting all three phenyl rings (Fig. 4c).

In addition, phenyl ring A of toremifene is fully buried and interacts with V66, L68, L515 and L558, ring B with L186, and ring C with V66 and A101. The ethyl chloride group interacts with L184, L186, M548 and L558, while the dimethylethanamine group points towards the main tunnel and is surrounded by polar/charged residues, including R64, E100, T519, T520 and D522 (Fig. 4c).

Ibuprofen is bound with its isobutyl group partially overlapping the ethyl chloride group of the toremifene but closer to L554. However, its phenyl ring does not overlap any of the rings of toremifene (Extended Data Fig. 5), but makes extensive interactions with M548 (Fig. 4d). The propanoic acid moiety is orientated to make a hydrogen bond to the side chain of R64 and hydrophobic interactions with Y517. Remarkably, ibuprofen was initially suggested to interact with EBOV GP by in silico screening, and predicted to dock in a pocket of the mucin domain12. A racemic mixture of ibuprofen was used for all experiments, however, we note that the S-isomer (which is also active as a painkiller) binds preferentially.

The flexible region, 521–526, of the fusion loop is stabilized in the two inhibitor-bound structures, but in different conformations compared to apo GP. The most notable conformation changes induced by toremifene are at side chains of M548 and L554, and M548 by ibuprofen (Fig. 4). The residues involved in inhibitor binding are highly conserved across filoviruses, with the exception of MARV (Extended Data Fig. 8), where the DFF lid and its preceding loop are replaced by a helix, and V66 and A101 are substituted by M50 and E85, respectively, partially blocking the binding site10.

The SERMs tamoxifen, 4-hydroxytamoxifen and clomiphene are less potent inhibitors, despite their chemical similarity to toremifene14,15. Compared to the ethyl chloride group of toremifene, the corresponding ethyl group in tamoxifen and chlorine in clomiphene are expected to make weaker interactions with L184, L186, M548 and L558. A partially bound 4-hydroxytamoxifen structure obtained by crystal soaking
(data not shown) shows the hydroxyl group makes close contacts with G67, shifting the whole inhibitor ~1.0 Å towards the solvent, weakening ring-stacking interactions with Y517 and having no contacts from the ethyl group to L184, L186 and L558 compared to toremifene. Our crystallographic results are in line with the inhibition data\textsuperscript{14–16} and our thermal-shift assay (Extended Data Fig. 1c, f). If toremifene and ibuprofen inhibit viral infection by causing premature conversion of GP to the post-fusion conformation or blocking receptor binding, we would expect them to abolish viral fusion. This was confirmed by measuring their effect on the fusion of EBOV GP pseudovirions as judged by a β-lactamase reporter assay (Extended Data Fig. 9). Benzotriphen, which decreases the \( T_m \) of GP by 4 °C, could not be soaked in our crystals, and needs further investigation (Extended Data Fig. 1f).

Our results pinpoint the binding site of toremifene and ibuprofen on the surface of the GP and reveal that they decrease the stability of the viral GP, and prevent viral fusion. The binding site is different to that predicted for ibuprofen\textsuperscript{12}, and the information on the binding modes of these compounds and the spare volume at the binding cavity can guide the design of more potent compounds. Finally, our readily grown well-diffracting crystals are suitable for fragment-based screening for different classes of binders for the development of new inhibitors to combat EBOV infection.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions Y.Z., J.R. and D.I.S designed the project. Y.Z. made the protein and grew the crystals together with J.R., collected X-ray data and determined the structures. K.H. helped with crystal mounting and data collection. D.M.J. and S.P. carried out cell imaging experiments. A.Z. and T.A.B. provided the cDNA. Y.Z., J.R., E.E.F. and D.I.S. analysed the results and wrote the manuscript in discussions with all authors.

Author Information The atomic coordinates and structure factors have been deposited with the RCSB Protein Data Bank under accession codes 5JQ3 (native GP), 5JQ7 (GP-toremifene) and 5JQ8 (GP-ibuprofen). Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to D.I.S. (dawe@strubi.ox.ac.uk).

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METHODS
No statistical methods were used to predetermine sample size. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

Protein cloning, expression and purification. Zaire EBOV (strain Mayinga-76) glycoprotein extracellular domain DNA was synthesized (UniProt entry KB-Q65320). The expression construct GPΔ contains two directly linked sections, amino acids 32–312 and 464–632, with a T42A mutation to eliminate N40 glycosylation. At the N terminus of the protein, the four amino acids ETGR were added from the expression vector pNecess37. At the C terminus, a foldon trimerization sequence from the bacteriophage T4 fibritin and a 6× His tag were added with the sequence: GSGYIPEAPRDGQAYVRKDGEWVLLSTFLGTHHHHHH. The endotoxin free pNecess-GPΔ plasmid was transiently transfected into the human embryonic kidney HEK293T (ATCC CRL11268) cells with polyethylenimine (PEI, molecular mass 25 kDa, Sigma). To inhibit the formation of complex glycosylation, the manniosidase inhibitor kifunensine (Cayman Chemical) was added to a final concentration of 51 μM. After 5 days of transfection, the conditioned media was collected, dialysed against PBS and incubated with talon beads (Takara Bio Europe SAS) at 15 °C for 1 h with gentle shaking. The beads were collected and washed with PBS plus 5–10 mM imidazole. The protein was eluted with 200 mM imidazole in PBS and further purified by size exclusion chromatography with a Superdex 200 HiLoad 16/600 column (GE Healthcare) and a buffer of 10 mM MES, pH 5.2, 150 mM NaCl.

Thermal-shift assay. 10 μl of solution containing 2 μM glycosylated EBOV GP protein, buffered by the addition of 10 μl of 850 mM sodium malonate at the desired pH, was mixed with 10 μl of 15 × SYPRO Orange dye (Thermo Fisher Scientific), along with 10 μl of 501 μM compound in 10% DMSO or just 10% DMSO. The mixture was made up to a total volume of 50 μl. Samples were placed in a semi skirted 96-well PCR plate (4 Titide), sealed and heated in an MS300Sp pQPCR machine (Stratagene, Agilent Technologies) from 24.5 to 98.5 °C at a rate of 1 °C min−1. Fluorescence changes were monitored with excitation and emission wavelengths of 485 ± 20 nm and 525 ± 20 nm, respectively. The data were collected and converted to percentage thermal stability T50 using the method of Greer et al.26

Data collection and structure determination. Data collection and structure determination were carried out using synchrotron radiation at the SRS Daresbury (U1371–U1372 (2005)). The crystals belong to space group P212121 with unit cell dimensions a = b = 114.0 Å and c = 307.0 Å approximately. The crystals were used for data collection and structure determination by molecular replacement using MOLREP37 using the GP structure of the GP-KZ52 Fab complex (PDB ID, 3CSY) as a search model. There is one GP molecule in the crystal asymmetric unit. The biological trimer is formed by a crystallographic three-fold axis. Structure refinement used REFMAC38 and models were rebuilt with COOT39. The apo structure was refined to 2.23 Å resolution with an Rfree value of 0.223 (Rfree = 0.251) and good stereochemistry. Close examination of the data from inhibitor-soaked crystals showed that only ternary complex and ibuprofen were fully bound with GP and structures were refined to resolutions of 2.69 Å and 2.68 Å, respectively. 4-hydroxytamoxifen was only bound with partial occupancy (data not shown). Data collection and structure refinement statistics are given in Extended Data Table 1. Structural comparisons used SHAP40, figures were prepared with PyMOL41.

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Extended Data Figure 1 | Thermal-shift essay. Representative thermal melt curves of EBOV GP with 10 μM compounds and 2% DMSO. 

a–d, Melting curves of EBOV GP with toremifene, ibuprofen or protein alone at pH 5.0, 6.0, 7.0 and 8.0, respectively. e, Small effects of SERM inhibitors tamoxifen, 4-hydroxytamoxifen and raloxifen on the melting temperature of EBOV GP shown at pH 5.2. f, Melt curves of EBOV GP with diacylglycerol kinase inhibitor, anastrozole and benztropine mesylate at pH 5.2. g, h, Shifts in melting temperature (ΔTm °C in absolute value) were plotted against different concentrations of toremifene (g) or ibuprofen (h) at pH 5.2. Data are mean ± s.d. (n = 4). The affinity constant Kd is calculated by a ligand binding 1:1 saturation fitting with the SigmaPlot version 13 (Systat Software Inc.).
Extended Data Figure 2 | Structural organization of EBOV GP and GP2 structure. a, Scheme showing the structural organization of EBOV GP. FL, fusion loop; NHR and CHR, N- and C-terminal heptad repeats; SP, signal peptide; TM, transmembrane helix. The GPΔ construct used for structure determination is made by deleting residues 313–463 of the GP mucin domain and residues 633–676. Residue 312 is directly linked to 464.

b, The GP2 trimer in the prefusion state (current structure). The trimer is shown as cartoon representation with the monomers coloured in red, green and blue, respectively. Disulfide bonds are shown as orange sticks.

c, The six-helix bundle of GP2 in the post-fusion state.

A foldon trimerization peptide and a 6×His tag are added at the C terminus. b, The GP2 trimer in the prefusion state (current structure). The trimer is shown as cartoon representation with the monomers coloured in red, green and blue, respectively. Disulfide bonds are shown as orange sticks. c, The six-helix bundle of GP2 in the post-fusion state.
Extended Data Figure 3 | The fusion loop. a, The fusion loop that connects β19 and β20 of GP2 projects onto a shallow depression on the surface of a neighbouring monomer. The fusion loop is shown as a red coil with side chains drawn as grey sticks, the neighbouring monomer is shown in semi-transparent surface representation. b, Comparison of the fusion loop in the apo GP (red and grey) obtained at pH 5.2 with that in the KZ52 Fab complex (cyan) obtained at pH 8.3.
Extended Data Figure 4 | Pockets and tunnels in EBOV GP trimer.
a, The several small pockets and three large tunnels in the GP trimer shown as grey surfaces. Protein backbones are drawn as ribbons and coloured as in Fig. 2 of the main text. A toremifene is bound at the entrance of each large tunnel and shown as yellow sticks. b, Close up view of a tunnel. Each tunnel is bordered by secondary structure elements from two neighbouring monomers.
Extended Data Figure 5 | The inhibitor-binding site. a, The DFF lid (residues 192–194, blue coil for main chain and sticks for side chains) nestles at the entrance of the large tunnel in the apo structure. The rest of the protein is shown as an electrostatic surface. The putative cathepsin cleavage site at residue 190 is indicated by an arrow. b, c, Toremifene (yellow sticks in b) and ibuprofen (cyan sticks in c) bind at the same site by expelling the DFF lid. In both panels, the inhibitor bound structure is shown in blue (GP1) and red (GP2), the apo GP in grey. d, Comparing the binding modes of toremifene and ibuprofen. The toremifene-bound structure is shown in blue and red, the ibuprofen bound structure in grey.
Extended Data Figure 6 | The environment of $\alpha_1'$ and $\alpha_1$ helices. The surfaces of $\alpha_1'$ and $\alpha_1$ helices, which undergo large conformational changes upon receptor binding, are protected by the 287–293 loop from the glycan cap domain and the N563 glycan from GP2 in the apo GP. The glycan is modelled as Man9GlcNAc2.
Extended Data Figure 7 | Chemical structures and electron density maps. a, b, The chemical structures of toremifene (a) and ibuprofen (b). c, d, |F_o − F_c| omit electron density maps for toremifene (c) and ibuprofen (d) contoured at 3σ.
Extended Data Figure 8 | Sequence alignment of filovirus GPs. Amino acid sequence alignment of 7 filovirus GPs around the inhibitor-binding site. The amino acids that form contacts with toremifene or ibuprofen are coloured in green. Numbering corresponds to the full length Zaire EBOV GP, conserved residues are shown in a red background. Secondary structure elements are labelled on the top.
Extended Data Figure 9 | Toremifene and ibuprofen inhibit fusion of Ebola GP pseudovirus particles. a, CCF2-loaded TZM-bl cells were exposed to EBOV pseudoparticles (EBOVpp) or control particles lacking envelope proteins (NoEnv) at 4 °C to synchronise binding and receptor engagement before fusion was initiated by shifting cells to 37 °C in the presence of toremifene (15μM and 1.5μM), ibuprofen (150μM and 15μM), or just the solvent (5% DMSO). After 2 h incubation, cells were loaded with the CCF2-AM FRET biosensor, fixed and the ratio of blue (440–480 nm, cleaved CCF2-AM) to green (500–540 nm, uncleaved CCF2-AM) fluorescence measured. Cells are pseudocoloured according to this ratio: blue represents no fusion, red represents fusion. Scale bar: 80 μm. b, The percentage of fusogenic cells (red versus blue) was calculated taking the average max value coming from the negative control as a threshold for fusion, data are means ± s.d. (n = 10). *P ≤ 0.05, ***P ≤ 0.001 (unpaired t-test, compared to the EBOV plus DMSO control). ns, not significant (P > 0.05). Error bars represent s.d.
## Extended Data Table 1 | Data collection and refinement statistics

|                      | Native GP       | GP-toremifene   | GP-ibuprofen   |
|----------------------|-----------------|-----------------|----------------|
| **Data collection**  |                 |                 |                |
| Space group          |                 | R32             |                |
| Cell dimensions      |                 |                 |                |
| \(a, b, c\) (Å)      | 114.3, 114.3, 307.4 | 113.5, 113.5, 306.9 | 113.8, 113.8, 306.2 |
| \(\alpha, \beta, \gamma\) (°) | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 |
| Resolution (Å)       | 94.2–2.23 (2.29–2.23)* | 51.2–2.69 (2.76–2.69) | 82.8–2.68 (2.75–2.68) |
| \(R_{merge}\)        | 0.204 (---)     | 0.079 (---)     | 0.143 (---)    |
| \(I / \sigma I\)     | 17.4 (1.3)      | 20.0 (1.9)      | 14.7 (1.5)     |
| Completeness (%)     | 100 (100)       | 99.9 (100)      | 99.9 (100)     |
| Redundancy           | 57.8 (15.4)     | 9.8 (8.6)       | 9.8 (8.3)      |
| **Refinement**       |                 |                 |                |
| Resolution (Å)       | 94.2–2.23       | 51.2–2.68       | 82.8–2.68      |
| No. reflections      | 36035/1865      | 20449/1090      | 20734/1107     |
| \(R_{work} / R_{free}\) | 0.226/0.241 | 0.201/0.245 | 0.199/0.235 |
| No. atoms            |                 |                 |                |
| Protein              | 3129            | 3034            | 3025           |
| Ligand/glycan/ion    | 143             | 151             | 140            |
| Water                | 119             | 53              | 73             |
| \(B\)-factors        |                 |                 |                |
| Protein              | 39              | 62              | 55             |
| Ligand/glycan/ion    | 99              | 115             | 96             |
| Water                | 54              | 70              | 55             |
| R.m.s. deviations    |                 |                 |                |
| Bond lengths (Å)     | 0.007           | 0.007           | 0.007          |
| Bond angles (°)      | 1.3             | 1.3             | 1.3            |

*Values in parentheses are for highest-resolution shell.