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Synthesis and SAR studies of novel benzodiazepinedione-based inhibitors of *Clostridium difficile* (*C. difficile*) toxin B (TcdB)

Jeffrey J. Letourneau[a,*], Ilana L. Stroke[a], David W. Hilbert[b], Andrew G. Cole[a,c], Laurie J. Sturzenbecker[a], Brett A. Marinelli[a], Jorge G. Quintero[a,c], Joan Sabalski[a], Yanfang Li[a], Linh Ma[a,d], Igor Pechik[a], Philip D. Stein[a,e], Maria L.Webb[a,f]

[a] *Venenum Biodesign, a member of Genesis Biotechnology Group, 8 Black Forest Road, Hamilton, NJ 08691, USA*
[b] *Femeris Women's Health Research Center, a member of Genesis Biotechnology Group, 2000 Waterview Drive, Hamilton, NJ 08691, USA*
[c] *Current address is Arbutus Biopharma Inc., 701 Veterans Circle, Warminster, PA 18974, USA*
[d] *Current address is Bristol-Myers Squibb, Route 206 and Province Line Road, Princeton, NJ 08543, USA*
[e] *Deceased*
[f] *Current address is Merck Research Labs, 2015 Galloping Hill Rd, Kenilworth, NJ 07033, USA*

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ABSTRACT

Synthesis and structure-activity relationships (SAR) of a novel series of benzodiazepinedione-based inhibitors of *Clostridium difficile* toxin B (TcdB) are described. Compounds demonstrating low nanomolar affinity for TcdB, and which possess improved stability in mouse plasma *vs.* earlier compounds from this series, have been identified. Optimized compound **11d** demonstrates a good pharmacokinetic (PK) profile in mouse and hamster and is efficacious in a hamster survival model of *Clostridium difficile* infection.

Clostridium difficile infection (CDI) is the most common cause of infectious hospital-acquired diarrhea, with significant morbidity, mortality and associated healthcare costs.[1,2] The major risk factor for CDI is antimicrobial therapy, which disrupts the normal gut microbiota and allows *C. difficile* to flourish. Treatment of CDI with antimicrobials is generally effective in the short-term, but recurrent infections are frequent and problematic,[3] indicating that improved treatment options are necessary. Symptoms of disease are largely due to two paralogous toxins, TcdA and TcdB, which are glucosyltransferases that hydrolyze UDP-glucose and transfer the glucose moiety to host Rho GTPases, resulting in irreversible inhibition.

To date, several experimental approaches specifically targeting the bacterial toxins have been explored, including development of toxin binding antibodies.[4] Bezlotoxumab, a human monoclonal antibody which binds TcdB and neutralizes its effects, was recently approved by the FDA and is indicated to reduce recurrence of CDI in adults who are receiving antibacterial drug treatment for CDI and are at a high risk of recurrence.[5] Another emerging approach towards possible treatment of CDI involves direct inhibition of the glucosyltransferase activity of *C. difficile* toxins.[6] We recently reported details of our hit-to-lead optimization effort around a novel series of benzodiazepinedione-based inhibitors of TcdB.[7]

This effort resulted in highly potent inhibitors **1** (IC_{50} = 1 nM) and **2** (IC_{50} = 3 nM) which were demonstrated to inhibit induction of apoptosis of mammalian cells (CHO cells) by either TcdB or TcdA (Figure 1). However, these compounds were unstable in mouse plasma leading to suboptimal mouse PK profiles, thus precluding further advancement into rodent models of CDI. As such, we initiated a lead optimization program, utilizing compounds **1** and **2** as our starting point, aiming to identify potent inhibitors with improved stability in mouse plasma and hence improved PK profiles suitable for advancement into rodent models of CDI.

Testing of compound **2** in a mouse plasma stability assay revealed a rapid disappearance of the parent compound, with amide **2** being undetectable after a 2-hour incubation in mouse plasma.[7] In addition, the formation of the potential hydrolysis product, carboxylic acid **3**, was assayed for in the mouse plasma stability testing and it was detected after a 2-hour incubation of amide **2** in mouse plasma. We reasoned that the likely cause of instability in mouse plasma for compound **2**, and related congeners, was enzyme-mediated hydrolysis (*e.g.*, by amidases) of the N-heteroaryl amide moiety (Figure 2).[8]

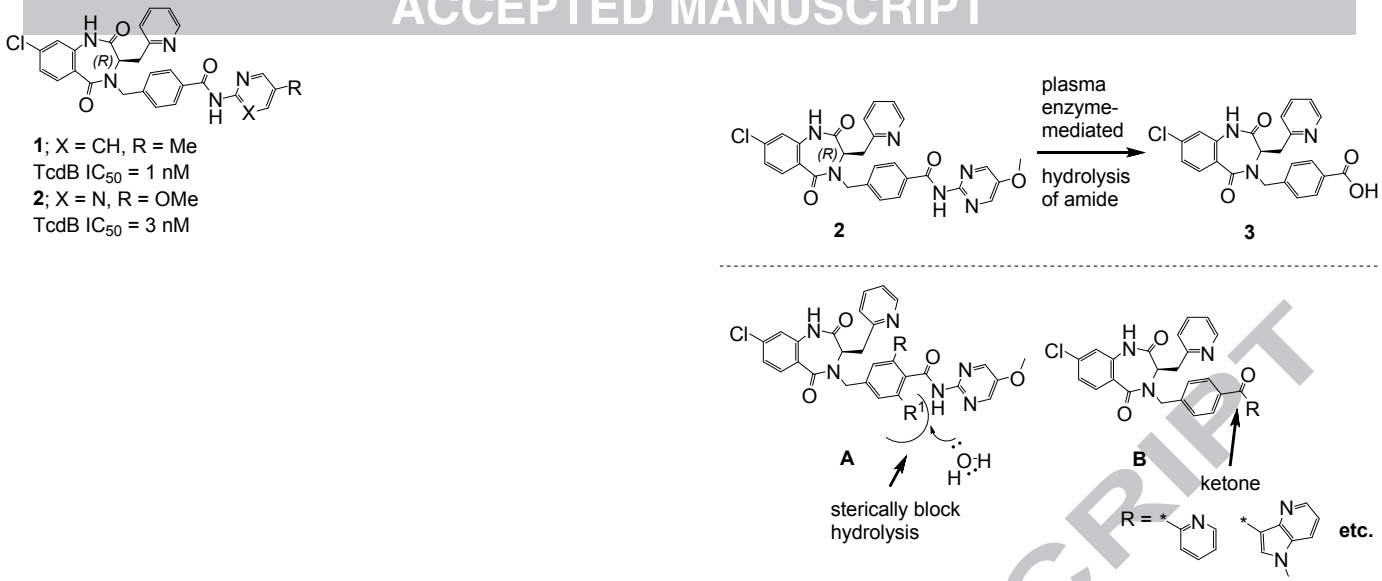


Figure 1. TcdB inhibitors

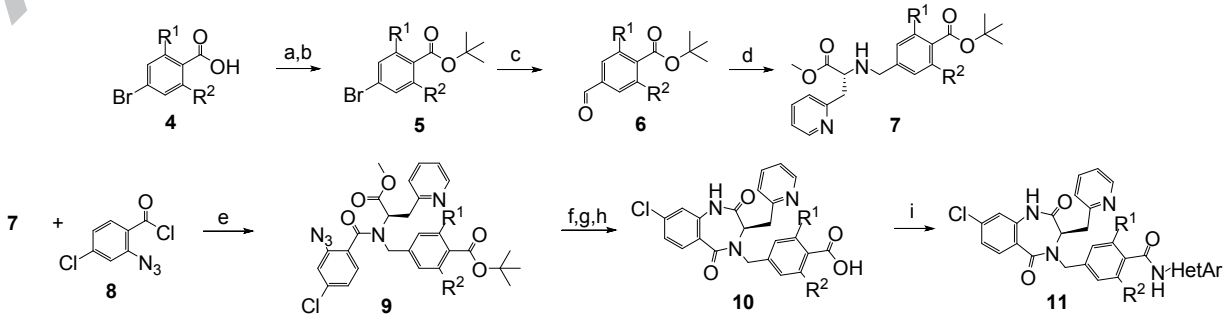
Described herein are efforts to identify potent inhibitors of TcdB with improved stability in mouse plasma, for possible advancement into rodent models of CDI. These efforts focused on structural changes in the region of the amide linkage that were intended to reduce hydrolysis. Two distinct strategies were employed to achieve this: 1) introduction of steric shields (*i.e.*, R^1 and/or R^2 substituents) onto the phenyl ring and next to the amide moiety in order to block the labile amide linkage (**A** in Figure 2); and, 2) replacement of the labile amide linkage with a ketone (**B** in Figure 2).

The compounds appearing in Tables 1 and 2 were synthesized *via* the general routes outlined in Schemes 1 and 2.[9] As illustrated in Scheme 1, the synthesis of amides **11** bearing substituents (R^1 and/or R^2) on the phenyl ring of the amide started with conversion of the appropriate 4-bromobenzoic acid to the acid chloride followed by reaction with potassium *tert*-butoxide to provide bromo-*t*-butylester **5**. Metal halogen exchange with *n*-BuLi at low temperature, and reaction of the resultant aryllithium intermediate with DMF provided the aldehyde **6**. Reductive amination of **6** with (*R*)-methyl 2-amino-3-(pyridin-2- yl)propanoate hydrochloride provided secondary amine **7**. Acylation with acid chloride **8**[10] gave azidobenzamide intermediate **9**. Conversion of **9** to the benzodiazepinedione-based carboxylic acid **10** was achieved *via* an aza-Wittig reaction[11] followed by acidic hydrolysis of the resultant iminoether intermediate and finally deprotection of the *tert*-butyl ester with TFA in DCM. Finally, arylamides **11** were prepared by reaction of carboxylic acid **10** with aryl amines ($ArNH_2$) using the coupling reagent HATU and heating in a microwave reactor. As shown in Scheme 2, the synthesis of heteroaryl ketones **13** started with conversion of carboxylic acid **3** to the corresponding Weinreb amide **12**. The Weinreb amide was then reacted with either lithiated-heteroaryls or heteroaryl Grignard reagents to provide the heteroaryl ketones **13**.

The compounds in Tables 1 and 2 were evaluated for their ability to inhibit the UDP-glucose hydrolysis activity of TcdB.[12]

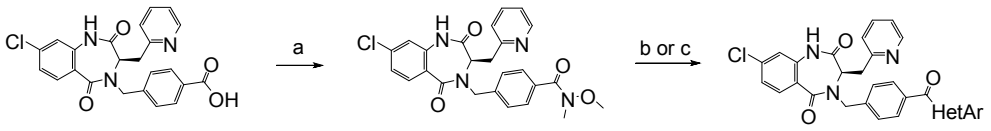
Figure 2. Plasma enzyme-mediated hydrolysis of amide **2** to give carboxylic acid **3**. Strategies to improve plasma stability: **A** - introduce steric shields (R^1 and/or R^2 around amide linkage; **B** - replace labile amide linkage with ketone.

We prepared a number of N-heteroaryl amide-based analogs containing phenyl substituents (R^1 and/or R^2) intended to block amide hydrolysis (Table 1). To determine whether or not substitution of the phenyl ring would be tolerated from a potency perspective we first prepared analogs of carboxylic acid **3** (IC_{50} = 0.18 μM) containing either a single chloro substituent (compound **10a**) or two chloro substituents (compound **10b**). Compound **10a** was only 2 to 3-fold less potent than compound **3**, but compound **10b** was more than an order of magnitude less potent than **10a**. Having previously established that replacement of the carboxylic acid moiety in **3** with certain N-heteroaryl amides significantly increases affinity for the target,[7] we then prepared a number of analogs containing a single substituent in the *ortho*-position with relation to the labile amide functionality. Substitution with chloro methyl, or methoxy, in combination with a variety of differently substituted N-pyridin-2-yl (R^2 = Cl; **11a-11e**, R^2 = Me; **11j-11n**, R^2 = OMe; **11q-11t**), N-pyrimidin-2-yl (R^2 = Cl; **11f, 11g**, R^2 = Me; **11o, 11p)** or N-pyrazin-2-yl (R^2 = Cl; **11h, 11i)** amides provided analogs with good binding affinity, with IC_{50} values ranging from 3 to 41 nM. In order to ascertain if this strategy could retard amide hydrolysis, we tested the stability of **11b** in mouse plasma. Indeed, 100% of parent compound remained after 2 hours following incubation with mouse plasma. In comparison, no parental **2** remained after 2 hours following incubation with mouse plasma.



Scheme 1. Reagents and conditions: a) oxalyl chloride, DMF (cat), DCM; b) KO*t*-Bu, THF, 0 °C to 23 °C, 16 h; c) *n*-BuLi, THF, -100 °C, 30 min; DMF, -100 °C, THF, 45 min; d) (*R*)-methyl 2-amino-3-(pyridin-2-yl)propanoate hydrochloride, Et_3N, MeOH, 30 min; $NaCNBH_3$, HOAc, MeOH, 16 h; e) DIPEA, DCM, DMAP (cat); f) $P(Bu)_3$, toluene, 70 °C; g) TFA/H_2O/THF (1:1:10); h) 20% TFA/DCM; i) $HetArNH_2$, HATU, DIPEA, NMP, 4Å molecular sieves, microwave @

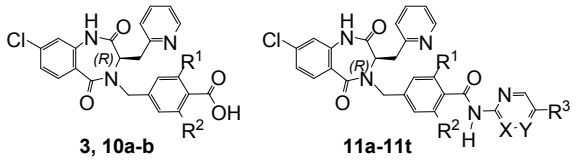
100 °C



3 **12** **13**

Scheme 2. Reagents and conditions: a) CDI, DCM; N,O-dimethylhydroxyl amine hydrochloride, DIPEA; b) XMg-HetAr (X = Cl or Br), THF, 0 °C or 23 °C; c) Li-HetAr, THF, -78 °C.

Table 1. SAR – N-heteroaryl amides containing steric shields



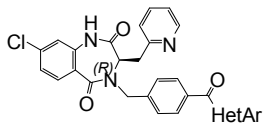
3, 10a-b **11a-11t**

Compd	*R^1*	*R^2*	*R^3*	*X*	*Y*	*TcdB, IC_{50}[a] (μM)*	*CHO cell TcdB, IC_{50}[a] (μM)*	*CHO cell TcdA, IC_{50}[a] (μM)*	*HLM/MLM, % rem. @ 15 min[b]*
3	H	H	-	-	-	0.18 (±0.08)	ND	ND	ND
10a	H	Cl	-	-	-	0.48 (±0.25)	ND	ND	ND
10b	Cl	Cl	-	-	-	7.4 (±1.3)	ND	ND	ND
11a	H	Cl	Me	CH	CH	0.005 (±0.001)	0.024 (±0.009)	0.58 (±0.11)	57/57
11b	H	Cl	OMe	CH	CH	0.005 (±0.002)	0.061 (±0.070)	0.76 (±0.072)	100/71
11c	H	Cl	F	CH	CH	0.004 (±0.000)	0.13 (±0.023)	1.8 (±0.36)	88/69
11d	H	Cl	CF_3	CH	CH	0.018 (±0.014)	0.25 (±0.14)	6.3 (±2.6)	94/92
11e	H	Cl	CN	CH	CH	0.008 (±0.002)	0.052 (±0.027)	ND	90/84
11f	H	Cl	Me	N	CH	0.004 (±0.001)	0.13 (±0.052)	1.5 [n=1]	79/33
11g	H	Cl	OMe	N	CH	0.004 (±0.001)	0.053 (±0.045)	0.92 (±0.45)	73/38
11h	H	Cl	Me	CH	N	0.009 (±0.004)	0.22 (±0.45)	2.1 (±0.46)	81/79
11i	H	Cl	OMe	CH	N	0.021 (±0.002)	0.49 (±0.19)	3.4 [n=1]	100/68
11j	H	Me	Me	CH	CH	0.007 (±0.002)	0.061 (±0.004)	0.86 (±0.11)	61/31
11k	H	Me	OMe	CH	CH	0.010 (±0.013)	0.071 (±0.021)	0.48 (±0.15)	68/51
11l	H	Me	F	CH	CH	0.008 (±0.007)	0.062 (±0.015)	0.86 (±0.14)	85/60
11m	H	Me	CF_3	CH	CH	0.020 (±0.017)	0.17 (±0.11)	2.4 (±1.2)	100/99
11n	H	Me	CN	CH	CH	0.003 (±0.001)	0.078 (±0.072)	ND	90/70
11o	H	Me	Me	N	CH	0.004 (±0.001)	0.13 (±0.063)	3.0 (±0.51)	74/44
11p	H	Me	OMe	N	CH	0.004 (±0.000)	0.029 (±0.016)	0.69 (±0.14)	82/39
11q	H	OMe	Me	CH	CH	0.009 (±0.002)	1.9 (±2.5)	11(±6.6)	62/49
11r	H	OMe	OMe	CH	CH	0.005 (±0.002)	0.13 (±0.082)	1.1 (±0.21)	78/71
11s	H	OMe	F	CH	CH	0.014 (±0.007)	1.1 (±0.56)	17 (±9.3)	94/67
11t	H	OMe	CF_3	CH	CH	0.041 (±0.022)	0.66 (±0.26)	31 (±7.4)	100/83

[a] Values are means of at least two experiments unless otherwise indicated, and standard deviation is given in parentheses; [n=1] indicates value is from a single experiment; ND = not determined. [b] Compound assayed in human liver microsomes (HLM) or mouse liver microsomes (MLM), percentage compound remaining unchanged after 15 min was determined.

Next, we investigated replacement of the N-heteroaryl amide moiety with various heteroaryl ketones (Table 2). As described elsewhere,[7] we initially designed N-heteroaryl amide-based inhibitors based on a hypothesis that the N-heteroaryl amide moiety could bind to the Mn^{+2} cofactor of TcdB *via* a bidentate chelate interaction. This hypothesis has been subsequently confirmed by x-ray crystallography (Pechik (2018), manuscript in preparation). Therefore the heteroaryl ketone-based analogs that were prepared each possessed strategic placement of a heteroatom in the heteroaryl moiety to allow possible bidentate coordination of the manganese cofactor in the enzyme (Figure 3). First we prepared the 2-pyridyl ketone **12a**, envisioning that it could coordinate the Mn^{+2} cofactor in a bidentate fashion akin to **B** as illustrated in Figure 3. This analog possessed moderate potency against TcdB (IC_{50} = 45 nM). Methylation of the pyridyl ring was tolerated at C(5) (**12c)** and at C(4) (**12d)** resulting in analogs with affinity at TcdB comparable to that of **12a**.

Table 2. SAR – Heteroaryl ketones

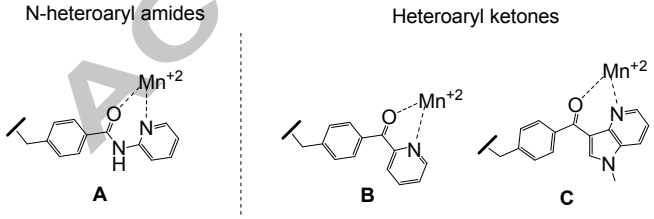


12a-12j

Compd	HetAr	TcdB, IC_{50}[a] (μM)	CHO cell TcdB, IC_{50}[a] (μM)	CHO cell TcdA, IC_{50}[a] (μM)	HLM/MLM, % rem. @ 15 min[b]
12a		0.045 (±0.014)	4.2 (±2.0)	37 (±1.0)	64/79
12b		0.34 (±0.06)	ND	ND	25/3
12c		0.038 (±0.011)	5.9 [n=1]	23 [n=1]	57/68
12d		0.074 (±0.073)	ND	ND	26/48
12e		0.19 (±0.14)	> 100 [n=1]	> 100 [n=1]	49/33
12f		0.23 (±0.08)	> 100 [n=1]	> 100 [n=1]	44/16
12g		0.006 (±0.004)	0.33 (±0.14)	5.3 (±0.81)	67/76
12h		0.002 (±0.001)	1.8 (±0.69)	18 (±2.3)	87/93
12i		0.008 (±0.006)	0.66 (±0.35)	8.1 (±2.8)	44/27
12j		0.061 (±0.015)	1.6 (±0.34)	ND	15/44

[a]Values are means of at least two experiments unless otherwise indicated, and standard deviation is given in parentheses; [n=1] indicates value is from a single experiment; ND = not determined. [b] Compound assayed in human liver microsomes (HLM) or mouse liver microsomes (MLM); percentage of compound remaining unchanged after 15 min was determined.

However methylation of the pyridyl ring at C(6) (**12b**) led to an 8-fold drop in potency *vs*. **12a**. Replacement of the 2-pyridyl ring with 1-methylimidazol-2-yl (**12e**) or 1-methylimidazol-4-yl (**12f**) led to a five-fold loss of potency in both instances. Next we investigated introduction of bicyclic heteroaromatics (**12g-j**) which we envisioned could coordinate the Mn^{+2} cofactor in a bidentate fashion analogous to **C** as shown in Figure 3. Azaindoles **12g** and **12h** and imidazopyridine **12i** all possessed excellent binding affinity at TcdB;



of these,

Figure 3. Proposed bidentate coordination of the Mn^{+2} cofactor in TcdB by a representative N-heteroaryl amide (**A**) or by representative heteroaryl ketones (**B** and **C**).

12h was the most potent (IC_{50} = 2nM). The triazolopyridine **12j** had decreased affinity (IC_{50} = 61 nM).

Most of the compounds in Tables 1 and 2 underwent further evaluation in a whole cell assay; in which their ability to inhibit induction of apoptosis in cultured mammalian cells (CHO) by full-length toxin (TcdB and/or TcdA) was determined.[13] A number of amide-based inhibitors (Table 1) with good binding affinity at TcdB demonstrated submicromolar activity in the cell-based assay in the presence of TcdB. Activity, albeit more modest, was also demonstrated in the cell-based assay in the presence of TcdA for this class of inhibitors. Methoxy substitution of the phenyl ring (R^2) generally had an adverse effect on activity in the whole cell assays *vs*. chloro or methyl substitution (*e.g.*, compare **11q** to **11a** and **11j**). Only two of the more potent ketone-based inhibitors (**12g** and **12i**)

demonstrated submicromolar activity in the cell-based assay in the presence of TcdB. *In vitro* microsomal stability, in the presence of human liver microsomes (HLM) or mouse liver microsomes (MLM), was also assessed for both amide and ketone-based inhibitors to identify possible candidates for progression into mouse pharmacokinetic (PK) experiments.

Table 3. Pharmacokinetic parameters for **11d** in CD-1 mice after *i.v.* and *p.o.* dosing

Route (dose)[a]	*i.v.* (1 mg/kg)	*p.o.* (10 mg/kg)
Cl (mL/min/kg)	28	
$T_{1/2}$ (h)	2.6	
V_z (L/kg)	6.2	
$AUC_{0\text{-}inf}$ (ng/mL h)	592	9423
C_{max} (ng/mL)	312	1533
T_{max} (h)		0.25
F (%)		159

[a]Vehicle for *i.v.* dosing is 40% PEG400/5% ethanol/ 55% water (v/v/v), and for *p.o*. dosing is 20% PEG400/80% (0.5% (w/v) CMC containing 0.25% (v/v) Tween 80) (v/v).

The main focus of our lead optimization program was to identify compounds for progression into mouse and hamster models of CDI. Therefore the pharmacokinetic profile of various analogs demonstrating both submicromolar activity in the whole cell assay in the presence of TcdB, and reasonable stability in the mouse microsome stability assay (*i.e.*, MLM stability > 50% remaining at 15 min), was determined after oral (*p.o.*) administration in CD-1 mice. Compound **11d** emerged as having particularly high exposure after oral dosing. Therefore full PK (*i.e.*, both intravenous (*i.v.*) and oral (*p.o.*) dosing) was subsequently obtained for this compound (Table 3). Compound **11d** showed moderate clearance (Cl) and high apparent volume of distribution during the terminal phase (V_z) leading to a moderate elimination half-life ($T_{1/2}$) of 2.6 hours. After oral dosing (10 mg/kg), **11d** exhibited rapid absorption (T_{max} = 0.25 h) and excellent oral bioavailability (F > 100%). Hamster PK was also determined after *p.o.* administration of compound **11d** (Table 4) since a hamster survival model is widely considered the gold standard amongst animal models for development of potential CDI therapeutics.[14] In hamsters, excellent exposure was observed with low apparent clearance (Cl/F) and an elimination half-life of 10 hours. Additionally, rapid absorption (T_{max} = 0.25 h) was observed, as it was in mice.

Table 4. Pharmacokinetic parameters for **11d** in hamsters after *p.o.* dosing

Route (dose)[a]	*p.o.* (10 mg/kg)
Cl/F (mL/min/kg)	7.8
$T_{1/2}$ (h)	10
AUC_{inf} (ng/mL h)	21477
C_{max} (ng/mL)	2747
T_{max} (h)	0.25

[a]Vehicle for *i.v.* dosing is 40% PEG400/ 5% ethanol/ 55% water (v/v/v), and for *p.o.* dosing is 20% PEG400/80% (0.5% (w/v) CMC containing 0.25% (v/v) Tween 80) (v/v).

Given its inhibitory activity at TcdB, activity in the whole cell assay, and favorable PK profiles in both mouse and hamster, compound **11d** was selected for further profiling *in vivo*. Compound **11d** was demonstrated to be efficacious in both mouse and hamster models of CDI, for which the details have been previously disclosed.[15] In a hamster survival model, oral administration of **11d** (50 mg/kg *b.i.d.*) for 7 days post-infection significantly increased survival with a median survival time of 12.3 days (Table 5).[16] In contrast, the median survival time in the vehicle group was only 2.3 days (with nine of ten animals expiring within three days). In comparison, oral administration of the positive control vancomycin (5 mg/kg *b.i.d.*) for 5 days post-infection significantly increased survival with a median survival time of 12.0 days.

Table 5. Summary of results from *in vivo* profiling of **11d** in a hamster survival model of CDI[15]

Compound	Dosing Regimen[a]	Duration of dosing	Median survival time	p-value [b]
11d[c]	50 mpk, b.i.d.	7 days	12.3	0.002
Vancomycin[d]	5 mpk, b.i.d.	5 days	12.0	0.006
Vehicle[c]	b.i.d.	-	2.3	

[a] Oral (*p.o.*) administration for all three groups; mpk = mg/kg; b.i.d. = twice daily.
[b] p-value is for comparison between treatment group (**11d** or vancomycin) and vehicle group; data were analyzed by log-rank (Mantel-Cox) test.
[c] Dosing vehicle is 20% PEG400/80% (0.5% (w/v) CMC containing 0.25% (v/v) Tween 80) (v/v).
[d] Dosing vehicle is DI water

In conclusion, a series of TcdB inhibitors, exemplified by lead compounds **1** and **2**, was further optimized with respect to plasma stability. In general, compounds showed good affinity for TcdB. Additionally, a number of analogs demonstrated good activity in a whole cell assay in the presence of either TcdB or TcdA and also possessed good stability in the presence of human or mouse liver microsomes. Compound **11d** exhibited a promising pharmacokinetic profile in both mouse and hamster and was profiled *in vivo* in mouse and hamster models of CDI. In a hamster survival model, oral administration of **11d** significantly extended life span *vs*. vehicle control. As such, compound **11d** and congeners represent an intriguing starting point for further development of TcdB inhibitors as a new therapeutic approach to CDI infection.

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Highlights

- Highly potent *C. diff.* toxin B (TcdB) inhibitors identified
- Improved plasma stability enabled compound progression into *in vivo* models of CDI
- Compound **11d** efficacious in hamster survival model of CDI upon oral dosing