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Decreasing HepG2 Cytotoxicity by Lowering the Lipophilicity of Benzo[d]oxazolephosphinate Ester Utrophin Modulators

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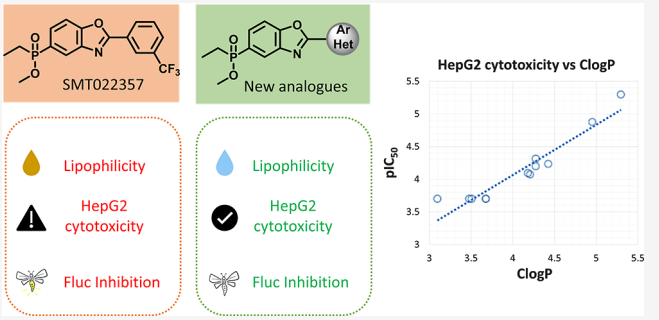
ABSTRACT: Utrophin modulation is a disease-modifying therapeutic strategy for Duchenne muscular dystrophy that would be applicable to all patient populations. To improve the suboptimal profile of ezutromid, the first-in-class clinical candidate, a second generation of utrophin modulators bearing a phosphinate ester moiety was developed. This modification significantly improved the physicochemical and ADME properties, but one of the main lead molecules was found to have dose-limiting hepatotoxicity. In this work we describe how less lipophilic analogues retained utrophin modulatory activity in a reporter gene assay, upregulated utrophin protein in dystrophic mouse muscle cells, but also had improved physicochemical and ADME properties. Notably, ClogP was found to directly correlate with pIC_{50} in HepG2 cells, hence leading to a potentially safer toxicological profiles in this series. Compound **21** showed a balanced profile (H2K EC_{50}: 4.17 μM, solubility: 477 μM, mouse hepatocyte $T_{1/2}$ > 240 min) and increased utrophin protein 1.6-fold in a Western blot assay.

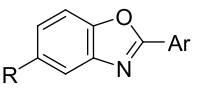


KEYWORDS: *Duchenne muscular dystrophy, utrophin upregulation, hepatotoxicity, phosphinate esters*

Duchenne muscular dystrophy (DMD) is an X-linked neuromuscular disorder caused by loss of function mutations on the dystrophin gene.[1] Though classified as a rare disease, it is one of the most common fatal genetic diseases affecting children.[1] Dystrophin is a critical component of the dystrophin-associated protein complex (DAPC) connecting the internal cytoskeleton to the surrounding extracellular matrix. In the absence of functional dystrophin, the muscles gradually degenerate, leading to inflammation, fibrosis, and failed cycles of regeneration.[2,3]

More than 30 years after the discovery of dystrophin,[4] there is still a paucity of treatment options for the disease, as the only disease-modifying therapeutics are targeting specific subpopulations and the standard of care is aimed mostly to alleviate the inflammation and other secondary effects. A promising therapeutic strategy that would be both disease modifying and could target all patient subpopulations is functional replacement of dystrophin with its structural paralogue utrophin.[2] Data from transgenic animal models[5−7] led to the initiation of drug discovery programs, the most advanced of which progressed to Phase 2 proof of concept clinical trials.

Ezutromid (Figure 1, **1**), formerly SMT C1100, a utrophin modulator initially discovered via a HTS using a firefly luciferase (Fluc) reporter gene assay,[8,9] led to promising results in animal models of DMD[10] and a safe profile in multiple



1 R: SO_2Et Ar: β-naphthalene
2 R: P(O)EtOMe Ar: *m*-CF_3-C_6H_5
3 R: P(O)EtOMe Ar: *p*-F-C_6H_5

Figure 1. Structures of ezutromid (**1**), SMT022357 (**2**), and SMT022332 ((+) enantiomer, **3**).

Phase 1 clinical trials.[11−13] Encouraging interim 24-week data in a Phase 2 trial (NCT02858362, Summit Therapeutics PLC, PhaseOut DMD) showed increase of utrophin protein and reduction of muscle fiber damage. However, the primary and secondary clinical end points were not met at the end of the trial and development of ezutromid was discontinued.[14] The lack of sustained efficacy has been attributed to extensive metabolism of ezutromid and induction of CYP1A (ezutro-

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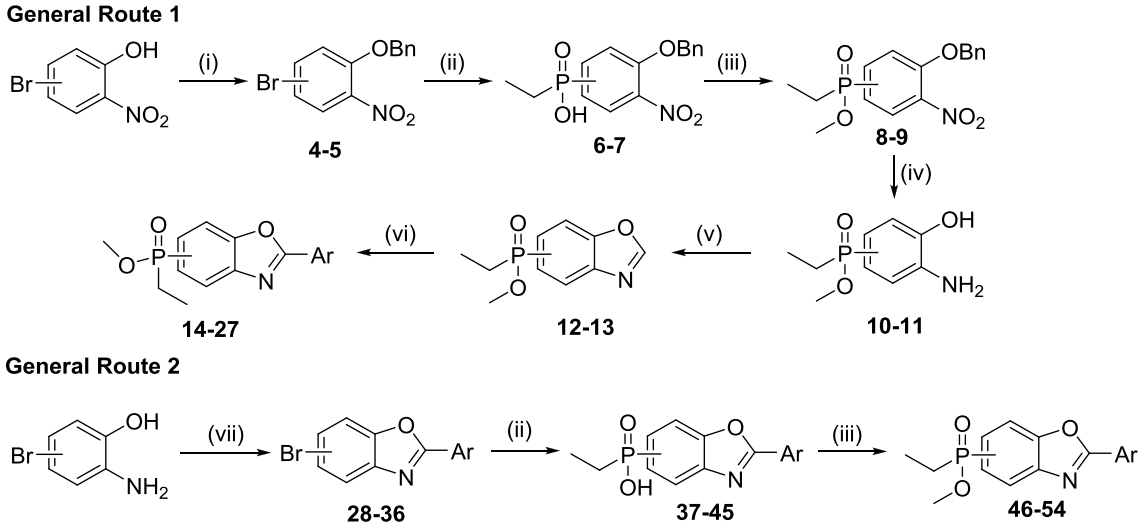
Scheme 1. General Synthetic Routes to Phosphinate Esters[a]



[a]Reagents and conditions: (i) BnBr, K_2CO_3, acetone, 60 °C, 6 h, (yields 97% and 74% for **4** and **5**, respectively); (ii) EtPH(O)OH, $Pd(OAc)_2$, Xantphos, DIPEA, DME/toluene, 90 °C, 2 h, (yields 71−98%); (iii) (1) $SOCl_2$, DMF, 70 °C, 1−2 h; (2) DIPEA, MeOH, DMAP, CH_2Cl_2, rt, 30 min (yields 41−85%) or MeOH, rt, 15 min for **9** (yield 81%); (iv) 10% Pd/C, EtOH/CH_2Cl_2, H_2, rt, 3 h (yields 94−96%); (v) $CH(OEt)_3$, PPTS, xylenes, 140 °C, 3 h (yields 91−95%); (vi) bromoaryl, $Pd(PPh_3)_2Cl_2$ or $Pd(dppf)Cl_2$, CuI, Cs_2CO_3, dioxane, 110 °C, 12 h (yields 7−40%) or bromoaryl, $Pd(OAc)_2$, N-Xantphos, 1 M LiHMDS, DME, 85 °C, 1 h, for **14a**, **15** (yields 22 and 12%, respectively) or chloroaryl, $In(OTf)_3$, dioxane, 100 °C, 24 h, (yield 26%); (vii) (1) ArCOCl, xylenes, 155 °C, 1 h; (2) MsOH, 155 °C, 2−3 h (yields 12−81%).

mid's main metabolic pathway),[15] which is further supported by reduction in ezutromid's exposure after repeated dosing in both healthy volunteers and DMD patients.[11−13]

A medicinal chemistry program was launched to improve ezutromid's suboptimal properties and led to a second generation of utrophin modulators.[16−18] Therein, a dual strategy was implemented to improve solubility by replacement of the sulfone moiety and to improve metabolic stability by replacement of naphthalene with halo- and heteroaryls. A promising lead molecule from this series (Figure 1, SMT022357, **2**) with an improved ADME profile increased levels of utrophin protein in skeletal, respiratory, and cardiac muscles, reduced regeneration, and improved muscle function when administered orally to *mdx* mice.[16] Further progression of SMT022357 (**2**) was halted, due to dose-limiting hepatotoxicity observed during subsequent maximum tolerated dose studies in rats. A structurally similar, but less lipophilic, analogue SMT022332 (Figure 1, (+)-enantiomer **3**) was instead progressed because of its encouraging activity and efficacy in *mdx* mice and its improved safety profile.[18]

During these studies, the relationship between lipophilicity and hepatotoxicity within this compound series was investigated. Several empirical studies have correlated lipophilicity with increased nonspecific binding and off-target effects, and a decreased chance of clinical success.[19,20] However, to our knowledge, there are few published studies that directly correlate lipophilicity with hepatotoxicity within a compound series.[21−25] Lipophilic compounds are more prone to extensive metabolism and thus may result more frequently in drug-induced liver injury (DILI).[26] As we observed extensive metabolism and *in vivo* hepatotoxicity in rodents with **2**, which translated in *in vitro* cellular hepatotoxicity for **2** and other related examples,[18] we felt that reducing lipophilicity would be a good strategy for optimizing the series. To achieve this, analogues that bear the -CHF_2 and -$OCHF_2$ groups and their pyridyl equivalents were synthesized as they are predicted to have lower lipophilicity than **2**.[27]

To access the phosphinate esters of the benzo[*d*]oxazoles, two main synthetic routes were devised (Scheme 1). In General Route 1, the phosphinate esters were synthesized first, followed by cyclization to the benzoxazole, and finally attachment of the C(2)-aryl via C−H activation. Bromo 2-nitrophenols were *O*-benzylated (**4**−**5**) and substituted with phosphinic acid (**6**−**7**), and the resulting acids were esterified to their corresponding ethyl phosphinate esters (**8**−**9**). Subsequent hydrogenation led to 2-aminophenols **10** and **11** in a one-step reduction/deprotection, which in turn gave the benzo[*d*]oxazoles **12** and **13** in a reaction with diethoxymethoxyethane in the presence of PPTS. The final products (**14**−**27**) were obtained with C−H activation using $Pd(PPh_3)_2Cl_2$ or $Pd(dppf)Cl_2$ and CuI as catalysts. In another variation $Pd(OAc)_2$, Xantphos, and 1 M LiHMDS, in DME, were used (**14**, **15**). This led to hydrolysis of the ester in the case of **12**, and the resulting phosphinic acid (**14a**) was reacted with MeOH to give the final product **14**.

General route 2 was described in detail in our previous work.[18] In brief, intermediate 2-arylbromobenzo[*d*]oxazoles (**28**−**36**) were derived from the condensation of 2-aminophenols with the respective acid chloride, followed by acid catalyzed dehydration. The final products were obtained from the bromobenzo[*d*]oxazoles in a Pd-catalyzed cross-coupling with ethyl phosphinic acid (**37**−**45**) and were then converted to the methyl phosphinate esters through formation of the phosphinic chloride and reaction with MeOH (**46**−**54**).

Utrophin modulation activity was assessed using a reporter gene assay in the previously reported cell line H2K-*mdx* utrnA-luc (H2K), a murine myoblast cell line that contains a stably integrated 8.4 kb fragment of the human utroA promoter linked to a firefly luciferase gene.[15,16,18] In the reporter gene assay (Table 1), most of the *o*-substituted analogues appeared to be inactive (**18**, **23**, **50**, **54**); however, the *o*-difluoromethyl

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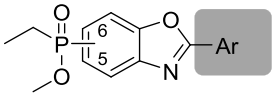
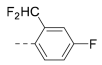
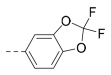
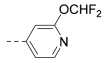
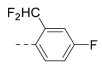
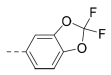
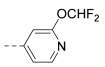
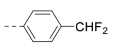
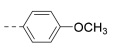
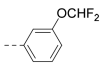
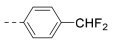
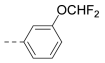
Table 1. Structure Activity Relationships (SARs) of C2 (Hetero)aryl Analogues



#	P-sub	Ar	H2K EC_{50}[a]	Fluc IC_{50}[b]	$logD_{7.4}$[c]
2	5		1.28 ± 0.25[d]	12.1 ± 0.1	3.80
3	5		0.244 ± 0.020	0.115 ± 0.025	2.80
14	5		1.26 ± 0.10	5.73 ± 0.07	2.80
15	5		0.89 ± 0.16	2.48 ± 0.56	3.09
16	5		0.58 ± 0.14	IA[e]	3.40
17	5		0.157 ± 0.038	0.088 ± 0.007	3.19
18	5		IA	IA	2.45
19	5		0.530 ± 0.015	23.6 ± 0.1	2.40
20	5		0.309 ± 0.042	>30	2.20
21	5		4.17 ± 0.59	IA	2.44
22	5		5.9 ± 1.1	IA	1.86
23	6		IA	IA	3.59
24	6		IA	>30	3.60
25	6		IA	>30	2.60
26	6		>50	IA	2.41
27	6		IA	IA	2.08
46	5		0.75 ± 0.28	IA	3.06
47	5		0.51 ± 0.16	0.19 ± 0.03	ND[f]
48	5		0.677 ± 0.085	>30	ND
49	5		IA	IA	ND
50	5		IA	IA	2.20
51	6		0.50 ± 0.16	IA	3.33
52	6		1.19 ± 0.33	IA	2.98
53	6		>50	IA	3.20
54	6		IA	IA	1.98

[a]Firefly luciferase reporter gene assay reported as EC_{50} ± SD (μM) of 3 biological replicates (3 technical replicates per experiment). [b]Firefly luciferase enzymatic assay reported as IC_{50} ± SD (μM) of 2 biological replicates (3 technical replicates per experiment). [c]logD is calculated with the shaking flask method at pH = 7.4 from the partition between n-octanol/water. [d]Reference: *Tetrahedron* **2020**, *76*, 130819. [e]IA, inactive. [f]ND, not determined.

analogues **14**, **16**, and **51** were active. Similarly, analogues bearing the phosphinate ester substituent in position 6 were inactive (**23**−**27**, **53**−**54**), apart from the *o*- and *p*-difluoromethyl analogues (**51**−**52**). Finally, pyridyl analogue **49** was also found to be inactive.

In our previous work,[18] confirmation of utrophin upregulation activity with an orthogonal assay was accomplished with labor-intensive Western blots. As we had previously demonstrated that ezutromid is a noncompetitive inhibitor of the firefly luciferase enzyme (Fluc),[28] and recognizing the complications Fluc inhibition can cause in the interpretation of reporter gene assays,[29] we sought a more facile counter-screen to include in our assay cascade. Quantification of utrophin protein using Western blots was still used for confirmation of utrophin upregulation activity, but the inclusion of a biochemical assay to directly measure inhibition of recombinant Fluc enzyme activity in our screening workflow allowed an early detection of compounds which exhibited interference in the H2K reporter assay.

Interestingly, despite their structural similarity with ezutromid,[29] several of the new analogues showed little or no inhibitory activity on Fluc (Table 1), with the exception of methoxy analogues **17** and **47**. Two difluoromethyl analogues (**14**, **15**) inhibited Fluc in the low micromolar range, and **19** was even less potent (IC_{50} of 23.6 μM). The previously reported analogue **3** was found to inhibit Fluc with an IC_{50} in the nanomolar range, while **2** was less potent with a low micromolar IC_{50}. None of the pyridyl-containing analogues inhibited Fluc. Interestingly, we observed a trend that less lipophilic compounds ($logD_{7.4}$ < 2.5) showed reduced or no activity in the Fluc assay, while several examples still showed activity in the H2K reporter assay. This finding is consistent

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with the work of others who have shown that this Fluc has a preference for binding lipophilic molecules.[30]

Decreasing lipophilicity significantly improved aqueous solubility, as was expected (Table 2). All the newly synthesized analogues were equally or more water-soluble than **2** and **3**. The extent of plasma protein binding varied between different analogues. Pyridyl derivatives were found to bind <90%, with the exception of **25**. All the new molecules were highly permeable in Caco-2 cells, and more importantly no efflux was noted for any of the new examples ([B-A]/[A-B] < 1). Finally, the metabolic stability of the compounds was found to vary considerably. Analogues **14**, **16**−**18**, **50**, **51**, and **54** were cleared very quickly in mouse hepatocytes. Interestingly, most of these analogues bear electron withdrawing groups in the *ortho* position, which may suggest a potential opening of the more electrophilic benzoxazole ring in these examples, enabling further CYP oxidation. On the other hand, **20**, **21**, **25**−**27**, and **49**, *m*-substituted pyridyl analogues, were the most metabolically robust. Apart from *o*-substituted analogues **50** and **54**, all pyridyl analogues had improved metabolic stability compared to their phenyl counterparts.

Table 2. Structure Property Relationships (SPR) of the Synthesized Analogues

			Caco-2[c]		mHep Stability	
#	Sol[a]	mPPB[b]	A-B	B-A	Cl_{int}[d]	$T_{1/2}$[e]
2	468	97	18.0	16.4	11.9	58
3	378	95	18.0	15.7	9.12	76
14	>250	97	16.2	11.2	>250	<3
15	>250	94	14.2	10.1	34.5	20
46	530	98			8.99	77
16	518	97			53.0	13
17	>250	96	15.7	12.6	>250	<3
18	366	97	18.8	15.7	>250	<3
19	576	98	12.7	11.7	4.49	154
20	733	86	22.3	20.6	<2.89	>240
21	477	75			<2.89	>240
22	440	74			<2.89	>240
23	469	97	12.3	11.5	18.2	38
24	574	98	17.2	16.2	4.49	154
25	834	93	21.8	16.9	<2.89	>240
26	603	80			<2.89	>240
27	487	77			<2.89	>240
47	265		13.3	10.7	12.4	56
48			13.3	9.37	54.4	13
49			12.3	11.0	<2.89	>240
50	854	89			184	4
51	446	97			>250	<3
52	556	96			8.77	79
53	8.4	96	18.1	15.9	19.0	37
54	699	76			142	5

[a]Aqueous kinetic solubility (μM). [b]mPPB, murine plasma protein binding (% bound). [c]Caco-2 permeability assay (P_{app} (10^{-6} cm/s)). [d]Murine hepatocyte intrinsic clearance (μL/min/10^6 cells). [e]Murine hepatocyte half-life (min).

Confirmation of utrophin modulation activity for representative analogues (**20**, **21**, and **52**) was determined using Western blot to quantify the increase of the utrophin protein levels in dystrophic mouse muscle cells (LUmdx), as before.[18] Pyridyl analogues **20** and **21** were selected because of their combination of potency in the H2K assay, lack of Fluc inhibition, and optimal ADME properties. Analogue **52** was selected as a representative haloaryl derivative, since it had a well-balanced overall profile. All three analogues were found to increase utrophin by approximately 1.3−1.6-fold (Figure 2).

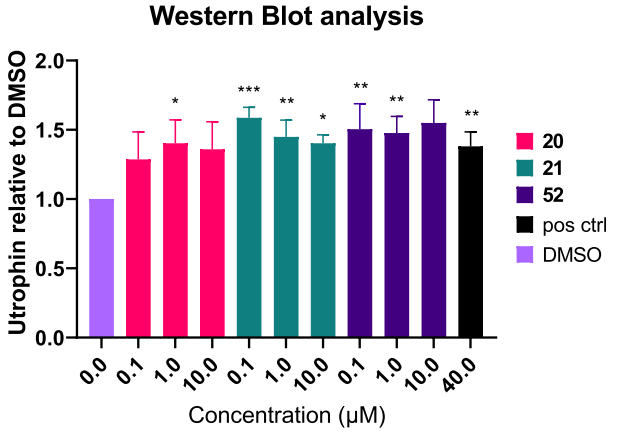

Figure 2. Treatment with **20**, **21**, and **52** increases utrophin protein in LUmdx cells. *p < 0.05, **p < 0.01, ***p < 0.001. pos ctrl = positive control.

The modest increase in utrophin levels in combination with the variability and low sensitivity of this assay are likely responsible for the apparent lack of a dose−response; however, these results demonstrate utrophin modulation in a cellular context, confirming the activity in this series of new analogues.

In an attempt to visualize the move from a suboptimal physicochemical property space, we plotted selected physicochemical properties of the previously synthesized phosphinate ester analogues against the new ones (Figure S1, Table S1). At a glance, it was observed that the optimal chemical space proposed by Gleeson (Figure S1a, gray area defined with clogP < 4 and MW < 400)[31] is mostly occupied by new analogues. These "not large/not greasy" molecules were found to have the best drug developability profiles according to a separate analysis by Ritchie et al.[32] Considering the more polar nature of some of the analogues, we went on to experimentally measure the logD of selected analogues and observed that indeed they move almost entirely into the desired area (Figure S1b).

The discovery that **2** was hepatotoxic during the maximum tolerated dose study in rats,[18] and the observation that **2** and related compounds showed cytotoxicity in vitro in liver cells, led us to probe the new analogues for their cytotoxic potential in HepG2 cells (Table 3) as an early indication of hepatotoxicity. Cytotoxicity was determined by quantification of cellular ATP levels, using an evolved luciferase known to have substantially reduced promiscuity.[33] Increased lipophilicity has been known to contribute to compound promiscuity and further toxicological outcomes.[19,20,34,35] From the results in Table 3 it was apparent that the most toxic compounds are those with high lipophilicity and low TPSA, in good agreement with other literature reports.[19]

Next, we sought to quantify the relationship between HepG2 cytotoxicity with key physicochemical properties. As our sample size described herein was rather small, we also expanded this analysis to include all synthesized compounds in this series (Table S2, Figures 3 and S2).

Plotting pIC_{50} against TPSA did not give a statistically significant correlation (data not shown); however, pIC_{50}

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Table 3. Cytotoxicity of Selected Compounds in HepG2 Cells

#	ClogP[a]	TPSA[a]	IC_{50} (μM)
2	4.43	62	58.4
3(e2)[b]	3.68	62	200
20	3.10	84	200
24	5.35	81	68.9
47	3.51	71	200
49	3.48	75	200
55[c]	5.19	62	0.02
56[c]	4.96	62	13.3
57[c]	5.30	62	5.04

[a]Generated with OSIRIS Datawarrior version 5.0.0 © 2002−2019 Idorsia Pharmaceuticals Ltd. [b]Cpd **27e2** in our previous work.[18] [c]Cpds **55**−**57** are reported in our previous work[18] and are esters of **2** with isopropanol, cyclopropanol, and cyclobutanol, respectively.

against ClogP derived equation S1 (n = 14, R^2 = 0.46, Figure S2a). Examining the graph, we observed two compounds that diverted from the group and considered removing them as potential outliers. Indeed, after removal of the two, the correlation between clogP and cytotoxicity appeared to improve as shown in equation S2 (n = 12, R^2 = 0.91, Figure 3).

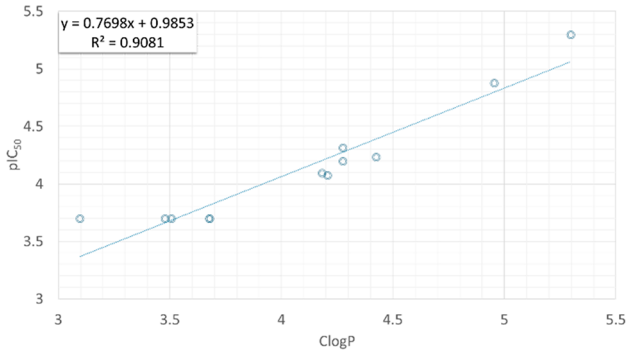

Figure 3. Correlation between cytotoxicity in HepG2 cells and lipophilicity as expressed from their pIC_{50} and clogP plot after the removal of two outliers (n = 12). Predicted clogP (a) was generated with OSIRIS Datawarrior version 5.0.0 © 2002−2019 Idorsia Pharmaceuticals Ltd.

The aberrant behavior of the two outliers may partly be explained by gaps in the ability of software to predict clogP successfully. Analogue **24** was predicted to be the most lipophilic compound with Datawarrior; however, it was almost one log unit less polar by the two other calculators (Table S3). Similarly, analogue **55** was significantly more lipophilic when other calculators were used. However, this analysis does not rule out the participation of other mechanisms contributing to the significantly higher cytotoxicity of this compound in HepG2 cells. Importantly utrophin modulation activity in H2K cells did not increase with clogP; plotting the ratio of EC_{50} in H2K cells and the IC_{50} in HepG2 cells showed only a weak but negative correlation (Table S2, Figure S2).

Aiming to improve the ADMET profile of existing benzo[*d*]oxazole phosphinate utrophin modulators, we implemented a strategy of reducing lipophilicity in the design of new analogues. A facile synthetic route was devised that allows faster access to analogues involving C−H activation of the intermediate benzoxazole phosphinate ester. The new analogues were shown to be in a more optimal chemical space and have better drug developability potential. The decrease in lipophilicity gave inactive or only weakly active analogues as Fluc inhibitors, while maintaining utrophin modulatory activity. We were able to show that cytotoxicity in HepG2 cells is directly proportional to lipophilicity and that reducing lipophilicity led to analogues that were only mildly or not cytotoxic at all in HepG2 cells.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00405.

Experimental details describing the biological assays and the synthesis and characterization of compounds. Computational and experimental calculations of physicochemical properties, graphs and equations. NMR and mass spectroscopy spectra of the synthesized compounds. (PDF)

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■ ABBREVIATIONS

ADMET, administration distribution metabolism excretion toxicity; CYP, cytochrome P450; DAPC, dystrophin associated complex; DIPEA, N,N-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMD, Duchenne muscular dystrophy; DME, dimethoxyethane; dppf, 1,1′-bis-(diphenylphosphino)ferrocene; Fluc, firefly luciferase; H2K, reporter cell line H2K-*mdx* utrnA-luc; HMDS, hexamethyldisilazane; LU*mdx*, dystrophic mouse cells; PPB, plasma protein binding; PPTS, pyridinium *p*-toluenesulfonate; SAR, structure−activity relationship; SPR, structure−property relationship; TPSA, topological polar surface area.

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