Sulfonamides as a New Scaffold for Hypoxia Inducible Factor Pathway Inhibitors

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Abstract

Solid tumors generally grow under hypoxic conditions, a pathophysiological change, which activates the expression of genes responsible for malignant, aggressive, and treatment-refractory properties. Hypoxia inducible factor (HIF) is the chief transcription factor regulating hypoxia-driven gene expression. Therefore, the HIF pathway has become a critical target for cancer therapeutics development. We screened a privileged library of about 10,000 natural-product-like compounds using a cell-based assay for HIF-dependent transcriptional activity and identified several arylsulfonamide HIF pathway inhibitors. Among these compounds, the most potent ones showed an IC_{50} of ~0.5 µM in the hypoxia-responsive element (HRE)-luciferase reporter system. Further studies are needed to fully elucidate the mechanism of action of this class of compounds and their structure-activity relationship.

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Keywords

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Hypoxia is a common occurrence in solid tumors largely due to inadequate vascularization,[1] which eventually leads to the development of tumor necrosis.[2, 3] Hypoxic conditions are well known to hinder the effectiveness of chemotherapy and radiation therapy.[4] There are many factors that affect the behavior of tumors under hypoxic conditions; chief among them is the hypoxia inducible factor (HIF) family, which regulates a large number of target genes related to cell growth, glycolysis, and angiogenesis. Proof-of-principle studies have already established that inhibition of the HIF pathway can lead to inhibition of malignant characteristics in a number of cancers, including gliomas.[5–8] As a result, this is an area of very active investigation in search of novel treatment options for cancer.[9, 10] In addition, many anti-cancer compounds used in the clinic or in preclinical development were found to indirectly inhibit the HIF pathway.[10–13] We have had a long-standing interest in the HIF pathway, and the development of novel experimental therapeutics based on the hypoxic status of tumors.[13–18] Aimed at identifying novel small molecule inhibitors of HIF-dependent transcription, we genetically engineered a human glioma cell line (LN229 glioblastoma)[20] to stably express an hypoxia-responsive element (HRE)-alkaline phosphatase reporter gene.[4, 12] The resulting cell line (LN229-HRE-AP) was used to screen a library of about 10,000 compounds from a 2,2-dimethylbenzopyran structural family[21] under normoxic and hypoxic growth conditions. The screening identified a number of sulfonamide structures that inhibited the reporter assay by 50% or more under hypoxia. In contrast, no compounds were identified that could activate the construct under normoxia. In the current report, we describe the identification and evaluation of arylsulfonamides as HIF-1 pathway inhibitors.

The library screened was generated from solid-phase combinatorial synthesis using split-and-pool chemistry with NanoKans and optical encoding, and careful attention to quality control.[21] This library contained about 10,000 compounds. The initial screening used a concentration of 10 μM with the test compounds dissolved in DMSO for stock solution preparation. Table 1 gives a partial list of structural scaffolds tested in this library. All compounds that reduced alkaline phosphatase induction under hypoxia by more than 50% were considered "hits" in the initial screen. All compounds identified as hits were re-synthesized and re-tested at 5 and 10 μM to confirm activity in the bioassay. A counter-screen was also used to verify that the compounds did not inhibit the reporter alkaline phosphatase enzyme per se (data not shown).

Following the initial library screen, several compounds with an arylsulfonamide scaffold were found to be among the most potent in the library. Thus a series of about 100 arylsulfonamide analogs were synthesized so as to obtain a preliminary indication of active vs. inactive compounds. Combined, these screening data showed that the arylsulfonamide scaffold afforded a high percentage of active compounds (Fig. 1).

Additional studies were conducted with a subset of these compounds showing the highest potency (reduction > 50%). This was done through the re-synthesis of all compounds in a purified form. To independently confirm the data from the original cell-based reporter system, as a precaution, a second confirmatory cell-based reporter system was engineered where a luciferase gene substituted the alkaline phosphatase gene. Luciferase is more sensitive and shows a better dynamic range, so it was anticipated to be a better discriminatory tool to distinguish between compounds with related activities. The selected compounds confirmed in the original alkaline phosphatase reporter assay were initially

tested in the new HRE-luciferase assay (at 2.5, 5 and 10 μM). In general, the data in the two reporter systems were concordant with the most active compounds having a sulfonamide group and a fused phenyl ring (Table 2). Overall it seems that the aryl ring of the arylsulfonamide analogs can tolerate a fairly high degree of modifications. For example, the different substitution patterns did not yield much difference in inhibitory activities between KCN49 (4-fluoro-3-trifluoromethoxy) and KCN1 (3,4-dimethoxy). The same is true for KCN65 (2,5-dichloro) and KCN66 (para-nitro), and KCN67 (3,4-dimethoxy) and KCN68 (3,5-dimethoxy). This aryl position seems to tolerate non-phenyl groups as well. For example, KCN63 with a naphthyl ring still possesses comparable activities to other phenylsulfonamides. For region 2, variations are also allowed. For example, when the region 2 group varies from phenyl (KCN1) to benzyl (KCN49), cyclohexyl (KCN60), and isobutyl (KCN63, KCN65–68) similar activities are observed, although their region 1 substituents are different and direct comparisons cannot be made. It seems that the combination of the modifications in regions 1 and 2 makes a more substantial difference than a singular modification (Table 2).

The best compounds were subsequently re-tested in the luciferase reporter assay in a dose-response fashion to establish an IC_{50} value of HIF-inhibitory activity (Fig. 2 and data not shown). We chose the commonly used four-parameter logistic function to describe the dose response relationship, in which the IC_{50}, the concentration of compound that is required for 50% inhibition in an assay, is the parameter of interest. The data was fitted into the nonlinear mixed effects[22] model by **nlme** library in R.[23] In this study, each compound was tested 5–21 times on different days, and we observed biological variations among those repeated experiments as expected. The nonlinear mixed effects model is an appropriate model that is able to address such between-variation of experiment-to-experiment by setting IC_{50} to have an associated random effect. The results indicate that 8 compounds displayed IC_{50} values in the high nanomolar to low micromolar range (Table 3), with KCN1, 49, 63, and 66 being the most promising.

In order to further confirm that these compounds indeed inhibit the HIF pathway, we conducted additional experiments probing the activity of the hypoxia-responsive promoter of the endogenous HIF transcriptional target gene *vascular endothelial growth factor (VEGF)* using cells stably transfected with a *VEGF* promoter-luciferase reporter. From Fig. 3, one can see that the tested compounds had no effect on the expression level of the *VEGF* promoter construct under normoxic conditions (21% oxygen) as expected, since HIF expression is low and its transcriptional activity is suppressed by factor inhibiting HIF[24] under normoxia (Fig. 4). On the other hand, under hypoxic conditions, KCN1, 49, 60, 63, 65, 66, 67, 68 at 10 μM were able to significantly inhibit transcription from the *VEGF* promoter in LN229-VEGF-Luc glioma cells.

For further mechanistic studies, we picked the representative compounds and a control (KCN-1, 49, 60, 63, 65, 66, 67 and KCN:85D5R) to probe their molecular basis of action using biochemical techniques. Given that HIF regulation typically occurs at the protein level, we determined whether the selected compounds had a direct effect on HIF-1α protein accumulation under hypoxia. HIF-1α levels were examined by Western blotting of cell extracts from cells grown under hypoxia in the presence or absence of inhibitor (20 μM). In addition to the selected arylsulfonamides, we also included as controls bortezomib and 103D5, two previously characterized HIF pathway inhibitors. As expected, the results with these control compounds show that bortezomib, a proteasome inhibitor leads to the accumulation of HIF-1α in an inactive form;[25] whereas 103D5, a HIF-1α translation inhibitor, leads to a blockage of HIF-1α accumulation under hypoxia.[11] It was found that some of the active compounds did slightly reduce the level of expression of HIF-1α at 20 μM (Fig. 4A), but a dose-response analysis (Fig. 4B) shows that this effect disappears at

lower concentrations ($<10\ \mu$M), suggesting that inhibition of HIF-1α expression is unlikely the cause of the strong inhibition seen against HIF-mediated transcription in the reporter assay ($IC_{50}<1\ \mu$M). Such results suggest that the compounds' main biological activity is not mediated by inhibiting HIF-1α gene expression, or affecting HIF-1α turnover through a blockage in translation of HIF-1α mRNA, or accelerated protein degradation. Instead, these findings hint at the HIF transcriptional complex being functionally inactive. Potential mechanisms may involve protein misfolding, incomplete protein modifications and/or lack of HIF complex assembly. Additional work is needed to further elucidate the precise mechanism of action of this class of HIF pathway inhibitors.

Hypoxia inducible factor has been recognized as a potential target for the development of anticancer agents. Aimed at discovering new structural classes of HIF pathway inhibitors, we screened a privileged library of about 10,000 compounds and identified an arylsulfonamide structural class as a promising scaffold for the further development of HIF pathway inhibitors. Among these compounds, the most potent ones showed an IC_{50} of ~0.5 μM in a luciferase reporter system. Representative compounds also inhibited the promoter of the *VEGF* gene under hypoxic conditions, consistent with HIF pathway inhibition. Further studies are needed to fully elucidate the mechanism of action of this class of compounds and their structure-activity relationship.

Acknowledgments

ABBREVIATIONS

AP	alkaline phosphatase
HIF	hypoxia inducible factor
HRE	hypoxia-responsive element
VEGF	vascular endothelial growth factor

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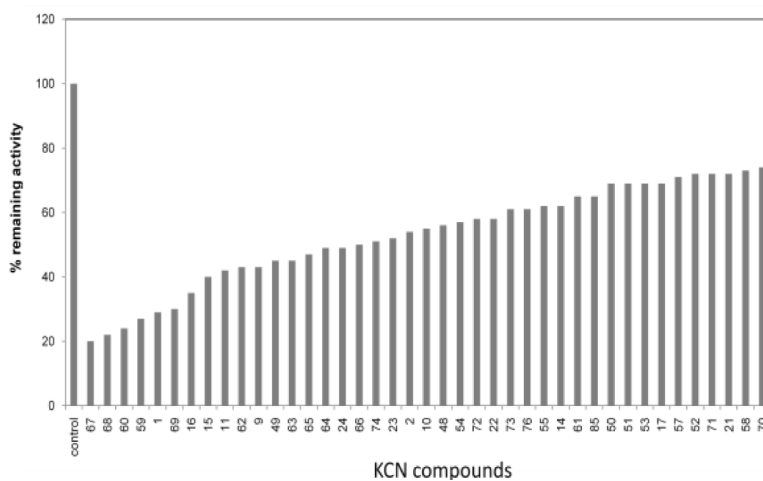
Figure 1.
Inhibitory effect of selected arylsulfonamides at 10 µM concentration on HIF activity in the HRE-alkaline phosphatase assay. LN229-HRE-AP cells were pre-treated with indicated compounds or a control with vehicle (1% DMSO) alone for 1 h, transferred to a hypoxia (1% O_2) incubator for 24 h. Thereafter, the cells were lysed and alkaline phosphatase activity was determined as previously described.[11] Data shown are from a single assay.
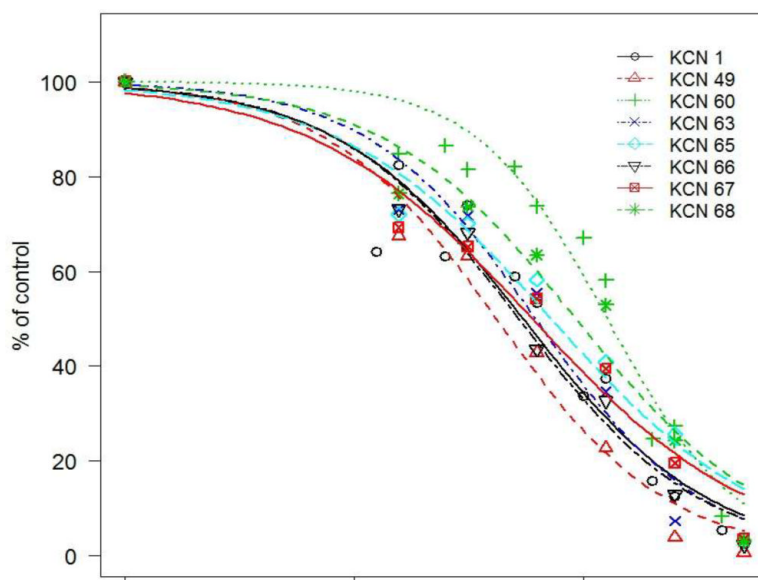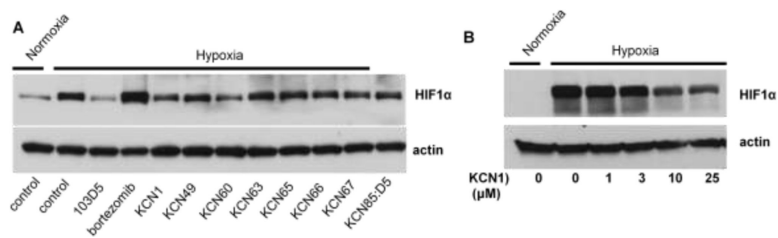
Figure 2.
Dose-response of a selected set of arylsulfonamide compounds using the HRE-luciferase reporter system. Cells were pre-treated with inhibitors as in Fig. 1, and luciferase activity was measured in cell extracts using a 20/20ⁿ Luminometer (Turner Biosystems). Data are expressed as average from at least 5 independent experiments carried out in triplicates.
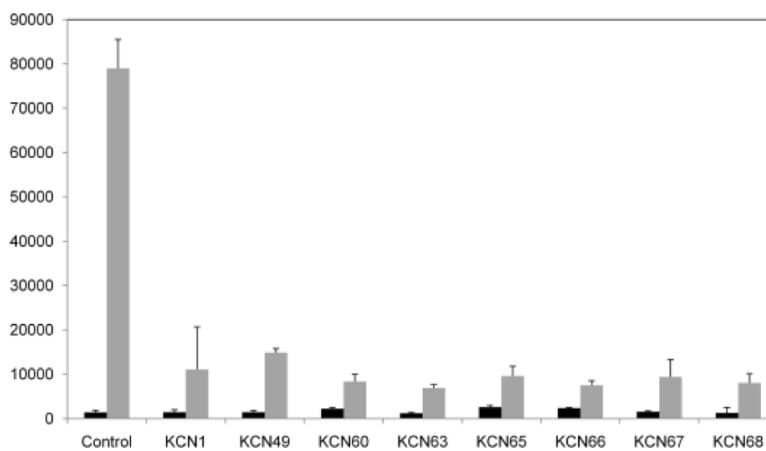
Figure 3.
Luciferase reporter assays showing the effect of arylsulfonamide HIF pathway inhibitors on the activity of a *VEGF* promoter-luciferase construct, stably transfected in LN229 glioma cells (LN229-VEGF-luc). Cells were pre-treated with inhibitors (10 µM final concentration) for 1 h in normoxia, followed by 24 hrs incubation in normoxia or hypoxia and luciferase measured in cell extracts as indicated in Fig. 2. Each value represents an average from triplicates +/− standard deviation.

Figure 4.
Western blots showing the effect of different HIF pathway inhibitors on hypoxic accumulation of HIF-1α in LN229 cells.
A. Cells were pre-treated with indicated inhibitors at 20 μM final concentration (bortezomib 100 nM) for 1 h before incubation in normoxia or hypoxia for 24 hrs.
B. Dose-response of KCN1 on HIF-1α levels. Cells were pre-treated with indicated concentrations of KCN1 for 1 h before incubation in normoxia or hypoxia for 6 hrs. Immunoblotting of HIF-1α and actin was as described earlier.[19]
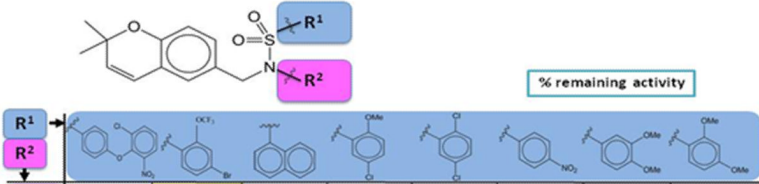
Table 1

Relative remaining HRE-activity in percentage at 10 μM of the indicated test compounds using the alkaline phosphatase reporter assay.

Table 2

Initial SAR studies of arylsulfonamide HIF-1 pathway inhibitors using HRE-luciferase reporter assay. ND, not done.

Table 3

Average IC$_{50}$ (from n independent experiments in μM) of selected arylsulfonamide HIF-1 pathway inhibitors as established in the HRE-luciferase assay. Molecular weight of compounds and predicted miLog P values (http://www.molinspiration.com/) are also shown.

Name	Mol. Wt.	Log P	IC$_{50}$ (n=)	Std. Error	95% CI[1]
KCN1	465.571	5.2	0. 593 (21)	0.063	(0.470, 0.716)
KCN 49	582.438	6.957	0.508 (5)	0.118	(0.277, 0.739)
KCN 60	471.619	5.798	1.267 (5)	0.127	(1.018, 1.516)
KCN 63	453.589	6.133	0.656 (5)	0.158	(0.346, 0.966)
KCN 65	454.419	6.258	0.731 (5)	0.191	(0.357, 1.105)
KCN 66	430.526	4.933	0.532 (5)	0.084	(0.367, 0.697)
KCN 67	445.581	4.62	0.672 (6)	0.177	(0.325, 1.019)
KCN 68	445.581	5.015	1.011 (6)	0.312	(0.399, 1.623)

[1]Confidence Interval