Protection against filovirus diseases by a novel broad-spectrum nucleoside analogue BCX4430

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Filoviruses are emerging pathogens and causative agents of viral haemorrhagic fever. Case fatality rates of filovirus disease outbreaks are among the highest reported for any human pathogen, exceeding 90% (ref. 1). Licensed therapeutic or vaccine products are not available to treat filovirus diseases. Candidate therapeutics previously shown to be efficacious in non-human primate disease models are based on virus-specific designs and have limited broad-spectrum antiviral potential. Here we show that BCX4430, a novel synthetic adenine analogue, inhibits infection of distinct filoviruses in human cells. Biochemical, reporter-based and primer-extension assays indicate that BCX4430 inhibits viral RNA polymerase function, acting as a non-obligate RNA chain terminator. Post-exposure intramuscular administration of BCX4430 protects against Ebola virus and Marburg virus disease in rodent models. Most importantly, BCX4430 completely protects cynomolgus macaques from Marburg virus infection when administered as late as 48 hours after infection. In addition, BCX4430 exhibits broad-spectrum antiviral activity against numerous viruses, including bunyaviruses, arenaviruses, paramyxoviruses, coronaviruses and flaviruses. This is the first report, to our knowledge, of non-human primate protection from filovirus disease by a synthetic drug-like small molecule. We provide additional pharmacological characterizations supporting the potential development of BCX4430 as a countermeasure against human filovirus diseases and other viral diseases representing major public health threats.

Members of the family Filoviridae include Ebola virus (EBOV), Marburg virus (MARV), Ravn virus (RAVV), Sudan virus (SUDV) and Bundibugyo virus (BDBV), all of which cause severe viral haemorrhagic fevers in humans. In nature, filoviruses are transmitted by physical contact between infected individuals, presumably via infected bodily fluids. Initial filovirus disease manifestations include fever, headache, vomiting and diarrhoea. Fatal cases are characterized by viraemia, elevated liver-associated enzyme levels, coagulopathy and haemorrhage. Filovirus disease outbreaks occur sporadically, most frequently in sub-Saharan Africa, with reported case fatality rates exceeding 90% (ref. 1). In 2012, simultaneous outbreaks involved MARV, SUDV and BDBV, all of which cause severe viral haemorrhagic fevers in humans. Nucleic-acid-based products, antibody therapies and therapeutic vaccines have successfully protected non-human primates from filovirus disease, but these approaches rely on virus-specific designs that inherently limit the spectrum of activity and potential utility of individual treatments. The development of a single therapeutic agent active against multiple filoviruses would provide a key, cost-effective component of public-health preparedness plans in outbreak-prone regions.

The broad-spectrum antiviral agent ribavirin, a trizole nucleoside effective against multiple pathogenic RNA viruses, is not active against filoviruses. Other small molecules—including the adenosine analogue 3-deazaneplanocin A (c5-Npc A) and T-705 (favipiravir), a substituted pyrazine compound—have conferred a high degree of protection against filoviruses in rodents but have not been reported to protect non-human primates.

BCX4430, a novel nucleoside analogue (Fig. 1a), was synthesized (Supplementary Information) as part of a small-molecule library designed as inhibitors of viral RNA polymerase activity. BCX4430 is designed to inhibit viral RNA polymerase activity indirectly through non-obligate RNA chain termination, a mechanism requiring anabolism of the parent compound to BCX4430-triphosphate (BCX4430-TP). Then, after pyrophosphate cleavage, incorporation of BCX4430-monophosphate (BCX4430-MP) into nascent viral RNA strands would be expected to cause premature termination of transcription and replication of viral RNA. In support of this proposed mechanism, BCX4430 is rapidly phosphorylated to BCX4430-TP in cultured cell lines and primary hepatocytes, similar to the natural adenosine nucleoside (Fig. 1b and Extended Data Fig. 1a). Addition of BCX4430 reduces expression of green fluorescent protein (GFP) in an artificial EBOV minigenome replication assay (Fig. 1c and Extended Data Fig. 1b), in which viroin structural proteins comprising the viral ribonucleoprotein complex mediate transcription and replication of an RNA replicon template containing a GFP-reporter cassette. BCX4430-TP inhibits hepatitis C virus (HCV) RNA polymerase transcriptional activity in a cell-free, isolated enzyme transcription assay (Fig. 1d) (an isolated filovirus RNA polymerase enzyme assay has yet to be reported) and induces premature termination of RNA chain synthesis by HCV RNA polymerase during template-directed primer-extension assays (Fig. 1e and Extended Data Fig. 1c). In virus-infected cells, BCX4430 reduces surface-expressed MARV and EBOV glycoprotein and reduces the production of intracellular and extracellular MARV RNA (Fig. 1f–h and Extended Data Fig. 1d). Additionally, HeLa cells incubated with ≥25 μM BCX4430 produce no detectable infectious MARV virus (concentration providing 90% inhibition (IC90) = 5.4 μM) (Fig. 1b).

Taken together, these assessments strongly support our hypothesis that BCX4430 inhibits viral RNA polymerase function by inducing RNA chain termination. Findings from primer-extension reactions suggest that termination occurs two bases after incorporation of BCX4430-MP, perhaps as a result of inhibitory stereochemical distortions of the nascent RNA chain. We observed no evidence of BCX4430-MP incorporation into human RNA or DNA on exposing human HuH-7 cells to concentrations of BCX4430 exceeding the MARV IC50 by more than tenfold (Extended Data Fig. 1f). The basis of the selectivity of BCX4430 for viral polymerases is not yet known.

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No L-polymerase encoding plasmid omitted from reaction. Replication of an EBOV minigenome RNA replicon in cultured cells (Figure 1).

To assess the spectrum of antiviral activity of BCX4430, we conducted additional high-content image (HCI)-based and neutral-red uptake antiviral assays using an array of other human viruses. BCX4430 exhibited antiviral activity against negative-sense RNA viruses representing the Filoviridae, Arenaviridae, Bunyaviridae, Orthomyxoviridae, Paramyxoviridae families, and positive-sense RNA viruses of the Flaviviridae and Coronaviridae families (Table 1). Antiviral effects were specific and did not result from cellular toxicity or anti-proliferative effects, as assessed by enumeration of live cells during all HCI assays and by the lactate dehydrogenase (LDH) release assay. In HeLa cells, the 50% cytotoxic concentration (CC50) of BCX4430 exceeded 500 μM as determined by the LDH assay, and consistently exceeded 100 μM using the HCI-based assay (Table 1). An antiviral effect was confirmed in infected human macrophages cultured from normal peripheral blood monocytes, using EBOV (Extended Data Fig. 1e). It is important to note that immortalized cell lines exhibit a different response to antiviral compounds, and expression of BCX4430 that could limit its utility in vivo during experimental or clinical infections.

In preparation for in vivo pharmacokinetics and efficacy testing, we sought to further characterize potential adverse pharmacological features of BCX4430 that could limit its utility in vivo or clinically. BCX4430 exhibited no mutagenicity when tested using the Ames assay at 5 mg per plate, produced no detectable chromosomal aberrations in human lymphocytes at a concentration of 787 μM, and did not inhibit the hERG ion channel current at 30 μM (data not shown). The compound is metabolically stable (half-life \((t_{1/2}) > 54\) min) when incubated with S9 liver fractions obtained from various animal species (Extended Data Table 1).

**Table 1 | BCX4430 antiviral activity**

| Virus family | Virus | Strain/variant | EC50 (μM) | EC90 (μM) | CC50 (μM) |
|--------------|-------|----------------|----------|----------|----------|
| Filoviridae  | MARV  | Musoke*         | 4.4      | 10.5     | 242      |
|              | MARV  | C67*           | 6.7      | 16.1     | 255      |
|              | MARV  | Angola*        | 5.0      | 12.3     | 242      |
|              | EBOV  | Kikwit*        | 11.8     | 25.4     | 100      |
|              | SUDV  | Boniface*      | 3.4      | 10.3     | >100     |
|              | EEEV  | SH3*           | >100     | >100     | >100     |
|              | EEEV  | FL93-939t      | >100     | >100     | >100     |
|              | WEEV  | Californiat*   | 21.3     | >30      | >100     |
|              | CHIKV | AF 15561*      | >100     | >100     | >100     |
| Bunyaviridae | RVFV  | ZH5018*        | 41.6     | 98.0     | >100     |
|              | LACV  | Wisconsin 1960t| 13.4     | 65.0     | >100     |
|              | MPRLV | HV97021050t    | 40.1     | 95.0     | >250     |
|              | ARENAVIRIDAE | LASV Josiah* | 43.0     | >100     | >100     |
|              | JUNV  | Romero*        | 42.2     | >100     | >100     |
|              | Paramyxoviridae | NIV Malaysia* | 41.9     | >100     | >100     |
|              | RSV   | 2305t         | 11.0     | 25.7     | >89      |
|              | MeV   | Chicago*       | 6.19     | 34.4     | >296     |
| Coronaviridae| MERS-CoV | Jordan N3* | 68.4     | >90      | >100     |
|              | SARS-CoV | Urbani*   | 57.7     | >95      | >500     |
|              | Orthomyxoviridae | Influenza | pH1N1t | 10.7     | 17.0     | >296     |
|              | Picornaviridae | HRV2 HGp* | 3.4      | 45.2     | >296     |
|              | Flaviviridae | YFV 17D* | 14.1     | 46.8     | >100     |
|              | JEV   | SA14t         | 43.6     | 93.4     | >100     |
|              | DENV2 | New Guinea Ct | 32.8     | 89.3     | >296     |

EC50, 50% effective concentration; EC90, 90% effective concentration. Definitions for virus abbreviations are provided in Methods.

* Antiviral activity assessed by high-content image analysis.

**Figure 1 | Pharmacological characterization of BCX4430.** a, BCX4430 chemical structure and properties. b, Conversion of \(^{3}H\)-BCX4430 or \(^{3}H\)-adenosine to diphosphate (DP) or triphosphate (TP) forms in Huh-7 cells (n = 1). c, Effect of BCX4430 on template-directed primer (\(^{5}P\)-\(^{3}G\)-G) extension assay. nt, nucleotides. d, Replication of an EBOV minigenome RNA replicon in cultured cells (n = 6). e, Effect of BCX4430 on replication of an EBOV minigenome RNA replicon in cultured cells (n = 6).

In mouse, rat, guinea pig and cynomolgus macaque, BCX4430 pharmacokinetics were characterized by rapid clearance from the plasma with a half-life of <5 min (Fig. 2b, Extended Data Fig. 4c and Extended Data Table 1).
Additionally, BCX4430 ameliorated haemorrhagic disease manifestations, and animals were administered with 15 mg kg$^{-1}$ Cynomolgus macaques were experimentally infected with a lethal dose of 150 mg kg$^{-1}$ (Fig. 3a, b). Notably, significant protection ($P < 0.05$) was also observed with doses of 150 mg kg$^{-1}$ initiated at times as long as 96 h after infection (Fig. 2a). Additionally, we demonstrated that mice administered BCX4430 either i.m. or orally were protected against lethal EBOV challenge and that mice receiving i.m. treatments were protected against lethal Rift Valley fever virus challenge (Extended Data Fig. 3c, d).

The in vivo post-exposure efficacy of BCX4430 was further verified in two guinea pig models of MARV disease in which animals were challenged either by intraperitoneal (i.p.) injection, or, because filoviruses can be transmitted via aerosolized particles, by exposure to aerosolized virus. In both the i.p. and aerosolized-virus challenge models, BCX4430 conferred significant post-exposure protection when treatment was initiated within 48 h of virus exposure (Extended Data Fig. 4a). In the respiratory exposure model, BCX4430 protected guinea pigs when treatment initiation was delayed to 72 h after infection, the longest delay tested (Extended Data Fig. 4b).

We further explored the post-exposure efficacy of BCX4430 using the cynomolgus macaque MARV disease model, which accurately reproduces filovirus disease manifestations observed in fatal human cases. Cynomolgus macaques were experimentally infected with a lethal dose of wild-type MARV (Musoke variant), derived from a human clinical isolate, and animals were administered with 15 mg kg$^{-1}$ BCX4430 twice daily by i.m. injection beginning 1–48 h after infection and continuing for 14 days. The six infection-control subjects succumbed by day 12 (Fig. 3a), after having developed viremia (Fig. 3b) and characteristic signs of filovirus disease, including behavioural inactivity, macular papular rash, increases in liver injury markers such as aspartate aminotransferase and bilirubin (Fig. 3c, d), and prolongations of prothrombin time (PT) and activated partial thromboplastin time (aPTT) (Fig. 3e, f). All animals treated with BCX4430 beginning 24 or 48 h after infection survived. Five out of six (83%) animals treated with BCX4430 beginning 1 h after infection survived (Fig. 3a). Consistent with the proposed antiviral mechanism of action, BCX4430 significantly and substantially reduced serum MARV burden (Fig. 3b), without inducing type I interferon responses (Extended Data Fig. 4d), as has been observed for c$^{-}$Npc A in mice. Additionally, BCX4430 ameliorated haemorrhagic disease manifestations, as evidenced by shorter PT and aPTT times and reduced laboratory indices of liver damage, such as serum AST and bilirubin concentrations, compared with vehicle treatment (Fig. 3c–f).

Outbreaks in 2012 involving MARV, SUDV and BDV reported in Uganda and the Democratic Republic of the Congo highlight the urgent need for development of an effective antiviral product to counter filovirus disease. For the first time, to our knowledge, we report the identification of a small molecule with efficacy against filovirus disease in non-human primates. Furthermore, we have provided evidence that BCX4430 exhibits broad-spectrum antiviral activity against other highly virulent RNA viruses (Table 1 and Extended Data Fig. 2). Additional evaluations are in progress to assess the in vivo efficacy of BCX4430 against EBOV and other highly virulent pathogens in non-human primates or other disease models that most closely recapitulate human disease.

BCX4430 was well tolerated, producing no overt signs of systemic toxicity or adverse local reactions in any of the efficacy studies. The substantial efficacy of BCX4430 observed with the i.m. route, which provides high bioavailability and rapid absorption, is conducive for use during outbreaks, potentially enabling administration by individuals lacking advanced medical training. In MARV-infected cynomolgus macaques,
we did not observe any diminution of protective effects with delay of BCX4430 treatment initiation up to 48 h after infection, suggesting that even greater delays of treatment, yet to be tested, may also yield significant protective benefit. In preparation for advancing BCX4430 into human phase I clinical trials in the United States, additional studies are in progress to support filing of an Investigational New Drug Application.

METHODS SUMMARY

BCX4430 was synthesized by BioCryst Pharmaceuticals. Cell-based infection assays were conducted using HCl-based analyses as described previously22 for filoviruses and Rift Valley fever virus. For other viruses, inhibition of virus-induced cytopathic effect (CPE) was assessed in cell-based assays using neutral-red uptake, as described previously22. Replication of EBOV minigenome RNA constructs was assayed by using a plasmid-based reconstituted replication/transcription system22. Inhibition of RNA transcriptional activity was assessed in an isolated HCV polymerase assay26 and inhibition of HCV polymerase RNA synthesis activity was evaluated using a template-driven 32P-GG primer extension assay. Mouse models of EBOV, MARV and RAVV disease have been described previously27,28. Experiments were conducted using mouse-adapted strains of EBOV (Mayinga variant) and RAVV (Ravn variant). A guinea pig MARV-Musoke parenteral challenge model and virus aerosolization methods used for guinea pig exposures have been previously described29,30. BCX4430 was administered in a vehicle of sterile 0.9% saline for injection for all in vivo applications. Animal infection experiments were performed in biosafety level 4 containment facilities at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID). All experimental treatment replication was conducted using biological replication, except for the virus-yard reduction assay (Fig. 1b), which relied on technical replication.

Research was conducted under an Institutional Animal Care and Use Committee approved protocol in compliance with the Animal Welfare Act, PHS Policy and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

Received 2 December 2012; accepted 13 January 2014.

Acknowledgements J. Kuhn and H. Juggins provided insightful discussions and critically reviewed the manuscript. R. Kincaid and G. Feuerstein provided advice and guidance for BCX4430 development efforts. These studies were in part supported by The Joint Science and Technology Office for Chemical and Biological Defense of the Defense Threat Reduction Agency (proposal #TMT10048_09_RD_T and CB3675) to S. B. Sawai. S. Radoszityski assisted with the EBOV minigenome replication assay. J. Reisman was essential to algorithm development of HCl image assessments. C. Basler provided the BHK-21-derived cell line constitutively expressing the T7 RNA polymerase. Plasmids encoding viral proteins and the EBOV minigenome replication vector were provided by P. Kranzusch and S. Whelan. Neutral-red uptake antiviral assays were conducted by: D. L. Barnard, G. W. Day, B. Gowan, J. G. Julander, B. Tarbet, D. F. Smee and J. D. Morrey of Utah State University under National Institute of Allergy and Infectious Diseases (NIAID) contract HHSN272201100019I. Cell-based assays were conducted by: D. L. Barnard, G. W. Day, B. Gowan, J. G. Julander, B. Tarbet, D. F. Smee and J. D. Morrey of Utah State University under National Institute of Allergy and Infectious Diseases (NIAID) contract HHSN272201100005I, and at the University of Alabama Birmingham under NIAID contract HHSN272201100016I. Cell-based metabolism studies were conducted by: C. Parker, X. Cheng, R. Upshaw and Y. Luo, A. Naica, E. E. Zumbren, H. B. Karpf, D. Dyer and J. Feagrr assisted with virus aerosolization. C. Cooper provided assistance with the culture of human macrophage cell culture and R. Zaman provided assistance with high-content image assessments. S. Tritsch assisted with Nipah virus antiviral assays. Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the US Army.

Author Contributions Y.S.B. and P.K. were responsible for the synthesis of BCX4430 and other small molecules. T.K.W. designed and supervised activities associated with rodent and non-human primate efficacy evaluations, evaluated study results, and wrote the manuscript. J.W., K.S.D., N.L.G. and S.A.V.T. conducted the rodent and non-human primate efficacy studies and cell-based sample analyses. R.G.P., G.P., C.J.R. and B.P.E. designed and executed cell-based filovirus assays and analysed these data. S. Banta, Y.S.B., D.M., W.P.S., B.R.T. and others designed and analysed data from cell-based antiviral assays. L.D. conducted quantitative PCR analysis. B.R.T. conducted statistical evaluations of in vivo study results. S.H. performed post-mortem analysis of all non-human primate subjects. Y.S.B. supervised the pharmacokinetics studies of BCX4430 and W.P.S. conducted pharmacokinetics data analysis. S. Banta conducted assessments of BCX4430 metabolite analysis and incorporation into host nucleic acids. X.C. conducted tissue and serum replicate experiments. J.K.W., D.M., L.S.W., B.R.T., Y.S.B., W.P.S. and S. Bava designed experiments, evaluated results and provided project oversight.

Author Information 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 S. Bavaire (sina.bawai.civ@mail.mil).

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Extended Data Figure 1 | Phosphorylation and antiviral mechanism of action of BCX4430. a, Conversion of BCX4430 to the active triphosphate (TP) form in cultured cell lines and fresh primary hepatocytes (n = 3–6). Right axis, values normalized to mean 24 h value for human hepatocytes. b, Expression of EBOV NP and VP35 in an EBOV minigenome RNA replicon assay, in BHK-21-derived cells (n = 6). Right three lanes, plasmids expressing the indicated viral protein were omitted from the transfection mix. Gel image cropped for clarity. c, RNA products synthesized by purified HCV polymerase, in a template-directed primer (32P-5'-GG) extension assay. d, Production of intra- and extracellular MARV RNA and cell-surface expression of viral glycoprotein in MARV-infected HeLa cells (n = 4) treated with BCX4430 either 18 h before infection, or 1, 12 or 24 h after infection. e, Expression of EBOV glycoprotein in monocyte-derived primary human macrophages (n = 4). f, Incorporation of 3H-BCX4430 (3H-4430) or 3H-adenosine (3H-AD) in human Huh-7 cells after 24 h incubation (n = 1). CPM, counts per min. Percentage inhibition assessed against the average of medium-only infection-control wells. Data in a are expressed as mean ± s.d. Data in d are expressed as mean ± s.e.m. Data in e are expressed as mean ± s.e.m.
Extended Data Figure 2 | Broad-spectrum antiviral activity of BCX4430. Antiviral activity was assessed in cell-based assays ($n = 3–5$; $n = 2$, MERS-CoV), either using high-content image-based analysis or neutral-red uptake, using cell lines described in Methods. Cells were pre-treated with BCX4430 for ~18 h before infection. Definitions of virus abbreviations are provided in Methods. Except for the top row, viruses are arranged in rows by taxonomic family. Percentage inhibition of BCX4430-treatment wells was assessed against the average of medium-only infection-control wells. Negative inhibition values were transformed to zero for curve fit analysis and display. Data are expressed as mean ± s.e.m.
Extended Data Figure 3 | Efficacy of BCX4430 in mouse disease models.

a, b, BCX4430 dose versus survival of RAVV-infected mice (a, n = 9–10). BCX4430 treatments (Tx) were administered for 9 days beginning 4 h before infection. Numbers in panel a indicate mg kg⁻¹ doses. c, Survival of mice (n = 10) infected with EBOV. BCX4430 was administered twice daily i.m. or orally at a dose of 150 mg kg⁻¹. d, Survival of mice (n = 10) infected with RVFV. BCX4430 was administered twice daily at doses of 5–150 mg kg⁻¹ by i.m. injection. *P < 0.05 for comparison of treatment versus vehicle survival curves by log-rank (Mantel–Cox) test.
Extended Data Figure 4 | In vivo activity of BCX4430 in guinea pigs and cynomolgus macaques. a, b, Survival of guinea pigs (n = 8 per group) infected by i.p. injection with MARV-Musoke (a) or by exposure to aerosolized MARV-Angola (b). BCX4430 (i.m., 50 mg kg$^{-1}$ twice daily) treatments (Tx) began at the indicated times before infection (BI) or post-infection (PI). c, Pharmacokinetics of BCX4430 in guinea pigs and cynomolgus macaques (n = 3) after single-dose i.m. administration. d, Individual animal maximal values of interferon-α2a in MARV-infected cynomolgus macaques. *P < 0.05 for comparison of treatment versus vehicle survival curves by log-rank (Mantel–Cox) test. Data in c are expressed as mean ± s.e.m. Horizontal bars in d represent group means.
### Extended Data Table 1  | In vitro metabolic stability of BCX4430 in liver S9 fractions

| Species | % Remaining of Initial (n=1) | 0 min | 10 min | 20 min | 30 min | 60 min | Half-life$^*$ (min) | CLint$^*$ (mL/min/mg protein) | Metabolites Detected$^d$ |
|---------|----------------------------|------|--------|--------|--------|--------|-------------------|-------------------------------|-----------------------------|
| Rat     | 100                        | 100  | 100    | 100    | 100    | 100    | > 60              | 0.0021                        | No                          |
| Dog     |                           | 100  | 100    | 100    | 100    | 100    | > 60              | 0.0092                        | No                          |
| Human   |                           | 100  | 100    | 100    | 100    | 100    | > 60              | 0.0128                        | No                          |
| Monkey  |                           | 100  | 100    | 100    | 100    | 100    | > 60              | 0.0050                        | No                          |
| Mouse   |                           | 100  | 100    | 100    | 100    | 100    | > 60              | 0.0084                        | No                          |

$^*$ Percentage remaining of test compound was calculated based on the peak area ratio of the test compound to the internal standard by LC-HRMS. Performance of positive biocontrol reagents testosterone and 7-hydroxycoumarin was assessed in parallel and met assay acceptability specifications.

$^*$ Half-life was calculated as \( t_{1/2} = \frac{0.693}{k} \), where \( k \) is the elimination rate constant in the equation describing first-order decay \( C_t = C_0 \times e^{-kt} \), and \( C_t \) and \( C_0 \) are the peak area ratios at time \( t \) and time 0, respectively. Data points were fitted to a first-order decay model by nonlinear regression using GraphPad Prism (version 5.04 or higher) without weighting or any user intervention.

$^d$ Intrinsic clearance (CLint) was calculated based on CLint = \( \frac{k \times P}{1} \), where \( k \) is the elimination rate constant and \( P \) is the protein concentration in the incubation.

Expected metabolite(s) detected.

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Extended Data Table 2  | BCX4430 and BCX4430-TP pharmacokinetics

| Species             | Dose (mg kg\(^{-1}\)) | Route | C\(_{\text{max}}\) ng mL\(^{-1}\) (plasma) | T\(_{\text{max}}\) | AUC ng\(\cdot\)hr mL\(^{-1}\) (plasma) | AUC ng\(\cdot\)hr g\(^{-1}\) (liver) | AUC adjusted for dose administered | T\(_{1/2a}\) |
|---------------------|------------------------|-------|---------------------------------------------|-----------------|-------------------------------------------|------------------------------------------|-----------------------------------|------------------|
| **Plasma PK parameters for BCX4430** |                       |       |                                             |                 |                                           |                                          |                                   |                  |
| Cynomolgus macaque  | 20                     | IM    | 22,333                                      | 5 min           | 13,109                                    | 655                                      | 10.1 min                         |                  |
| Guinea pig          | 50                     | IM    | 56,333                                      | 5 min           | 20,545                                    | 411                                      | 5.6 min                          |                  |
| Rat                 | 2                      | IV    | 6,890                                       | 5 min           | 1,070                                     | 535                                      | 1.9 min                          |                  |
| Mouse               | 2                      | IV    | 2,943                                       | 5 min           | 619                                       | 310                                      | 1.9 min                          |                  |
| **Liver PK parameters for BCX4430-TP** |                       |       |                                             |                 |                                           |                                          |                                   |                  |
| Rat                 | 30                     | IM    | 53,511                                      | 4 h             | 766,982                                   | 25,566                                   | 6.2 h                            |                  |
| Mouse               | 150                    | IM    | 320,695                                     | 8 h             | 4,566,000                                 | 30,373                                   | 4.3 h                            |                  |

Pharmacokinetics parameters were calculated from plasma BCX4430 and liver BCX4430-TP levels. Liver BCX4430-TP C\(_{\text{max}}\) and area under the curve (AUC) parameters are expressed in parent drug equivalents.