Published in final edited form as:

Bioorg Med Chem Lett. 2015 September 15; 25(18): 3897–3899. doi:10.1016/j.bmcl.2015.07.040.

N-Aryl Benzenesulfonamide Inhibitors of [³H]-Thymidine Incorporation and β -Catenin Signaling in Human Hepatocyte-derived Huh-7 Carcinoma Cells

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Abstract

Structure-activity relationships (SAR) in 2,5-dichloro-*N*-(2-methyl-4-nitrophenyl)benzenesulfonamide (FH535) were examined as part of a program to identify agents that inhibit the Wnt/ β -catenin signaling pathway that is frequently upregulated in hepatocellular carcinoma (HCC). FH535 was reported as an inhibitor of both β -catenin in the Wnt signaling pathway and the peroxisome proliferator-activated receptor (PPAR). A β -catenin/T-cell factor (TCF)/Lymphoid-enhancer factor (LEF)-dependent assay (*i.e.*, luciferase-based TOPFlash assay) as well as a [³H]-thymidine incorporation assay were used to explore SAR modifications of FH535. Although replacing the 2,5-dichlorophenylsulfonyl substituent in FH535 with a 2,6-dihalogenation pattern generally produced more biologically active analogs than FH535, other SAR modifications led only to FH535 analogs with comparable or slightly improved activity in these two assays. The absence of a clear SAR pattern in activity suggested a multiplicity of target effectors for *N*-arylbenzenesulfonamides.

Graphical Abstract

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Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and represents a significant health care problem as the third most common cause of cancer-related deaths worldwide^{1,2}. The prevalence of HCC differs greatly by geographical location, reflecting variations in the main risk factors. Most cases of HCC (80%) arise in the Asian Pacific and sub-Saharan African regions, where the dominant risk factor is chronic infection with hepatitis B virus³. Elsewhere, the incidence reflects increasing infection with hepatitis C virus (HCV) and alcohol use, although obesity-related non-alcoholic fatty liver disease (NAFLD) is becoming an important risk factor in developed countries^{4,5}. HCC involves the dysregulation of multiple signaling pathways including the RAS/RAF/MAPK, PI3K/AKT/mTOR, HGF/c-MET, IGF, VEGF, PDGF and Wnt/ β -catenin pathways. Among them, dysregulation of the Wnt/ β -catenin pathway is by far the most difficult to treat⁶⁻⁹.

Chemical agents that target the Wnt signaling pathway provide the means for probing the intricacies of this pathway and ultimately providing drugs for treating liver, pancreatic and colorectal cancers that frequently exhibit upregulated Wnt signaling. The reported activity^{10,11} of 2,5-dichloro-*N*-(2-methyl-4-nitrophenyl)benzenesulfonamide (FH535, Figure 1) as an inhibitor of both β -catenin in the Wnt signaling pathway and the peroxisome proliferator-activated receptor (PPAR) prompted our investigation of the specific molecular target or targets interdicted by FH535. To address this problem, we required a biologically active, biotinylated version of FH535, and in the absence of published, structure-activity relationships, we explored modifications of FH535 that inhibited both [³H]-thymidine incorporation and activation of a β -catenin-dependent reporter gene in human hepatocyte-derived carcinoma Huh-7 cells and that provided suitable, chemical “handles” for biotin incorporation.

In defining the modifications to FH535 compatible with biological activity, we began by removing, replacing or relocating various substituents with comparable electron-donating or withdrawing capability. We synthesized numerous analogs of FH535 given the availability of variously substituted sulfonyl chlorides and anilines (Fig. 1).

With respect to the substituents in the N-aryl ring, replacement of the C-4 nitro group or the C-2 methyl group in FH535 (**1a**) with combinations of a carbomethoxy group and either a methyl or halogen as in **1b**, **1c**, and **1d**, respectively, diminished biological activity at 10 μ M concentrations relative to FH535 in the [3 H]-thymidine incorporation assay (Table 1). On the other hand, reduction of the carbomethoxy group in **1b** to the primary alcohol **1e** or replacement of the N-(2-methyl-4-nitrophenyl) group in FH535 with a 4-nitro-1-naphthyl group as in **1f** led to more active compounds than FH535 by a factor of 2.5 and 1.6, respectively, at this same concentration.

Modification of the halogenation pattern in the 2,5-dichlorobenzenesulfonyl portion of FH535 revealed that either the 2,6-difluorosulfonyl or 2,6-dichlorosulfonyl structures exhibited enhanced activity in the 3–10 μ M range (Table 1; data shown only for 10 μ M concentrations). For example, among the 2,6-dichloro analogs of FH535, the N-2-methyl-4-hydroxymethylphenyl 2,6-dichlorobenzenesulfonamide (**1h**) exhibited an activity 2 times that of the activity displayed by FH535 (**1a**). In the same vein, the 2,6-difluorinated analog **1i** and non-halogenated analog **1j** also possessed activities that exceeded that of FH535 (**1a**). Other 2,6-difluorinated analogs **1l–1m**, 2,5-difluorinated analogs **1n**, 2,6-dichlorinated analogs **1o–1r**, or the non-halogenated analogs **1s–1u** with varied, representative substituents on the N-aryl ring, which were among many that were explored (data not shown), were at best equipotent or diminished in activity relative to FH535 (Table 1).

The liver tumor microenvironment¹² is a complex mixture of tumor cells within the extracellular matrix as well as stromal cells, infiltrating immune cells and secreted proteins, all of which contribute to and participate in the carcinogenic process. Prior work in our groups established that FH535 affected, at the very least, β -catenin in the Wnt signaling pathway^{9,10}. The use of an assay relying on [3 H]-thymidine incorporation was problematic in the sense that thymidine incorporation in DNA occurs far downstream from the presumed and desired FH535 protein targets in the Wnt pathway. The absence of an unmistakable SAR pattern among the analogs in Table 1 as well as many others synthesized in the course of this study (data not shown) suggested that FH535 has targets other than β -catenin and PPAR, or at the very least, differential effects on these two targets.

We also employed a β -catenin/T-cell factor (TCF)/Lymphoid-enhancer factor (LEF)-dependent assay (*i.e.*, luciferase-based TOPFlash assay) to evaluate these FH535 analogs. In this case, we assessed TCF/LEF-dependent promoter activation due to β -catenin translocation to the nucleus. This assay monitors the ability of FH535 to decrease luciferase levels as a surrogate for β -catenin inhibition and represents a more direct assay than [3 H]-thymidine incorporation for Wnt pathway regulation. As expected and as shown in Table 1, the [3 H]-thymidine incorporation and the TOPFlash assays exhibited only partial agreement. For example, methyl 2-(2,5-dichlorophenylsulfonamido)-5-fluorobenzoate (**1c**) showed little activity in the [3 H]-thymidine incorporation assay but activity comparable to FH535 in the

TOPFlash assay. The converse was also true: *N*-4-hydroxymethyl-2-methylphenyl 2,6-dichlorobenzenesulfonamide (**1h**) showed enhanced activity in the [³H]-thymidine incorporation assay relative to FH535 but minimal activity in the TOPFlash assay. In summary, we developed analogs of FH535 with activities in a [³H]-thymidine incorporation assay and/or a TOPFlash assay that were equipotent or slightly increased relative to the activity of FH535. Replacing the 2,5-dichlorophenylsulfonyl substituent in FH535 with a 2,6-dihalogenation pattern generally produced more biologically active analogs than FH535, but none of the modifications made in the *N*-phenyl substituent, including replacement with various heterocycles, led to FH535 analogs with improved potency presumably because FH535 and its analogs may have either a multiplicity of target effectors or a few effectors that do not have robust structural requirements beyond the *N*-aryl benzenesulfonamide substructure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

CL and DSW were supported by R01 CA172379 from the NIH and by the Kentucky Lung Cancer Research Program. BTS and JJ were supported by R01 DK074816 from the NIH. DSW was supported by the Office of the Dean of the College of Medicine and by NIH Grant Number P30 RR020171 from the National Institute of General Medical Sciences to L. Hersh, PI.

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Figure 1. FH535 (1a) and synthesis of N-aryl benzenesulfonamides 1
Reagents: *a*, ArNH₂, NaH, THF (Method A) or ArNH₂, Py (Method B).

Table 1
Percentage inhibition of $[^3\text{H}]$ -thymidine incorporation in Huh-7 cells by FH535 analogs at 3 μM concentrations.

FH535 Analog	C-2	C-3	C-4	C-5	C-6	C-2'	C-3'	C-4'	C-5'	C-6'	$[^3\text{H}]$ -Thymidine incorporation ratio in Huh-7 cells at 10 μM relative to DMSO	% Inhibition at 10 μM	Ratio of % inhibition of analog to % inhibition by FH535 at 10 μM	TOPFlash Assay (10 μM)	TOPFlash Assay as a percentage Decrease Relative to Control
Control											100 $\pm$ 10			31.2 $\pm$ 4.5	
1a	Cl	H	H	Cl	H	CH ₃	H	NO ₂	H	H	64 $\pm$ 3.7	36	1.0	8.5 $\pm$ 2.1	73
1b	Cl	H	H	Cl	H	CH ₃	H	CO ₂ CH ₃	H	H	82 $\pm$ 4.2	18	0.5		
1c	Cl	H	H	Cl	H	CO ₂ CH ₃	H	F	H	H	93 $\pm$ 5.6	7	0.2	12.1 $\pm$ 0.9	61
1d	Cl	H	H	Cl	H	CO ₂ CH ₃	H	Cl	H	H	106 $\pm$ 14	0	0.0		
1e	Cl	H	H	Cl	H	CH ₃	H	CH ₂ OH	H	H	33 $\pm$ 4.8	67	1.9		
1f	Cl	H	H	Cl	H	1-(4-NO ₂)C ₁₀ H ₆					52 $\pm$ 6.4	48	1.3		
1g	Cl	H	H	H	Cl	CH ₃	H	CO ₂ CH ₃	H	H	71 $\pm$ 1	29	0.8		
1h	Cl	H	H	H	Cl	CH ₃	H	CH ₂ OH	H	H	27 $\pm$ 5.1	73	2.0	27.3 $\pm$ 3.5	13
1i	F	H	H	H	F	CH ₃	H	CH ₂ OH	H	H	62 $\pm$ 1.5	38	1.1		
1j	H	H	H	H	H	CH ₃	H	CH ₂ OH	H	H	47 $\pm$ 2.3	53	1.5		
1k	F	H	H	H	F	OC ₆ H ₅	H	H	H	H	66 $\pm$ 3.2	34	0.9		
1l	F	H	H	H	F	H	OCH ₂ C ₆ H ₅	H	H	H	57 $\pm$ 21	43	1.2		
1m	Cl	H	H	H	Cl	H	H	COC ₆ H ₅	H	H	67 $\pm$ 12	33	0.9		
1n	F	H	H	F	H	1-(4-NO ₂)C ₁₀ H ₆					69 $\pm$ 3.51	31	0.9	11.2 $\pm$ 1.0	64
1o	Cl	H	H	H	Cl	OC ₆ H ₅	H	H	H	H	62 $\pm$ 10	38	1.1	17.2 $\pm$ 0.9	45
1p	Cl	H	H	H	Cl	H	OCH ₂ C ₆ H ₅	H	H	H	69 $\pm$ 6.9	31	0.9	14.8 $\pm$ 0.7	53
1q	F	H	H	H	F	H	H	COC ₆ H ₅	H	H	67 $\pm$ 5.9	33	0.9		
1r	Cl	H	H	H	Cl	1-(4-NO ₂)C ₁₀ H ₆					74 $\pm$ 27	26	0.7	16.6 $\pm$ 1.1	47
1s	H	H	H	H	H	H	OCH ₂ C ₆ H ₅	H	H	H	71 $\pm$ 12	29	0.8		
1t	H	H	H	H	H	H	H	COC ₆ H ₅	H	H	62 $\pm$ 2.3	38	1.1	16.8 $\pm$ 0.9	46
1u	H	H	H	H	H	1-(4-NO ₂)C ₁₀ H ₆					53 $\pm$ 6.9	42	1.2	8.7 $\pm$ 0.1	72