nature portfolio

Peer Review File

BWC0977, a broad-spectrum antibacterial clinical candidate to treat multidrug resistant infections

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

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1. Activity against ciprofloxacin-resistant isolates and against Klebsiella.
I can not find in Table 1 nor in the supplementary material details about activity against ciprofloxacin-resistant isolates. Please present a table of MICs of BWC0977 against ciprofloxacin-resistant and ciprofloxacin-susceptible isolates for the same species so the reader can judge whether ciprofloxacin resistance has any impact on BWC0977 MICs.
Klebsiella: Table 1 shows the MIC90 of BWC0977 against Klebsiella is 2 and Supp Table 3B shows Klebsiella MIC range up to 32. Please present data on mechanisms of resistance of Klebsiella and other Enterobacterales which have elevated MICs?

2. Phase I Clinical Trials - Page 6, final paragraph. Please clarify details which may be confusing to a reader. NCT05088421 is a completed single and multiple dose study according to clinicaltrials.gov. Therefore, since it has been completed, please describe the results of the multiple ascending dose part of this trial since the trial was both single and multiple ascending dose as described on clinicaltrials.gov. On clinicaltrials.gov it states that 44 of an expected 64 healthy volunteers were recruited. Please explain why the full cohort of 64 participants was not recruited. Also the CONSORT diagram provided by the authors shows 40 patients recruited which is also at variance with the 44 indicated on clinicaltrials.gov. NCT05942820 is listed on clinicaltrials.gov as a currently recruiting single and multiple dose study. Please explain in the text why another virtually identical Phase I trial is necessary.

3. Page 8. Discussion, 2nd last paragraph. Much of this is speculative and depends very much on the results of further clinical trials. Phrases such as "treatment of choice" are simply "spin" at this point. I would suggest deleting most of this material and replacing it with "The in vitro spectrum of activity of BWC0977 and results from the other preclinical studies presented here, are supportive of BWC0977 advancing into clinical trials for cUTI, HABP and VABP and complicated intra-abdominal infections. The spectrum of activity against pathogens of CF patients (B. cepacia, MRSA and P. aeruginosa), coupled with its high pulmonary drug levels also support trials in patients with CF."

Other points:

Page 2, 2nd paragraph, 1st line: the word "promising" could be deleted as it could be construed as promotional.

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and topline results released.

Page 3, 3rd paragraph, discussion on compound 6. It is not entirely clear from the text if loss in MIC means the MIC is lower or higher. Please re-word to improve clarity. Same issue for compound 5 vs 7.

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Page 4, final paragraph. How does the in vitro resistance frequency of BWC0977 compare with comparator drugs? (If these experiments were not performed by the investigators they should be available in previously published literature).

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Table 1. Generic names for avycaz and vabomere should be presented.

Suppl Table 6. Is the cipro MIC correct as written as 0.05 (should it be 0.06 or 0.5?) and 0.4 (should it be 0.5 or 4?) since these are not standard double dilution figures

Reviewer #2 (Remarks to the Author):

The authors have presented a manuscript about BWC0977, a novel bacterial topoisomerase inhibitor antibiotic. As it appears to be the first manuscript specifically on this compound, it is wide-ranging, starting from initial structural discovery through in vitro activity, in vivo studies, and the first-in-human study. All of the included information is very thorough and what is to be expected in an investigator's brochure. A new antibiotic like this is definitely exciting, and an appropriate topic for the journal.

Medical/pharmaceutical chemistry and compound discovery are not my area of expertise, so I do not have much to say on that portion of the manuscript.

1. Please avoid colloquialisms such as "big pharma" and "medchem" throughout.
2. Please make sure all abbreviations and bacterial species have been defined in the first appearance in the main body and capitalization is consistent throughout.
3. Introduction: At this point, I'm not sure I would consider the AMR crisis "emergent." Consider

rewording.
4. Introduction: Consider naming some of the “global priority” pathogens that BWC0977 is active against. It might be early, but as a reader I’d like to get some sort of idea where the rest of the paper is heading.
5. Introduction: Please change “serious threat” to either “a serious threat” or “serious threats.”
6. Results, antibacterial spectrum, p4: Consider restructuring the sentences with MIC90 values. The sentences should still make sense with the parenthetical text removed, and it currently does not. I suggest something like “…1 µg/mL against A. baumannii, 1 µg/mL against P. aeruginosa…” etc.
7. Results, killing kinetics, p4: Seems a bit odd to put “16X to 0.5X” when normally you’d put the smaller number first. Consider rearranging.
8. Results, Figure 2D: I’m not sure why “LUNG” is in all capital letters. You say the isolates used for this figure are MDR with different mechanisms, but that’s not exactly something I want to jump to the supplemental for. Consider a general description in the relevant results section of the main body (p5). Additionally, Supplemental Table 6 does not explicitly state each strain’s resistance mechanisms. I don’t have strain numbers memorized, and I doubt many people do. Readers are largely not going to know a specific strain’s resistance mechanisms if you do not tell them. You shouldn’t point people towards this table implying that that’s where they could find that information, then not provide it.
9. Results, ADME, p5: You state that BWC0977 has low potential for DDI since it does not inhibit or induce certain CYPs, but what about BWC0977 as a victim? You state that it is primarily metabolized by CYP3A4; wouldn’t interactions with CYP3A4 modulators affect BWC0977 PK?
10. Results, ELF, p6: A lot of this section feels like it belongs in the methods rather than results.
11. Discussion: The discussion feels a bit disjointed. Once the reader arrives to the middle of page 8 (“Given the global spread…”) it feels like a conclusion, so the mention of CF in the next paragraph feels a bit out of left field, especially since this is the first (and last) mention of it. Of the posters I've been able to find on BWC0977, CF is not mentioned there either. It is therefore unclear if treatment of CF-related infections is one of the indications Bugworks is going for. Consider making the CF paragraph the final paragraph, not mentioning CF at all, or rearranging the discussion another way.
12. Supplemental, neutropenic murine thigh model methods: Not sure why you specify “3/sex/time point” when only male mice were used.

Reviewer #3 (Remarks to the Author):

Paper Review Report

The manuscript “BWC0977, a novel broad-spectrum antibacterial clinical candidate to treat multidrug-resistant infections” evaluated both in vitro and in vivo efficacy of BWC097 against multidrug-resistant Gram-negative pathogens by several methods. Due to widespread antimicrobial resistance and a lack of new compounds for the treatment of Gram-negative bacterial infection in particular, the effort pursued by the authors and others to develop new antibiotics or repurpose existing molecules should be acknowledged and celebrated. Authors investigated in vitro microbiology methods such as minimum inhibitory concentration (MIC), time-killing kinetic assays to determine antimicrobial activity of BWC0997 against a multi-drug panel of Gram-negative bacteria. Then, the authors used several models to study

the BWC097 mechanism of action, followed by PK-PD and in vivo efficacy studies using many mouse models. I have some suggestions and comments for the editor and authors' consideration.

Suggestions

1. Use the phase: multidrug-resistant Gram-negative infections rather than multidrug-resistant infections because no data demonstrated activity against Gram-positive bacteria
2. Re-organise method sections to be logical orders starting with MIC determination, time-killing kinetics, protein binding, mechanism studies, PK studies, in vivo efficacy

Comments

Method

1. Section "Determination of the Minimum Inhibitory Concentration (MIC): The method described did not convince me as the following points
• Author mentioned CLSI guidelines, but citation is incorrect.
• which inoculum (please state in detail), store conditions such as how many per cent of glycerol in which media.
• Which compound dissolves in DMSO (need to define DMSO)? Results are shown in table 1 (results) with several antibiotics, and they dissolve in different solvents, not just DMSO.
• 150 ul of bacterial suspension was added to 96-well plates (state in detail of provider, reference number of plates), and then 3 ul of the drug made up a total volume of 153 ul. How could you get 4 ug/ml to 7.5 ug/ml?
2. Section "Determination of MIC90 of BWC097…..". It is not necessary to stand as one section, it is very simply MIC90 calculated based on total tested isolates.
3. Section of "Killing kinetics assay".
• Citation is incorrect.
• 30 ul of samples were taken to serially dilute for plating at 7-time points, as shown in supplementary figure 1. Therefore, a total volume of 180 ul was taken out after 12 h. The authors did not mention the total volume. However, time killing kinetics assay need to be done in at least volume of 1 ml, many reports did in 2 ml or 10 ml that gave enough condition for bacteria grown when many times of samples withdrawn.
4. Section "Dose finding studies of BWC0977 in the neutropenic murine thigh infection model."
• The method was not well described for the reviewer to understand, such as the number of mice in the group, infection doses of bacteria, bacteria preparation, and mice monitoring progress did not mentioned.
• Which polymyxin authors used, polymyxin B or colistin, need to be clearly stated
• The very huge concerns:
o No animal ethic statement throughout any mouse trials, either in vivo efficacy trials or PK studies. No in vitro safety studies (cell toxicity) and in vivo safety mouse trials were presented to indicate the doses listed in Table 1 (method) were safe for mice before efficacy testing.
o There are many reports in the literature mention toxicity of either colistin or PMB at high doses, such as 10.1128/AAC.00070-14

• What is the reason for combining meropenem with polymyxin as a positive control (table 1 method)
• No consistent labelling order tables, tables 1 and 2 in the method, then tables 1 and 2 in results.
• Could you please provide clinical record sheets for each animal trial?

Results

Table 1:
• No unit for MIC
• Is BWC0977 a bactericidal or bacteriostatic compound?
Supplementary figure 1:
• Legend should be under the figure.
• The legend was not easy for the reader to understand.
• The method motioned initial bacterial is 10^7 CFU/mL. Better to present the initial inoculum rather than starting at 0
Figure 2:
• Reviewer expected to see data of in vivo efficacy of BWC0977 against each bacteria listed in table 1 (method) with two set of data including CFU count and survival analysis that are very important to show how BWC0977 works in vivo.
• There is no statistical analysis to indicate significant differences between untreated group and BWC0977 treated groups to show BWC0977 works.
• In method (table 1), author mentioned using meropenem and combination meropenem with polymyxin as comparators, but I did not found in figure 2.

Comments from Reviewer #1:

1. The manuscript describes the lead optimization and identification, preclinical development and Phase I clinical trials of BWC0977. Given its in vitro spectrum of activity this could be a very important new antibiotic (but there is a long road of clinical trials to confirm this!). In general, the material is well presented but I have found two areas where more explanation is necessary and one where the discussion seems premature and somewhat promotional in nature:

   Activity against ciprofloxacin-resistant isolates and against Klebsiella. I cannot find in Table 1 nor in the supplementary material details about activity against ciprofloxacin-resistant isolates. Please present a table of MICs of BWC0977 against ciprofloxacin-resistant and ciprofloxacin-susceptible isolates for the same species so the reader can judge whether ciprofloxacin resistance has any impact on BWC0977 MICs.
   Klebsiella: Table 1 shows the MIC90 of BWC0977 against Klebsiella is 2 and Supp Table 3B shows Klebsiella MIC range up to 32. Please present data on mechanisms of resistance of Klebsiella and other Enterobacterales which have elevated MICs?
   Response: At the outset, I would like to take this opportunity to thank the reviewer #1 for the words of appreciation for this work and accepting to evaluate our manuscript.
   Regarding the point of providing data on the inhibitory effect on fluoroquinolone-resistant strains, I would like to draw your attention to Figure 5 {Molecular docking to identify unique residues of (A) Gyrase and (B) Topoisomerase IV involved in key interactions with BWC0977. (C) non-overlapping MICs (µg/ml) between ciprofloxacin (•) and BWC0977 (Δ)}. While the docking studies clearly shows uniqueness at the sites of interaction between the target enzymes and BWC0977 Vs ciprofloxacin, the scatter plot of non-overlapping MICs against the 4 Gram-negative pathogens (E. coli, A. baumannii, P. aeruginosa and K. pneumoniae) demonstrates the growth inhibitory activity of BWC0977 on fluoroquinolone-resistant strains indicating the lack of cross-resistance and that cipro-resistant isolates are still susceptible to BWC0977. The data set used here is from studies conducted at JMI Laboratories.

   Please find attached the excel file (20240319 Molecular_data_High_MIC_Kpn_Eclo) with data on the mechanisms of resistance of Klebsiella and Enterobacter spp. which have elevated MICs for BWC0977.

Collection Number	Organism	BWC0977 MIC (µg/ml)	Positive Molecular Tests
981643	Enterobacter cloacae species complex	8	aac(6`)-IIc, ACT-15-like, arr-3, mph(A), OXA-1_OXA-30, qnrB4, qnrS1, SHV-12, sul1, sul2, TEM-1, VIM-1
997590	Klebsiella pneumoniae	8	aac(6`)-Ib-cr, aph(6)-Ia, aph(6)-Id, ble, CTX-M-15, dfrA14, NDM-1, OMPK35(T74I,P93T,V183I,G211S,V241I), OMPK36(V178P,A183_T184insLSP,G189T,Q191L,V202L,F207Y,H218N,T222L,D223G,N227S,N227_L228insK,V229A,N232R,L311I,V349I), OMPK37(V19A,S88T,V295G,D350G,S353K,K356E), oqxA, OXA-1_OXA-30, qnrB1, SHV-11, sul2, TEM-1, tet(A)
1009094	Klebsiella pneumoniae	32	aac(6`)-Ib-cr, aadA2, ant(2``)-Ia, aph(6)-Ia, aph(6)-Id, CARB-2_PSE-1, catB3, CTX-M-15, dfrA12, dfrA14, dfrA19, fosA, FOX-5, mph(A), mph(E), msr(E), OMPK35(T74I,P93T,V183I,G211S,V241I), OMPK36(A183_T184insLSP,G189T,Q191L,V202L,F207Y,H218N,T222L,D223G,N227S,N227_L228insK,V229A,N232R,L311I,R345H,V349I), OMPK37(V19A,S88T,V295G,D350G,S353K,K356E), oqxA, qnrA1, SHV-28, sul2, TEM-1
1009716	Klebsiella pneumoniae	8	aac(3)-IIa, aac(6`)-Ib-cr, aadA1, aadA2, aph(3`)-Ia, aph(6)-Ia, aph(6)-Id, catA2, cmlA5, CTX-M-15, dfrA12, dfrA14, floR, KPC-2, oqxA, oqxB, OXA-10, OXA-1_OXA-30, qnrB1, qnrS1, SHV-11, sul1, sul2, TEM-1, tet(A)
1009744	Klebsiella pneumoniae	8	aac(3)-IIa, aac(6`)-Ib-cr, aadA1, aadA2, aph(3`)-Ia, aph(6)-Ia, aph(6)-Id, catA2, cmlA5, CTX-M-15, dfrA12, dfrA14, floR, KPC-2, oqxA, oqxB, OXA-10, OXA-1_OXA-30, qnrB1, qnrS1, SHV-11, sul1, sul2, TEM-1, tet(A)
1012739	Klebsiella pneumoniae	8	dfrA22, fosA, OMPK35(T74I,P93T,V183I,G211S,V241I), OMPK36(A190W,Q191S,Y198F,V202L,F207W,D224E,N227S,L228V,L228_V229insP,V229A,N232R,T254S,E304_G305insR,L311I,V349I), OMPK37(V19A,S88T,V295G,D350G,S353K,K356E), oqxA, qnrB17, SHV-12, sul1
1012979	Enterobacter cloacae species complex	32	ACT-17-like, dfrA1, LAP-2, qnrS1, sul1, TEM-1, tet(A)
1017710	Klebsiella pneumoniae	8	aac(3)-IIa, aac(6`)-Ib-cr, aph(6)-Ia, aph(6)-Id, CTX-M-15, dfrA14, KPC-3, oqxA, oqxB25, OXA-1_OXA-30, qnrB1, SHV-11, sul2, TEM-1, tet(A)
1018867	Klebsiella pneumoniae	8	aac(6`)-Ib, aadA2, ant(3``)-Ia, ble, CTX-M-15, dfrA12, NDM-1, OMPK35(T74I,P93T,V183I,G211S,V241I,Q273X), OMPK36(G134_D135insD,A190W,Q191S,Y198F,V202L,F207W,D224E,N227S,L228V,L228_V229insP,V229A,N232R,T254S,E304_G305insR,L311I,D340E,S342D,N346K,V349I), OMPK37(V19A,S88T,T261A,V295G,D350G,S353K,K356E), oqxA, oqxB32, OXA-232, OXA-9, pbp1a(T639A), pbp1b(A407V,Q829_P830insQQ), pbp2, pbp3, SHV-1, sul1, TEM-1, tet(B)
1023121	Klebsiella pneumoniae	32	aac(6`)-Ib-cr, aac(6`)-Il, aadA1, arr-3, catB3, dfrA1, OXA-1_OXA-30, qnrA1, SHV-187, sul1, VIM-12
1030808	Enterobacter cloacae species complex	8	aac(3)-IIa, aac(6`)-Ib, aac(6`)-IIc, ACT-45, aph(6)-Ia, aph(6)-Id, arr, catA2, CTX-M-9-like, dfrA19, OMPC(L211I,G228R), OMPF(Y48N,A49D,G50S,F113L,D114R,Y115S,G116X), OXA-48, pbp1a(Y23I,I29V,I138M,V188I,E189D,E241D,Q242K,N245S,G295V,D301Q,S329G,S338D,T339S,L350R,T358D,T365M,L366M,D377E,I379V,L440M,E510Q,R557H,I587V,A613P,N623D,D628N,V640I,Q644R,P645Q,G647L,V648A,N676S,D749T,S783A,V784A,V799I,V802I,E819A), pbp1b(D32E,M79I,E128Q,S136T,N188D,L416M,F494Y,K554R,V587T,A596P,V772I,M777V,S789A,Q798E,N822E,R835K), pbp2(E103N,N112T,V274I,N347T,V419I,E490D,F511Y,R563K), pbp3(R2K,I20V,R152K,H162R,S268A,N277S,T287A,A298S,E378D,V440I,I458V,V563I), qnrA1, SHV-12, sul1, TEM-1
1036904	Klebsiella pneumoniae	8	aac(6`)-Ib-cr, aadA5, ant(2``)-Ia, ant(3``)-Ia, armA, catA1, CTX-M-15, dfrA1, dfrA17, mph(E), msr(E), OMPK35(T74I,P93T,V183I,G211S,V241I,P255_E256del,E257Q,D258P,N259L,H260R,F261W,A262X), OMPK36(A183_T184insLSP,G189T,Q191L,V202L,F207Y,H218N,T222L,D223G,N227S,N227_L228insK,V229A,N232R,T254S,E304_G305insR,L311I,V349I), OMPK37(V19A,S88T,N230G,M233Q,T234H,Q235Y,N237H,N237_A238insTERY,R239K,E244D,N274S,D275T,D275_G276insSSTNGG,V277I,V295G,D350G,S353K,K356E), oqxA, oqxB, OXA-1_OXA-30, OXA-48, pbp1a(T639A), pbp1b(A407V,Q819P,Q820P,Q821P,Q829_Q829del), pbp2, pbp3, qnrS1, SHV-11, TEM-1, tet(A)

2. Phase I Clinical Trials - Page 6, final paragraph. Please clarify details which may be confusing to a reader. NCT05088421 is a completed single and multiple dose study according to clinicaltrials.gov. Therefore, since it has been completed, please describe the results of the multiple ascending dose part of this trial since the trial was both single and multiple ascending dose as described on clinicaltrials.gov. On clinicaltrials.gov it states that 44 of an expected 64 healthy volunteers were recruited. Please explain why the full cohort of 64 participants was not recruited. Also, the CONSORT diagram provided by the authors shows 40 patients recruited which is also at variance with the 44 indicated on clinicaltrials.gov. NCT05942820 is listed on clinicaltrials.gov as a currently recruiting single and multiple dose study. Please explain in the text why another virtually identical Phase I trial is necessary.
Response: NCT05088421 (BW Study No: C001-2020-001): This was our first-in-human (FIH) trial, where we completed a single ascending dose (SAD) study successfully in (5 cohorts x 8 subjects = 40 healthy volunteers). After concluding Part A, we had started the Part B multiple ascending dose (MAD) study. However, in the very first cohort in Part B, we observed infusion site reactions (ISR) in the 4 volunteers after they had received 3-5 doses of either BWC0977 or placebo (adverse event (AE) seen in the placebo and active arms). The

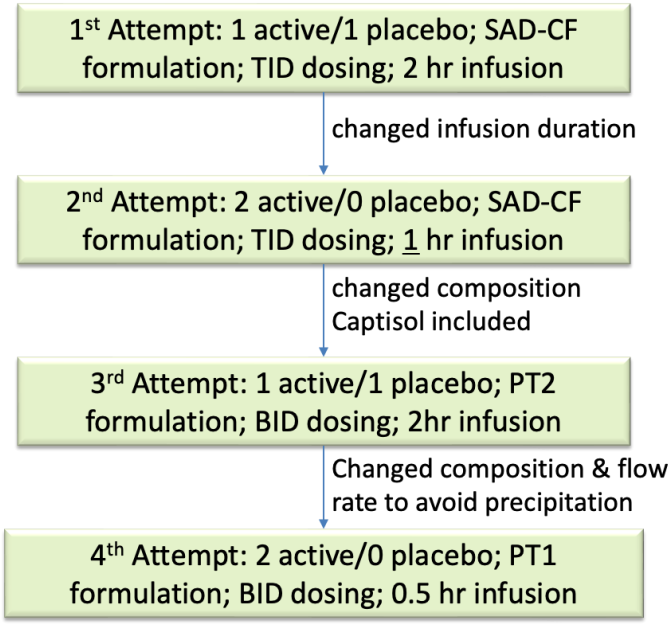
trial had to be halted to enable the development of a new formulation. Hence, on the CT.gov portal, we declared that 44 volunteers were recruited, and the trial had to be halted, we are writing to CT.gov administrators to modify the trial status reported as "completed" to "terminated".

In the CONSORT diagram as well, we had captured only SAD safety findings, since in the MAD cohort we haven't completed all the intended dose administration (21 doses over 7 days) to explore the systemic safety effects of BWC0977 following intravenous administration.

Based on extensive pre-clinical testing of revised BWC0977 formulation for local tolerance in rats and dogs, we re-opened the trial, Bugworks Study No C002-2023-01 ie NCT05942820. The study design remained the same as the first trial with the primary (safety) and secondary (PK) outcomes the same as before.

NCT05942820: In the 2nd trial C002-2023-01, with PT1 formulation (see schematic below), two subjects completed 12/14 and 14/14 doses as planned with no evidence of erythema or phlebitis and with minimal to no pain following drug administration. Due to the earlier trial experience, the Principal Investigator (PI) conducted a bedside ultrasound at the end of the dosing, where a small non-occlusive thrombus was seen proximal to the infusion site in both volunteers. There were no systemic adverse events. The blood profile for biochemical parameters for various organ functions including coagulation parameters were found to be normal. There was no evidence of a thrombus on the other arm where the volunteers were cannulated for PK sampling. Yet, due to the evidence of thrombus at the infusion site, we decided not to progress further until we get to the root cause of thrombus formation. It was important to rule out that BWC0977 per se did not have a clotting risk (the extensive pre-clinical GLP toxicology studies in rats and dogs did not reveal any evidence of such a risk). Therefore, we have stopped C002-2023-01 and we are back to the drawing board to rework on developing a better tolerated formulation.

We have modified the status of the trial on CT.gov from recruiting to "Terminated", post review from CT.gov reviewers, this updated information will go public.



3. Page 8. Discussion, 2nd last paragraph. Much of this is speculative and depends very much on the results of further clinical trials. Phrases such as "treatment of choice" are simply "spin" at this point. I would suggest deleting most of this material and replacing it with "The in vitro spectrum of activity of BWC0977 and results from the other preclinical studies presented here, are supportive of BWC0977 advancing into clinical trials for cUTI, HABP and VABP and complicated intra-abdominal infections. The spectrum of activity against pathogens of CF patients (B. cepacia, MRSA and P. aeruginosa), coupled with its high pulmonary drug levels also support trials in patients with CF."

   Response: We agree with the reviewer's suggestion and have accordingly made the change in the manuscript. This modification does read well and conveys the final take home message more effectively.

   Other points:

4. Page 2, 2nd paragraph, 1st line: the word "promising" could be deleted as it could be construed as promotional.
   Response: We appreciate the feedback. Accordingly, we have replaced the term promising to 'novel'.

5. Page 2, 2nd paragraph, 3rd from last line: The Phase III trial topline results were released on November 1, 2023. (See gardp.org). The sentence could be modified to reflect that the trial has been completed and topline results released.
   Response: We appreciate the feedback. The below given text is now included in the main manuscript. The revised text would read like: "A second NBTI, Zoliflodacin is a spiropyrimidinetrione developed by Entasis Therapeutics has completed a Phase-3 trial as an oral treatment for uUG. In this study, Zoliflodacin demonstrated statistical non-inferiority of microbiological cure at the urogenital site versus treatment with intramuscular injection of ceftriaxone and oral azithromycin".
6. Page 3, 3rd paragraph, discussion on compound 6. It is not entirely clear from the text if loss in MIC means the MIC is lower or higher. Please re-word to improve clarity. Same issue for compound 5 vs 7.
   Response: We appreciate the feedback. Corrections are made in the manuscript for better clarity and understanding.

7. Page 4, second paragraph. It is unfortunate that the comparator drugs for the MDR isolates only included two agents (cefiderocol, meropenem-vaborbactam) expected to have some activity against carbapenem-resistant isolates. Meropenem-vaborbactam will only have activity against KPC producers. Please include results from other comparator agents if these are available. Otherwise, there may be the impression of "cherry picking" comparators.
   Response: The MIC_{90} data provided here is a CARB-X sponsored study using close to 3,000 geographically diverse isolates and conducted at IHMA Laboratories, Switzerland. The data table demonstrates that BWC0977 MICs are potent compared to other antibiotics tested. The two tables (Table 1 and Supplementary Table 2) have data for 20 comparators in case of Gram-negative pathogens and 8 comparator antibiotics for Gram-positive pathogens, respectively. The broad-spectrum of MIC, lack of cross-resistance and potency of BWC0977 against a globally diverse set of isolates with various drug resistance backgrounds are demonstrated in this study.

8. Page 4, final paragraph. How does the in vitro resistance frequency of BWC0977 compare with comparator drugs? (If these experiments were not performed by the investigators they should be available in previously published literature).

   Response: As a control, ciprofloxacin resistance frequency was determined in parallel in this study. The resistance frequency of ciprofloxacin at 4x MIC in E. coli ATCC 25922 was found to be $5*10^{-9}$, P. aeruginosa ATCC 27853 was $<1*10^{-9}$ and A. baumannii ATCC 19606 was $1*10^{-9}$. Therefore, the resistance frequencies of ciprofloxacin and BWC0977 for the Gram-negative bacteria listed above are comparable.

9. Page 5. ADME. Please be specific with the figure for plasma protein binding in humans. The range is OK for animals but let's see the specific human figure.
   Response: In humans, the PPB observed is 94%. We will modify the text accordingly.

Species	% bound
Mice	86
Human	94
Beagle dog	94
SD rat	93
Guinea pig	84
Cynomolgus Monkey	91

10. Page 6, line 1. Please add "in mice and rats" so the heading will read "Epithelial lining fluid concentrations in mice and rats".

    Table 1. Generic names for avycaz and vabomere should be presented.

    Response: We appreciate the feedback. The corrections have been made in Table 1.

    Suppl Table 6. Is the cipro MIC correct as written as 0.05 (should it be 0.06 or 0.5?) and 0.4 (should it be 0.5 or 4?) since these are not standard double dilution figures.

    Response: We appreciate the feedback. The table has been modified to reflect the values as per CLSI guidelines. We shall now replace with the table given below in the Figures and Tables document (Suppl. Table 6)

	MIC (µg/ml)			
Strain Name	**BWC0977**	**Colistin**	**Ciprofloxacin**	**Meropenem**
A. baumannii ATCC19606	0.06	0.5	0.4	0.25
A. baumannii SAC002 (Ciprofloxacin & carbapenem-resistant)	0.125	1	>16	>16
E. coli ATCC BAA-2469 (Ciprofloxacin & carbapenem-resistant)	0.06	1	>16	16
E. coli ATCC BAA-2471 (Ciprofloxacin & carbapenem-resistant)	0.5	0.5	>16	>32
E. coli SEC-015 (Ciprofloxacin & carbapenem-resistant))	0.25	1	>8	>8
K pneumoniae SKB067 (Colistin, Ciprofloxacin & carbapenem-resistant)	1	16	>16	>16
K. pneumoniae ATCC 13883	0.06	1	0.06	0.06
K. pneumoniae KPNIH1 (Ciprofloxacin & carbapenem-resistant)	1	2	>16	>32
K. pneumoniae MKP103 (Ciprofloxacin-resistant)	1	2	>16	<0.25
P. aeruginosa ATCC 27853	0.25	2	0.5	0.5
P. aeruginosa SPA041 (Ciprofloxacin-resistant)	1	2	>16	0.5

Origin of the strains used in the above studies: A. baumannii ATCC 19606, E. coli ATCC BAA-2469, E. coli ATCC BAA-2471, K. pneumoniae ATCC 13883 and P. aeruginosa ATCC 27853 were purchased from the American Type Culture Collection (ATCC, USA). K. pneumoniae KPNIH1 and MKP103 strains were purchased from Manoil Laboratory, University of Washington, USA. E. coli SEC-015, A. baumannii SAC002, K. pneumoniae SKB067, P. aeruginosa SPA041 were clinical isolates obtained from St. John's Hospital, Bangalore, India.

Comments from Reviewer #2:

The authors have presented a manuscript about BWC0977, a novel bacterial topoisomerase inhibitor antibiotic. As it appears to be the first manuscript specifically on this compound, it is wide-ranging, starting from initial structural discovery through in vitro activity, in vivo studies, and the first-in-human study. All of the included information is very thorough and what is to be expected in an investigator's brochure. A new antibiotic like this is definitely exciting, and an appropriate topic for the journal.
Response: We are very thankful to reviewer #2 for accepting to review our manuscript and for providing valuable insights and feedback. We are also happy to note the positive words of appreciation for the work presented in this manuscript.

Medical/pharmaceutical chemistry and compound discovery are not my area of expertise, so I do not have much to say on that portion of the manuscript.

1. Please avoid colloquialisms such as "big pharma" and "medchem" throughout.

Response: We agree with the reviewer's views and have accordingly made changes in the manuscript.

2. Please make sure all abbreviations and bacterial species have been defined in the first appearance in the main body and capitalization is consistent throughout.

Response: We agree with the reviewer's views and have gone through the entire manuscript and ensured to the best of our abilities the abbreviations and bacterial species have been defined in the first appearance in the main body and capitalization is consistent throughout.

3. Introduction: At this point, I'm not sure I would consider the AMR crisis "emergent." Consider rewording.

Response: We agree with the reviewer's views and have accordingly made changes in the manuscript.

4. Introduction: Consider naming some of the "global priority" pathogens that BWC0977 is active against. It might be early, but as a reader I'd like to get some sort of idea where the rest of the paper is heading.

Response: We agree with the reviewer's views and have accordingly made changes in the manuscript.

5. Introduction: Please change "serious threat" to either "a serious threat" or "serious threats."

Response: We agree with the reviewer's views and have accordingly made changes in the manuscript.

6. Results, antibacterial spectrum, p4: Consider restructuring the sentences with MIC90 values. The sentences should still make sense with the parenthetical text removed, and it currently does not. I suggest something like "…1 µg/mL against A. baumannii, 1 µg/mL against P. aeruginosa…" etc.

Response: We agree with the reviewer's views and have accordingly made changes in the manuscript.

7. Results, killing kinetics, p4: Seems a bit odd to put "16X to 0.5X" when normally you'd put the smaller number first. Consider rearranging.

Response: We agree with the reviewer's views and have accordingly made changes in the manuscript.

8. Results, Figure 2D: I'm not sure why "LUNG" is in all capital letters. You say the isolates used for this figure are MDR with different mechanisms, but that's not exactly something I want to jump to the supplemental for. Consider a general description in the relevant results section of the main body (p5). Additionally, Supplemental Table 6 does not explicitly state each strain's resistance mechanisms. I don't have strain numbers memorized, and I doubt many people do. Readers are largely not going to know a specific strain's resistance mechanisms if you do not tell them. You shouldn't point people towards this table implying that that's where they could find that information, then not provide it.

Response: We agree to this point and have accordingly made changes in the manuscript (Figure 2D) and in Supplementary Table 6.

9. Results, ADME, p5: You state that BWC0977 has low potential for DDI since it does not inhibit or induce certain CYPs, but what about BWC0977 as a victim? You state that it is primarily metabolized by CYP3A4; wouldn't interactions with CYP3A4 modulators affect BWC0977 PK?

Response: We are planning to undertake detailed in vitro-DDI studies and based on the understanding emerging from those studies, we intend to perform an in-vivo DDI study in humans. Similar thoughts are also on CYP3A4 modulators interacting with BWC0977. Post which we would be able to respond to your comment, the data currently in hand suggest it has low potential for DDI of BWC0977.

10. Results, ELF, p6: A lot of this section feels like it belongs in the methods rather than results.

Response: We have modified the above-mentioned section accordingly.

11. Discussion: The discussion feels a bit disjointed. Once the reader arrives to the middle of page 8 ("Given the global spread…") it feels like a conclusion, so the mention of CF in the next paragraph feels a bit out of left field, especially since this is the first (and last) mention of it. Of the posters I've been able to find on BWC0977, CF is not mentioned there either. It is therefore unclear if treatment of CF-related infections is one of the indications Bugworks is going for. Consider making the CF paragraph the final paragraph, not mentioning CF at all, or rearranging the discussion another way.

Response: A similar observation was made by reviewer #1. We have made the necessary modifications in the manuscript accordingly.

"The in vitro spectrum of activity of BWC0977 and results from the other preclinical studies presented here, are supportive of BWC0977 advancing into clinical trials for cUTI, HABP and VABP and cIAI".

"The spectrum of activity against pathogens from CF patients (B. cepacia, MRSA and P. aeruginosa), coupled with its high pulmonary drug levels also support trials in patients with CF".

12. Supplemental, neutropenic murine thigh model methods: Not sure why you specify "3/sex/time point" when only male mice were used.

Response: We stand corrected. We shall make the necessary corrections in the supplementary methods document.

Comments from Reviewer #3:
Paper Review Report
The manuscript "BWC0977, a novel broad-spectrum antibacterial clinical candidate to treat multidrug-resistant infections" evaluated both in vitro and in vivo efficacy of BWC097

against multidrug-resistant Gram-negative pathogens by several methods. Due to widespread antimicrobial resistance and a lack of new compounds for the treatment of Gram-negative bacterial infection in particular, the effort pursued by the authors and others to develop new antibiotics or repurpose existing molecules should be acknowledged and celebrated. Authors investigated in vitro microbiology methods such as minimum inhibitory concentration (MIC), time-killing kinetic assays to determine antimicrobial activity of BWC0997 against a multi-drug panel of Gram-negative bacteria. Then, the authors used several models to study the BWC097 mechanism of action, followed by PK-PD and in vivo efficacy studies using many mouse models. I have some suggestions and comments for the editor and authors' consideration.

Response: We thank the reviewer for reviewing our manuscript and offering valuable and critical feedback. We are grateful to note that our work to discover novel antibiotics is of great value and would contribute towards rebuilding the lean pipeline of antibiotics.

Suggestions

1. Use the phase: multidrug-resistant Gram-negative infections rather than multidrug-resistant infections because no data demonstrated activity against Gram-positive bacteria.

Response: We would like to draw your attention to Supplementary Table 2, Supplementary Table 3C. Here, we have shown exquisite antibacterial potency against Gram-positives pathogens. Owing to BWC0977's broad-spectrum activity against a diverse panel of MDR isolates (including Gram-negatives, Gram-positives, biothreat and cystic fibrosis pathogens) with varying mechanisms of drug resistance, we have referred BWC0977 as a novel agent to treat multidrug-resistant infections.

2. Re-organise method sections to be logical orders starting with MIC determination, time-killing kinetics, protein binding, mechanism studies, PK studies, in vivo efficacy

Response: We agree with the reviewer's suggestion and have accordingly made changes in method section with regards to re-organising the sections.

Comments

Method

1. Section "Determination of the Minimum Inhibitory Concentration (MIC): The method described did not convince me as the following points

• Author mentioned CLSI guidelines, but citation is incorrect.

Response: We have now corrected the citation. Thanks much for pointing the discrepancy in the citation.

CLSI, 2018. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; 11th Edition; CLSI document M07-A11: Clinical Laboratory Standards Institute, Wayne, Pennsylvania USA.

• which inoculum (please state in detail), store conditions such as how many per cent of glycerol in which media.

Response: We appreciate the suggestion; the text will be modified to: The inoculum used for all the experiments was derived from a single seed lot that is maintained as glycerol stocks at -80^{0}C and are sub-cultured on LB plates for isolated colonies. Single colony of

each strain is inoculated and grown in the appropriate MIC test medium (cation adjusted Mueller Hinton broth). The final glycerol concertation is kept at 20% (v/v). We have incorporated these details in the methods section.

• Which compound dissolves in DMSO (need to define DMSO)? Results are shown in table 1 (results) with several antibiotics, and they dissolve in different solvents, not just DMSO.

Response: Point noted. In the supplement section, we have now included the complete information on the primary solubilising agent for all antibiotics described in this manuscript.

Antimicrobial Agent	Solvent
Amikacin	Water
Ampicillin	Phosphate buffer pH 8.0, 0.1 M
Avibactam sodium	Water
Azithromycin	95% Ethanol
Aztreonam	Saturated sol sodium bicarbonate
Cefepime hydrochloride	Phosphate buffer pH 6.0, 0.1 M
Cefpodoxime free acid	0.1% (11.9mM) aqueous sodium bicarbonate
Ceftazidime pentahydrate	sodium carbonate 10% of the weight
Ceftriaxone sodium	Water
Ciprofloxacin hydrochloride	Water
Clindamycin hydrochloride	Water
Colistin sulfate	Water
Daptomycin	Water
Doxycycline hyclate	Water
Eravacycline dihydrochloride	Water
Gentamicin sulfate	Water
Levofloxacin	1/2 vol of water + 0.1 M NaOH dropwise to dissolve
Linezolid	Water
Meropenem	Water
Penicillin G potassium	Water

• 150 ul of bacterial suspension was added to 96-well plates (state in detail of provider, reference number of plates), and then 3 ul of the drug made up a total volume of 153 ul. How could you get 4 ug/ml to 7.5 ug/ml?

Response: We are thankful to the reviewer for pointing out this discrepancy. This has been corrected in the manuscript.

The revised section will read as follows: 147 µl of bacterial suspension was added to 96-well plates (ThermoFisher Scientific, Catalog No. 130188), followed by 3 µl of the drug solution to make up the total volume to 150 µl. 10 concentrations were set up with a start concentration of 4µg/ml, and two-fold serial dilution leading to final concentrations of 2µg/ml, 1µg/ml, 500ng, 250, 125, 62.5, 31.25, 15.6 and 7.8ng/ml. We shall correct the line as 4ug to 7.8ng/ml instead of 7.5ug/ml.

2. Section "Determination of MIC90 of BWC097…..". It is not necessary to stand as one section, it is very simply MIC90 calculated based on total tested isolates.

Response: We agree with your views and have accordingly made changes in the manuscript. Now the MIC_{90} section is placed as a part/continuum to MIC section.

3. Section of "Killing kinetics assay".

• Citation is incorrect.

Response: The citation is our own publication [Hameed, P. et al., Nitrothiophene carboxamides, a novel narrow spectrum antibacterial series: Mechanism of action and Efficacy. Sci Rep. 8, 7263 (2018)]. The procedure is given at the end of the section titled "Determination of MIC and MBC" within the methods section.

• 30 ul of samples were taken to serially dilute for plating at 7-time points, as shown in supplementary figure 1. Therefore, a total volume of 180 ul was taken out after 12 h. The authors did not mention the total volume. However, time killing kinetics assay need to be done in at least volume of 1 ml, many reports did in 2 ml or 10 ml that gave enough condition for bacteria grown when many times of samples withdrawn.

Response: Sorry for not providing the clear protocol that was followed. In the procedure we followed, we prepared multiple wells of each concentration with a total volume of 150 µl (column wise) in the same plate. Sampling was done for the same concentration every time from a different well at the various time points.

4. Section "Dose finding studies of BWC0977 in the neutropenic murine thigh infection model."

• The method was not well described for the reviewer to understand, such as the number of mice in the group, infection doses of bacteria, bacteria preparation, and mice monitoring progress did not mentioned.

Response: We agree with the comments and have accordingly made changes in the Methods section.

• Which polymyxin authors used, polymyxin B or colistin, need to be clearly stated

Response: Polymyxin B was used throughout (EMD Millipore Corp, USA).

• The very huge concerns:

o No animal ethic statement throughout any mouse trials, either in vivo efficacy trials or PK studies.

Response: All laboratory animal experiments were conducted under the UK Home Office project License PAC022930 which was renewed in 2022 as PP3585942. These licenses and all animal experiments conducted under them are approved by the University of Liverpool Animal Welfare Ethics Review Board. All in vivo study protocols (neutropenic rats) were conducted after obtaining permission from the Committee for the purpose of the control and supervision of experiments on animal (CPCSEA), New Delhi, India and the Institutional Animal Ethics Committee (IAEC).

No in vitro safety studies (cell toxicity) and in vivo safety mouse trials were presented to indicate the doses listed in Table 1 (method) were safe for mice before efficacy testing.
Response: Mouse tolerability studies were conducted prior to any mouse efficacy experiments using BWC0977. These studies involved dosing a single mouse per dose with an escalating dose on q8h regimen. Mice were closely monitored, and all observations recorded. Mice were euthanized after 24 hours and if no/minimal observations were recorded, the next dose was administered to another mouse. The following doses were tested: 30, 60, 90, 120 and 160mg/kg/dose. Observations were minimal (scratching / grooming at the injection site, redness at injection site) for doses up to, and including 120mg/kg/dose. Severe observations were observed following 1st dose of 160mg/kg/dose leading to the mouse being euthanized, therefore, the highest tolerated dose to move forward into efficacy studies was 120mg/kg/dose.

o There are many reports in the literature mention toxicity of either colistin or PMB at high doses, such as 10.1128/AAC.00070-14
Response: Meropenem and polymyxin B were not combined. There were 2 separate positive controls in these studies. The Polymyxin B tolerability studies have previously been conducted under the Home Office license PAC022930 with a maximum tolerated dose of 75mg/kg q24h or 25mg/kg q8h.

• What is the reason for combining meropenem with polymyxin as a positive control (table 1 method)
Response: We apologise for the confusion. Instead of listing them separately, the + sign was given which did not convey the correct message. What we actually meant was that these two antibiotics (polymyxin B and colistin) were used as controls, separately. We have now corrected the table to reflect this message.

• No consistent labelling order tables, tables 1 and 2 in the method, then tables 1 and 2 in results.
Response: Labelling is now changed in the Supplementary Table # as appropriate.

• Could you please provide clinical record sheets for each animal trial?
Response: We have provided the entire raw data set for the in vitro killing kinetics, in vivo rat and mouse efficacy studies as an Excel file with multiple sheets. We believe this Excel file provides more value to the reader than the clinical record sheets.

Results

Table 1:

• No unit for MIC

Response: The heading for the table has the unit information.

Determination of antibacterial potency [MIC_{90} (µg/ml)] of BWC0977 and other known antibiotics at International Health Management Associates (IHMA) against a global collection of Gram-negative multi drug-resistant clinical isolates.

• Is BWC0977 a bactericidal or bacteriostatic compound?

Response: BWC0977 is a bactericidal compound. Both in vitro and in vivo studies have shown BWC0977 to exhibit bactericidal properties against all the pathogens tested.

Supplementary figure 1:

• Legend should be under the figure.

• The legend was not easy for the reader to understand.

Response: Thanks for pointing this out. We have now included the same.

Time kill kinetics is determined by enumerating the number of survivors at various time points following compound exposure. Bacteria (~10^7CFU/ml; A_{600} of 0.1) is exposed to various concentrations (2-fold serial dilutions, 9 concentrations, with start of either 256 to 1µg/ml, 64 to 0.25µg/ml, 32 to 0.125 µg/ml, 16-0.06 µg/ml, 8 to 0.03 µg/ml, 4 to 0.015µg/ml, or 2 to 0.008 µg/ml µg/ml) of BWC0977 in a 96-well microtiter plate.

At various time intervals, (0, 1, 3, 6, 9, 12, 24 hours), 30µl/150ul of treated cultures was removed, serially diluted, and plated for bacterial survivors on LB agar plates following incubation at 37°C for 16-18 hours. The extent of cidality was monitored against a panel of susceptible and MDR strains of E. coli, A. baumannii, K. pneumoniae, P. aeruginosa and E. cloacae. The viable colony forming units (CFUs) were enumerated and the plots generated using Graph Pad Prism. Data was expressed as the $\Delta\log_{10}$ CFU at each drug concentration. There was significant cidality observed over time but limited rise in kill with increasing concentrations.

• The method motioned initial bacterial is 10^7 CFU/mL. Better to present the initial inoculum rather than starting at 0

Response: The y-axis denotes $\Delta\log_{10}$CFU/ml for various time points for each drug concentration. The '0' denotes the start at time '0' which is equivalent to no kill ($\Delta\log_{10}$CFU/ml=0) and then the $\log_{10}$ reduction in CFUs are captured beyond this point.

Figure 2:

• Reviewer expected to see data of in vivo efficacy of BWC0977 against each bacteria listed in table 1 (method) with two set of data including CFU count and survival analysis that are very important to show how BWC0977 works in vivo.

Response: All raw data pertaining to the in vitro and in vivo studies is now provided as an Excel file with multiple sheets.

Please note, we do not assess the survival rates as the experiment is run only for 24 hours, hence survival analysis was not performed.

• There is no statistical analysis to indicate significant differences between untreated group and BWC0977 treated groups to show BWC0977 works…
Response: For all mice studies, a Tukey's test was used to do a pair-wise comparison of overall mean differences of log_{10}CFU/gm. Results showed that each fractionated dose is significantly different as compared to control (p=0.0006, 0.0015, 0.0062), but no fractionated dose is significantly different between each other (p=0.8624, 0.2862, 0.6685) in terms of log_{10}CFU/gm.
In addition, for the rat studies, a one-way ANNOVA using GraphPad Prism software was used to run the statistical analysis.

• In method (table 1), author mentioned using meropenem and combination meropenem with polymyxin as comparators, but I did not find in figure 2.
Response: We apologise for the confusion. Meropenem and polymyxin were separate positive control groups within the same experiment but never used as a combination – Suppl. Methods Table 2 has been amended below.

Suppl. Methods Table 2. Efficacy of BWC0977 in neutropenic murine dual thigh infection model.

Strain	Dose range (mg/kg) and doses (mg/kg) administered via SC q8h	Positive control q8h, SC
P. aeruginosa ATCC 27853	1-100 (1, 5, 10, 30, 60, and 100)	Meropenem 200mg/kg
P. aeruginosa NCTC 13437	5-120 (5, 10, 30, 60, 90, and 120)	Meropenem 200mg/kg
P. aeruginosa NCTC 13921	10-120 (10, 30, 60, 90, and 120)	Meropenem 200mg/kg and Polymyxin 25mg/kg*
A. baumannii NCTC 13301	10-120 (10, 30, 60, 90, and 120)	Meropenem 200mg/kg and Polymyxin 25mg/kg*
A. baumannii ATCC 17978	10-120 (10, 30, 60, 90, and 120)	Meropenem 200mg/kg and Polymyxin 25mg/kg*
A. baumannii NCTC 13421	2.5-120 (2.5,7.5,10,30,60, and 120)	Meropenem 200mg/kg and Polymyxin 25mg/kg*
K. pneumoniae NCTC 13465	10-120 (10, 30, 60, 90, and 120)	Meropenem 200mg/kg and Polymyxin 25mg/kg*
K. pneumoniae ATCC 43816	10-120 (10, 30, 60, 90, and 120)	Meropenem 200mg/kg and Polymyxin 25mg/kg*
K. pneumoniae NCTC 27736	2.5-120 (2.5,7.5,10,30,60,90 and 120)	Meropenem 200mg/kg and Polymyxin 25mg/kg*
E. coli ATCC -BAA-2523	10-120 (10, 30, 60, 90, and 120)	Meropenem 200mg/kg and Polymyxin 25mg/kg*
E. coli NCTC 13462	10-120 (10, 30, 60, 90, and 120)	Meropenem 200mg/kg and Polymyxin 25mg/kg*

*Meropenem and Polymyxin B represent 2 separate positive control groups within the same experiment

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Thank you for providing further explanation about the clinical trial status. This information should be provided for readers as well.
Please describe in the text: (1) what was seen in the first MAD cohort, (2) the need for reformulation, (3) what occurred in the new trial. This should be paraphrased from the information you provided to me in response to reviewers, as below:
NCT05942820: In the 2nd trial C002-2023-01, with PT1 formulation (see schematic below), two subjects completed 12/14 and 14/14 doses as planned with no evidence of erythema or phlebitis and with minimal to no pain following drug administration. Due to the earlier trial experience, the Principal Investigator (PI) conducted a bedside ultrasound at the end of the
dosing, where a small non-occlusive thrombus was seen proximal to the infusion site in both volunteers. There were no systemic adverse events. The blood profile for biochemical
parameters for various organ functions including coagulation parameters were found to be normal. There was no evidence of a thrombus on the other arm where the volunteers were cannulated for PK sampling. Yet, due to the evidence of thrombus at the infusion site, we decided not to progress further until we get to the root cause of thrombus formation. It was important to rule out that BWC0977 per se did not have a clotting risk (the extensive preclinical GLP toxicology studies in rats and dogs did not reveal any evidence of such a risk).
Therefore, we have stopped C002-2023-01 and we are back to the drawing board to rework on developing a better tolerated formulation.

Reviewer #3 (Remarks to the Author):

The reviewer thanks the authors' responses and the editors for inviting me to review this manuscript. After considering the authors' responses to my concerns I would suggest accepting to publish.

Best wishes!

Reviewer #4 (Remarks to the Author):

This is an important piece of work that reports the discovery, pre-clinical evaluation, and clinical studies of a new broad-spectrum antibiotic. It is urgent to develop a new class of antibiotics to tackle the rising cases of drug-resistant infections. BWC0977 is an important candidate drug being developed by Bugworks. While the pre-clinical development studies and clinical studies have been well described in the manuscript, and the authors have adequately responded to most of the comments made by the reviewers, the early discovery and medicinal chemistry modifications of the hit compound 1 to obtain the candidate drug BWC0977 are very poorly described in the manuscript. As this is an important part of the study, it is vital to convey the full rationale and decision-making process behind the medicinal

chemistry modifications to make the study truly impactful. Here are a couple of important points about the medicinal chemistry that the authors should address in a suitably revised manuscript.

Line 88: The authors mentioned that "The chemical library consisted of ~3000 compounds selected for structural diversity from commercial databases." The identity of the chemical library should be provided.
Line 91: The hit-to-lead optimization of the chemical series is provided in Figure 1, which raises a number of questions.
a) The substitution of the terminal amine (NH2) group of the initial hit compound 1 with a fluoroindolinone group is not a standard and obvious medicinal chemistry modification. The fluoroindolinone group is not present in any NBTIs reported in the reference 9 paper by J&J. What is the rational basis of this modification?
b) Was the design guided by molecular modelling?
c) How many structural analogues of compound 1 were synthesized to obtain compound 2?
d) The detailed medicinal chemistry modifications should be provided in a supplementary figure.
e) Similarly, why was a specific stereomodification (S-configuration) of the linker necessary? Is this specific stereomodification of the chemical scaffold guided by structural studies? Is this essential for the on-target activity of these compounds?

Line 322: Molecular docking is an important tool to evaluate the molecular-level interaction between a ligand and its target. However, it is a static interaction study, whereas the biological system is dynamic. Therefore, the data obtained using molecular docking alone should be treated with caution. It is useful to conduct molecular dynamics (MD) simulations for a reasonably long period (e.g., 1 microsecond) and compare the binding poses at the start and end of the simulation to evaluate if the interactions observed during the initial docking are temporary or if the complex formed is a fairly stable interaction, reflecting the interactions within the biological system. The authors should conduct at least a 1 microsecond MD simulation of BWC0977 and TopIV and report the findings in the revised manuscript.

Minor point:
Line 53: Fluoroquinolones were not introduced in the 1970s. The first quinolone, nalidixic acid, was introduced in the 1960s, and the first fluoroquinolone antibiotic, norfloxacin, was introduced in the 1980s.

Response to Reviewers' comments following Resubmission

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author): Thank you for providing further explanation about the clinical trial status. This information should be provided for readers as well. Please describe in the text: (1) what was seen in the first MAD cohort, (2) the need for reformulation, (3) what occurred in the new trial. This should be paraphrased from the information you provided to me in response to reviewers, as below:

NCT05942820: In the 2nd trial C002-2023-01, with PT1 formulation (see schematic below), two subjects completed 12/14 and 14/14 doses as planned with no evidence of erythema or phlebitis and with minimal to no pain following drug administration. Due to the earlier trial experience, the Principal Investigator (PI) conducted a bedside ultrasound at the end of the dosing, where a small non-occlusive thrombus was seen proximal to the infusion site in both volunteers. There were no systemic adverse events. The blood profile for biochemical parameters for various organ functions including coagulation parameters were found to be normal. There was no evidence of a thrombus on the other arm where the volunteers were cannulated for PK sampling. Yet, due to the evidence of thrombus at the infusion site, we decided not to progress further until we get to the root cause of thrombus formation. It was important to rule out that BWC0977 per se did not have a clotting risk (the extensive preclinical GLP toxicology studies in rats and dogs did not reveal any evidence of such a risk). Therefore, we have stopped C002-2023-01 and we are back to the drawing board to rework on developing a better tolerated formulation.

Authors response: Agreed and changes as suggested incorporated in the manuscript

Reviewer #3 (Remarks to the Author):

The reviewer thanks the authors' responses and the editors for inviting me to review this manuscript. After considering the authors' responses to my concerns I would suggest accepting to publish.

Best wishes!

Authors response: Thank you

Reviewer #4 (Remarks to the Author):

This is an important piece of work that reports the discovery, pre-clinical evaluation, and clinical studies of a new broad-spectrum antibiotic. It is urgent to develop a new class of antibiotics to tackle the rising cases of drug-resistant infections. BWC0977 is an important candidate drug being developed by Bugworks. While the pre-clinical development studies and clinical studies have been well described in the manuscript, and the authors have adequately responded to most of the comments made by the reviewers, the early discovery and medicinal chemistry modifications of the hit compound 1 to obtain the candidate drug BWC0977 are very poorly described in the manuscript. As this is an important part of the study, it is vital to convey the full rationale and decision-making process behind the medicinal chemistry modifications to make the study truly impactful. Here are a couple of important points about the medicinal chemistry that the authors should address in a suitably revised manuscript.

Authors response: We would like to thank this reviewer for appreciating the novelty of our work, for thorough reviewing, for pointing out the errors, and for providing valuable suggestions to improve the overall quality of the work presented in the manuscript. We believe that the feedback provided by this reviewer on the medicinal chemistry aspect will provide improved clarity to the readers. We are grateful for reviewers' time and effort in helping us improve our research. We are confident that the revisions made have enhanced the clarity and impact of our work.

The authors have incorporated most of the suggestions into the revised manuscript and provided a point-by-point response to reviewer-4 queries.

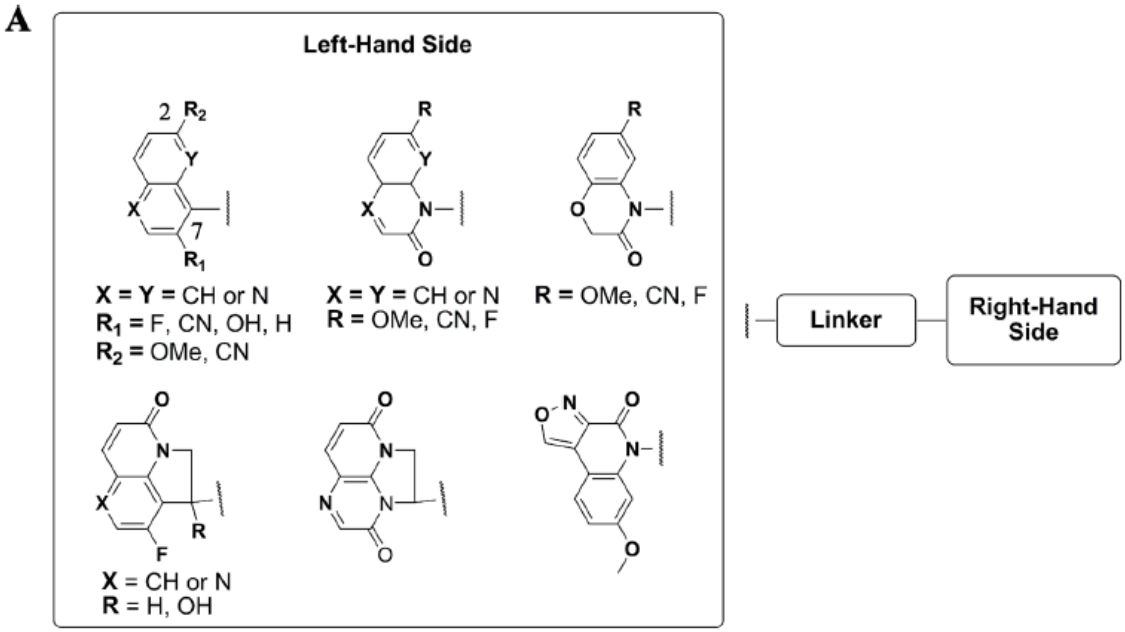
Line 88: The authors mentioned that "The chemical library consisted of ~3000 compounds selected for structural diversity from commercial databases." The identity of the chemical library should be provided.

Authors Response: Thank for the suggestion. As suggested the chemical procurement data-base source provided in the manuscript and the revised text is as follow: "The chemical library consisted of approximately 3000 compounds selected for structural diversity from commercial databases such as eMolecule, the REAL database from Enamine, Aurora Fine Chemicals catalogue and custom synthesized fragments. The diverse set was procured from various libraries, including Enamine Diversity Library, Aurora Building Blocks 1, Lifechemical Screening and Fragment Library, Analyticon Focused Library, and Ottava Chemical Fragment and Diversity Library."

Line 91: The hit-to-lead optimization of the chemical series is provided in Figure 1, which raises a number of questions.

a) The substitution of the terminal amine (NH2) group of the initial hit compound 1 with a fluoroindolinone group is not a standard and obvious medicinal chemistry modification. The fluoroindolinone group is not present in any NBTIs reported in the reference 9 paper by J&J. What is the rational basis of this modification?

Authors Response: The generic chemical structures of novel bacterial topoisomerase inhibitors (NBTIs) consist of three parts: a bicyclic or tricyclic heteroaromatic left-hand side (LHS), which makes intercalation between the DNA base pairs; a bicyclic right-hand side (RHS), which interacts with the topoisomerase protein (Gyrase and Topo IV); and a linker region joining the RHS and LHS. Figure 3A on page 4 of reference 36 [Two Decades of Successful SAR-Grounded Stories of the Novel Bacterial Topoisomerase Inhibitors (NBTIs)] J Med Chem. 63, 5664–5674 (20200)] provides the generic NBTI architecture with details of the LHS ring system, and the same is reproduced below



The above figure illustrates that NBTI 's LHS ring system generally contains heteroaromatic rings such as methoxy, cayano, and fluoro substituted quinoline, naphthyridine, and quinoxaline rings attached to a linker via carbon or nitrogen linkage. The rationale behind the fluoroindolinone modification is that, to differentiate from the published LHS ring, we have designed the fluoro substituted oxindole (fluoroindolinone) as a novel LHS core that can mimic the quinolinone ring system and can interact with DNA base pairs (purine bases).

b) Was the design guided by molecular modelling?

Authors Response: Yes, it is guided by both medicinal chemistry and the molecular modelling understanding. The literature survey and structural similarity analyses revealed that screening hit (compound1) has high similarity to known Novel bacterial topoisomerase inhibitors (NBTI) published in the literature. Deciphering of the structure activity relationship of NBTI class from the literature and rational medicinal chemistry designer approach using Gyrase crystal structure information led to identification of novel lead compound1 with fluoro-oxindole LHS ring system in such a way that it interacts efficiently with dsDNA portion of gyrase complex to bring enzyme inhibition.

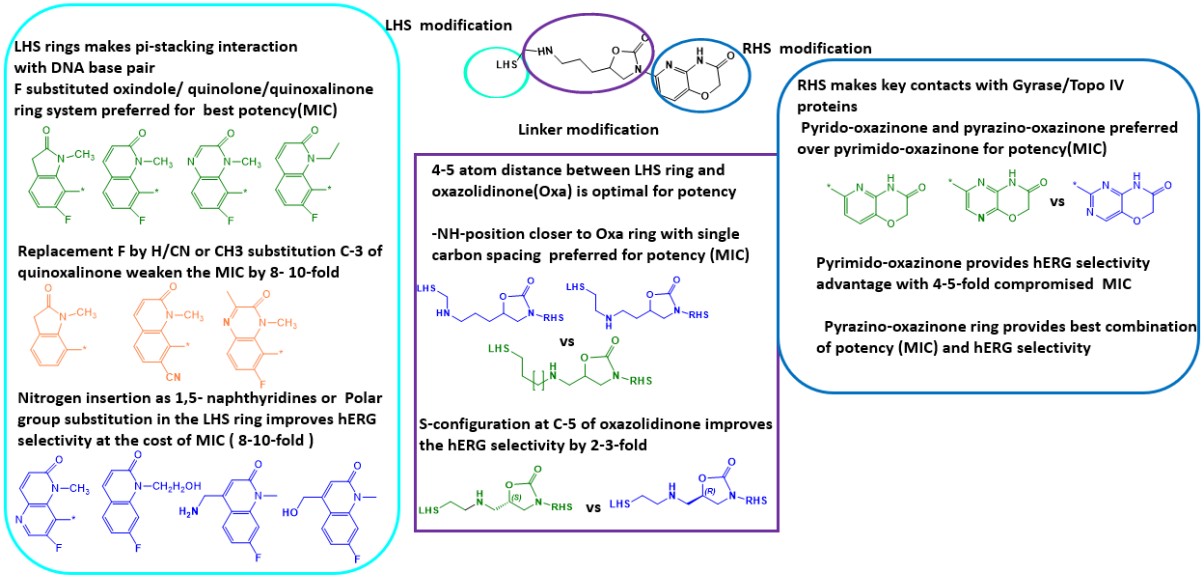
To bring more clarity to the readers, we have also added the following additional information in the discussion part of the main manuscript: "A review of the literature and structural similarity analyses showed that the screening hit (compound 1) is very similar to novel bacterial topoisomerase inhibitors (NBTI) that have been published in the literature.[36, 37] Deciphering the structure activity relationship of the NBTI class from the literature, the initial medicinal chemistry design strategy focused on the design of a novel LHS ring system capable of interacting with DNA base pairs."

c) How many structural analogues of compound 1 were synthesized to obtain compound 2?

Authors Response: Indeed, it is the second compound from the screening hit. As stated above we have deciphered the NBTI class SAR and rationally designed compound to bring potent activity.

d) The detailed medicinal chemistry modifications should be provided in a supplementary figure.

Authors Response: Response: We have included a detailed SAR modification leading to the discovery of BWC0977 as a supplementary figure.



e) Similarly, why was a specific stereo-modification (S-configuration) of the linker necessary? Is this specific stereo-modification of the chemical scaffold guided by structural studies? Is this essential for the on-target activity of these compounds?

Authors Response: The stereochemistry of the C-5 carbon of the oxazolidinone linker does not have any impact on enzymatic potency or antibacterial activity, but the S-configuration provides 2-4-fold improvement in off-target selectivity (hERG).

The above explanation was discussed in the manuscript (Line 128 to 132): "Both compounds exhibited similar broad-spectrum MIC, but with 4-fold difference in hERG inhibition, indicating that chirality at C-5 carbon of oxazolidinone ring does not influence the Gram-negative MIC activity, but impacts the hERG inhibition. Compounds with S-configuration (compound 9) showed 2.2-fold increase in hERG IC50 versus R-configuration 131 (IC50 60µM for compound 8 and 131µM for compound 9) (Suppl Table 1)."

Line 322: Molecular docking is an important tool to evaluate the molecular-level interaction between a ligand and its target. However, it is a static interaction study, whereas the biological system is dynamic. Therefore, the data obtained using molecular docking alone should be treated with caution. It is useful to conduct molecular dynamics (MD) simulations for a reasonably long period (e.g., 1 microsecond) and compare the binding poses at the start and end of the simulation to evaluate if the interactions observed during the initial docking are temporary or if the complex formed is a fairly stable interaction, reflecting the interactions within the biological system. The authors should conduct at least a 1 microsecond MD simulation of BWC0977 and TopIV and report the findings in the revised manuscript.

Authors Response: We have conducted a 100 nanosecond MD simulation and compared the poses at the start and end of the simulation. The interactions observed are stable over the observed period. The poses are presented as a figure in the Supplementary section.

Minor point:

Line 53: Fluoroquinolones were not introduced in the 1970s. The first quinolone, nalidixic acid, was introduced in the 1960s, and the first fluoroquinolone antibiotic, norfloxacin, was introduced in the 1980s.

Authors Response: Thanks for pointing out the error. We have corrected it in the updated manuscript.

REVIEWERS' COMMENTS

Reviewer #4 (Remarks to the Author):

The authors have addressed the comments and made the necessary changes to the manuscript. Currently the supplementary figure 2A which they added to show the medicinal chemistry modification has not been cited in the relevant discussion section. They should add the following sentence after the med-cheem discussion in the main manuscript.

The SAR modification leading to the discovery of BWC0977 is detailed in Supplementary Figure 2A.

The manuscript can be accepted after this minor modification.

NCOMMS-23-62263B_Response to Reviewer

Reviewer #4 (Remarks to the Author):

The authors have addressed the comments and made the necessary changes to the manuscript. Currently the supplementary figure 2A which they added to show the medicinal chemistry modification has not been cited in the relevant discussion section. They should add the following sentence after the med-cheem discussion in the main manuscript.

The SAR modification leading to the discovery of BWC0977 is detailed in Supplementary Figure 2A.

The manuscript can be accepted after this minor modification.

Response to Reviewer #4
Accepted and incorporated in the manuscript as suggested