

PRELIMINARY COMMUNICATION

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Discovery and SAR exploration of *N*-aryl-*N*-(3-aryl-1,2,4-oxadiazol-5-yl)amines as potential therapeutic agents for prostate cancer

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Abstract

A new chemical series of antiproliferative compounds was identified *via* high-throughput screening on DU-145 human prostate carcinoma cell line (hit compound potency - 5.7 μ M). Exploration of the two peripheral diversity vectors of the hit molecule in a hit-targeted library and testing of the resulting compounds led to SAR generalizations and identification of the 'best' pharmacophoric moieties. The latter were merged in a single compound that exhibited a 200-fold better potency than the original hit compound. Specific cancer cell cytotoxicity was confirmed for the most potent compounds.

Background

Prostate cancer is the number one cancer diagnosed in men today. While it occurs to certain extent throughout the world (least commonly in Eastern/Southern Asia), it is viewed as the major public health threat in Western Europe and, especially, the United States [1]. In the US alone, it has been estimated [2] that 186,295 new cases of prostate cancer (mostly - among men over fifty) were diagnosed in 2008, accounting for 25% of all cancers diagnosed in men that year and 10% of the total cancer-related mortality. Appropriate diet (including dietary supplements) and exercise are currently the common themes for prostate cancer prevention while classical treatments are limited to surgery, radiation therapy, and hormone therapy. Chemotherapy of late-stage prostate cancer is still largely experimental; however, it may lead to increased survival in the future [3]. Specifically, small molecules as well as antibodies targeted at disrupting vital signaling pathways in cancerous cells have a potential to provide new basis for innovative treatment of proliferative disorders as prostate cancer in the years to come [4].

Results and discussion

The present study was a part of an ongoing effort [5] in our laboratories to find novel antiproliferative agents as potential treatments for prostate cancer. It was aimed at identifying new small heterocyclic molecules in Chemical Diversity Research Institute collection (parts of that can be accessed at <http://www.chemdiv.com>) that would be specifically inhibitory to DU-145 human prostate carcinoma cell line (a 'classical' cell line of androgen-independent prostate cancer [6]) while exhibiting no non-specific (general) cytotoxicity. High-throughput screening of a highly diverse set of over 5,000 compounds comprising over 200 chemical classes led to several confirmed hit classes.

Among these, one hit compound, **1** (Figure 1), that exhibited inhibition of DU-145 cell proliferation in dose-response manner, attracted our attention due to its drug-likeness [7], structural simplicity and the presence in its structure of two distinct types of peripheral appendages (thus allowing for informative SAR exploration).

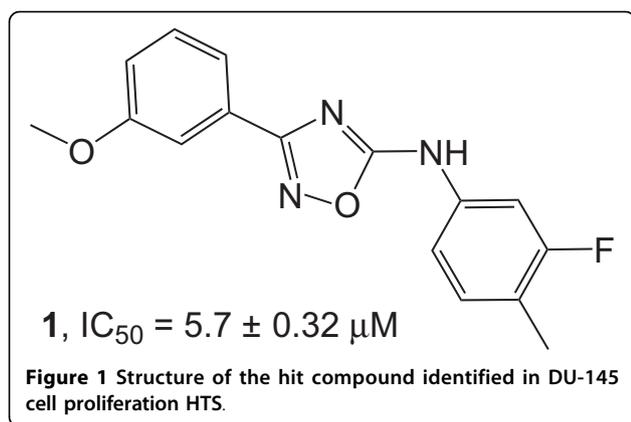
Herein we report on the synthesis and potency characterization of twenty analogs of the hit compound **1** leading to the initial SAR generalizations in this chemical class and to significant improvement of the inhibitory potency as well as to confirmation that the most potent compounds synthesized in this work inhibit DU-145 proliferation in a cell-specific manner rather than *via* general cytotoxicity.

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To confirm that the observed activity of **1** was indeed associated with the *N*-aryl-*N*-(3-aryl-1,2,4-oxadiazol-5-yl)amines chemotype and to obtain initial SAR clues for further potency optimization, we synthesized a library of 176 analogs **2** of the hit compound. According to the literature [8], *N*-aryl-*N*-(3-aryl-1,2,4-oxadiazol-5-yl)amines **2** can be prepared in moderate yields by reaction of 2 eq. of carbodiimides with amidoximes (**3**). The target products **2** were isolated and purified chromatography on silica gel using 5-25% EtOAc in hexanes as a mobile phase. The amidoximes **3** were conveniently synthesized in nearly quantitative yields from corresponding benzonitriles (R1CN) (Scheme 1). Scheme 1 Preparation of *N*-aryl-*N*-(3-aryl-1,2,4-oxadiazol-5-yl)amines **2** [8]. *Reagents and conditions*: (a) hydroxylamine hydrochloride (1 eq.), EtOH, reflux, 5-10 h; (b) aq. Na_2CO_3 , extraction with EtOAc; (c) 0.2 M solution of **3** in dry DMF, 2.1 eq. of R2-N = C = N-R2 carbodiimides, 120°C, 18-36 h. Compounds **2** were tested at 10 μM concentration for inhibition of DU-145 cell proliferation. 19 compounds with inhibition >50% were selected for dose-response experiments to determine IC_{50} values. The results are presented in Table 1.

There was a clear preference of electron-rich or unsubstituted phenyls in the 'western' aryl portion of the molecule with notorious presence of *meta*-methoxy substituent in 14 active compounds out of 19 compounds for which dose-response curves were obtained. As to the 'eastern' arylamino portion, the best activity was observed for *para*-methoxyanilino substituent (**2k** and **2s**). Notably, presence of electron-withdrawing or *ortho*-substituents in the arylamino portion of **2** rendered such compounds less active or completely devoid of inhibitory potency. This observation is illustrated by several examples provided in Figure 2.

These SAR observations are useful in determining the 'activity' chemistry space for future optimization of the series. To test our generalizations, we decided

to combined the *para*-methoxy substituent in the 'eastern' arylamino portion of the molecule with another beneficial feature - namely, *meta*-methoxy substituent in the 'western' aryl portion (*vide supra*). Indeed, the activity of the synthesized SAR-guided compound **2t** (Figure 3) improved nearly three-fold compared to **2k**, thus demonstrating the synergy of effects of these two substituents on the inhibitory potency of the series.

Having improved the antiproliferative potency of the series by two orders of magnitude, we then investigated the specificity of the cytotoxicity of the most potent compounds. The general, non-specific cytotoxicity of the most potent three representative compounds (**2j**, **2s** and **2t** with IC_{50} values of 0.55, 0.38 and 0.029 μM , respectively) was measured on HepG2 and DU-145 cell lines using a known cytotoxic compound, digitonin, as a positive control (Figure 4). None of these compounds exhibited cytotoxicity in excess of 20% at concentrations as high as 50 μM , while digitonin itself caused 100% cell death in nanomolar concentration range. Thus, the observed antiproliferative activity of the most potent compounds in the series is not due to non-specific cytotoxicity but must be attributed to a specific cellular target and is selective towards human prostate carcinoma cells.

Methods

Cell sources: the prostate cancer Du-145 cell line was purchased from the American Type Cell Collection (HTB-81). Du-145 was cultured in RPMI-1640 complemented with 10% fetal bovine serum and 2 mM L-glutamine. To our delight, 80% of the library screened exhibited >20% inhibition of DU-145 proliferation. The hepatocellular carcinoma HepG2 cell line was purchased from the American Type Cell Collection (HB-8065) and was cultured in DMEM complemented with 10% fetal bovine serum and 2 mM L-glutamine.

Assays description

1. Biological assay to determine inhibition of proliferation of the DU-145 cells

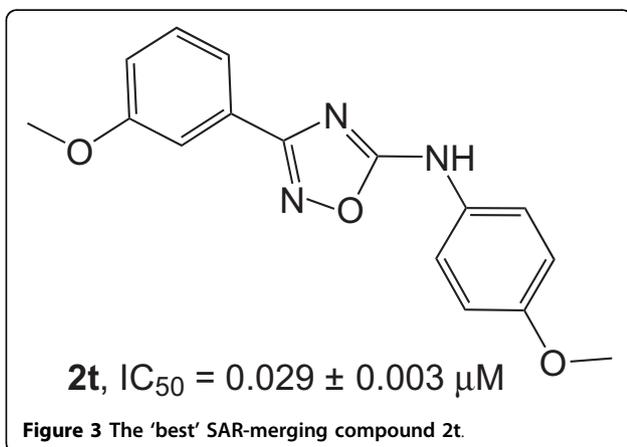
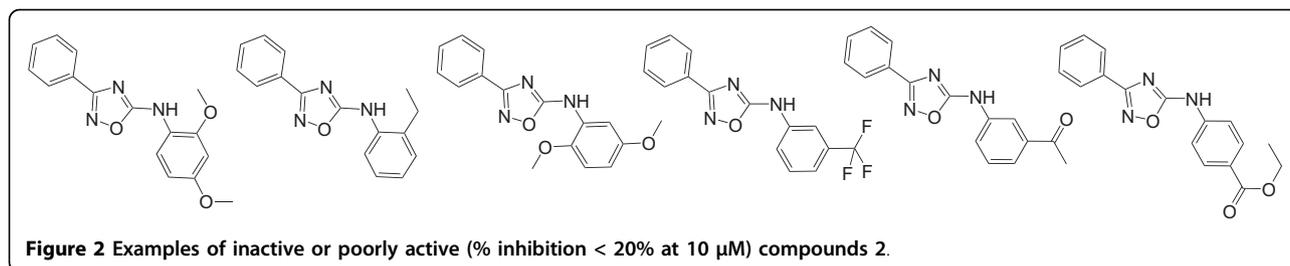
Du-145 cells were plated in 384-well plate at the density of 4000 cells per well. 4 mM solutions of compounds in DMSO were diluted 100 times with medium and added to cells to a final concentration of 20 μM (40 μL of cell culture plus 40 μL of compound solution, final concentration of DMSO - 0.5%). Taxol at the final concentration of 1 μM was used as a positive control. The cells were incubated with compounds for 3 days. Alamar Blue was then added to the cells to a final concentration of 50 μM . After incubation for 4-6 hours at 37°C, plate fluorescence was read using fluorescence plate reader Wallac 1420 (530 nm excitation filter, 590 nm emission

Table 1 Structures, preparative yields (from 3), and DU-145 proliferation inhibition data of compounds 2a-s.

Compound	R1	R2	Yield, %	IC ₅₀ , μM ^a
2a ^b			62	2.2 ± 0.30
2b			68	0.86 ± 0.10
2c			70	1.6 ± 0.25
2d			29	2.6 ± 0.35
2e			43	1.0 ± 0.01
2f			43	1.0 ± 0.12
2g			73	2.7 ± 0.22
2h			37	1.6 ± 0.14
2i			77	0.64 ± 0.025
2j			56	0.55 ± 0.010
2k			81	0.084 ± 0.005
2l			54	2.7 ± 0.50
2m			46	0.96 ± 0.08
2n			72	0.93 ± 0.009
2o			75	1.8 ± 0.30
2p			82	2.1 ± 0.17
2q			65	0.91 ± 0.07
2r			66	1.5 ± 0.09
2s			55	0.38 ± 0.024

^aIC₅₀ values have been evaluated for standard error. The values reported are the mean of 2 determinations, standard error provided.

^bCompound 2a referred in this table is identical with the hit compound 1 in the text and figure 1.



filter). Proliferation inhibition was calculated using formula:

$$\%INH = 100 * ((F_{negative} - \text{readout signal}) / (F_{negative} - F_{positive})),$$

where

$F_{negative}$: DMSO added to the cells (viable cells) and

$F_{positive}$: taxol (1 μM) added to cells (cell count on 1st day of incubation)

2. Cytotoxicity assay

For cytotoxicity experiments, higher seeding densities (10,000 or 20,000 cells per well) were used to more accurately observe potentially diminishing number of

cells. This is in contrast with the measurement of anti-proliferative activity where initial seeding density (4,000 cells per well) was chosen to allow more room for cell proliferation. Thus, Du-145 or HepG2 cells were plated in 384-well plate at the density of 10,000 or 20,000 cells per well, respectively. Serial dilutions (200×) of the tested compounds in DMSO were prepared. After that compounds were diluted 100 times in medium and added to the cells (40 μL of cell culture plus 40 μL of compound solution, final concentration of DMSO - 0.5%). The cells were incubated with compounds overnight. Known toxic compound digitonin was used as control. The next day Alamar Blue was added to the cells to final concentration of 50 μM. After incubation for 4-6 hours at 37°C, plate fluorescence was read using fluorescence plate reader Wallac 1420 (PerkinElmer) (530 nm excitation filter, 590 nm emission filter). Compound cytotoxicity was calculated using formula:

$$\%TOX = 100 * ((F_{negative} - \text{compound signal}) / (F_{negative} - F_{positive})),$$

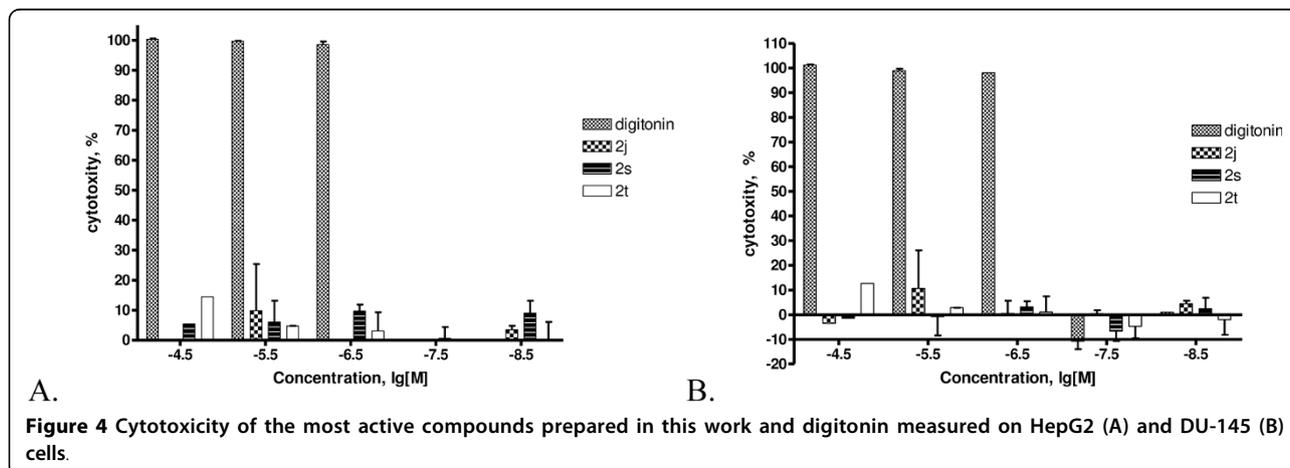
where

$F_{negative}$ - DMSO was added to cells (viable cells)

$F_{positive}$ - digitonin was added to cells (dead cells)

Conclusions

In conclusion, we have undertaken hit expansion and SAR exploration of a new antiproliferative chemical



series, *N*-aryl-*N*-(3-aryl-1,2,4-oxadiazol-5-yl)amines. This study led to identification of the new 'best' peripheral moieties which, when combined in the same compound (**2t**) led to significant improvement of the DU-145 proliferation inhibitory potency (>200-fold compared to the initial hit compound **1**). We also demonstrated that the observed antiproliferative activity of the compounds belonging to the studied chemotype was not due to non-specific cytotoxicity. The compound **2t** represents a promising new lead for development of novel therapeutic agents for treatment of androgen-independent prostate cancer. The specific cellular mechanism of action of this compound remains to be investigated and will be presented in subsequent communication.

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Authors' contributions

MK has formulated the research idea and prepared the manuscript draft version, KAR prepared the manuscript for submission and coordinated further formalities, AVS coordinated efforts of all ChemDiv co-workers within the project flow, RK coordinated efforts of the ChemDiv lead discovery team, EG carried out the chemical and biological studies, OS participated in the data collection and calculation of IC₅₀ values and %TOX, YL performed final data check, AAG conceived of the study, participated in its design and coordination. All authors have read and approved the final manuscript.

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