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Design and synthesis of 4-HPR derivatives for rhabdoid tumors

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ABSTRACT

Rhabdoid tumors (RTs) are aggressive pediatric malignancies with poor prognosis that arise due to loss of the *hSNF5/INI1* tumor suppressor. Molecular studies indicate that *cyclin D1*, a downstream effector of *INI1* is up regulated in RT, and is essential for this tumor formation. Previously we demonstrated that 4-HPR, a synthetic retinoid that targets Cyclin D1, is a potential chemotherapeutic agent for RT. To facilitate further chemical development of this retinoid, and to determine its active moiety, we synthesized small chemical libraries of 4-HPR and tested their cytotoxic effect on RT cells. We synthesized 4-HPR (**1**) and the derivatives (**5a**–**5n**) starting from retinoic acid. First, retinoic acid was converted to acid chloride derivatives, then in the presence of DMF, base, and aniline derivatives, we synthesized the corresponding 4-hydroxy phenyl amine derivatives (**5a**–**5n**). This procedure gave 70–90% yield. Then, the 4-HPR derivatives were tested for their ability to inhibit RT cells using an in vitro cell survival assay. We found that the 4-hydroxy group at para-position is essential for cytotoxic activity against RT cells. Furthermore, we identified a few derivatives of 4-HPR with higher cytotoxic potencies than 4-HPR. In addition, we demonstrate that either chloro, fluoro or iodo derivatives at meta-position of phenyl ring retain the cytotoxic activity. Interestingly, substitution of iodo-moiety at meta-position (**5j**) substantially increased the efficacy ($IC_{50} \sim 3$ μM, Fig. 1D). These results indicate that chemical modification of 4-HPR may result in derivatives with increased therapeutic potential for RTs and that halogen substituted 4-HPR that retain the activity can be synthesized for further therapeutic and diagnostic use.

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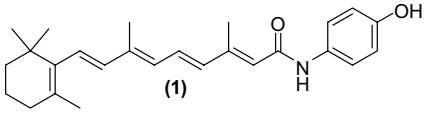
Rhabdoid tumors (RTs) are rare, but highly aggressive and mostly incurable pediatric malignancies that arise in brain, kidneys and soft tissues.[1,2] RTs most commonly occur in children younger than five years of age with a peak incidence between birth and three years of age. Irrespective of their location, all RTs are characterized by the presence of sheets or nests of rhabdoid cells and exhibit biallelic deletions and/or mutations in the *INI1/hSNF5* gene, located at chromosome 22q11.2.[3–6] Current therapeutic regimens for RTs involve empirically selected combinations of chemotherapeutic agents that are highly toxic and rarely curative, and hence the survival rate for children with RTs remains poor.[1,2,7–9] Thus, there is a dire need to develop novel therapeutic strategies for RTs, preferably based on the understanding of molecular factors responsible for the genesis, growth, and survival of these tumors. Previously, we demonstrated that Cyclin D1 is essential for the genesis and survival of RTs and that chemotherapeutic agents such as 4-HPR, which target Cyclin D1, are effective in inhibiting the growth of RTs in preclinical models.[10,11]

4-HPR [*N*-(4-hydroxyphenyl) retinamide, or fenretinide structure 1] is a synthetic retinoid that has Structure 1 low toxicity and potent chemopreventive effects in preclinical cancer models, and suppresses in vitro tumor cell growth at low μ molar concentrations (IC_{50}'s ranging from 1 to 15 μM).[12] 4-HPR is under phase III clinical trials for many cancers. In pilot clinical studies with pediatric neuroblastoma patients, 4-HPR has demonstrated prolonged stabilization of disease.[13–16] It has also been largely studied as a chemo-preventive agent in patients at risk for breast cancer and in animal models of carcinogen-induced epithelial tumors.[17–20] Recent results of a fifteen-year follow-up study of phase III trials using 4-HPR to prevent second breast cancer indicated that it significantly reduced the risk in premenopausal women.[21] Taken together, these preclinical and clinical studies indicate that 4-HPR is a promising anticancer drug.



(1)

N-(4-hydroxyphenyl)retinamide (4-HPR)

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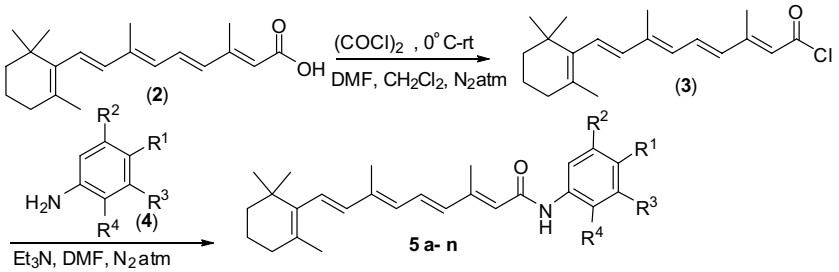
Cell culture studies of 4-HPR demonstrated that this compound induces apoptosis in vitro by various mechanisms including (i) activation of retinoid receptors, RAR β and γ; (ii) induction of ceramide-dependent cytotoxicity; (iii) generation of free radical oxygen species; (iv) increase of NOS expression resulting in NO-dependent cytotoxicity; and (v) increase of mitochondrial permeability transition.[13,14,16,17,19] Furthermore, over expression of Cyclin D1 sensitizes breast-cancer cells to 4-HPR.[22] Based on this observation, and based on the fact that RTs are dependent on Cyclin D1, we tested the effect of 4-HPR and found that it inhibits the survival of RT cells by inducing G1 arrest and caspase 3/7-mediated apoptosis, and inhibits xenografted RT growth in vivo.[10] These results were correlated to down-modulation of Cyclin D1.[10]

Activity of 4-HPR derivatives. There are several reports indicating that synthetic analogues of 4-HPR are more active or equally active compared to 4-HPR in tumor cell toxicity studies. It was reported that a non-hydrolysable carbon linked analogue of 4-HPR (*N*-benzyl hydroxyl retinamide, 4-HBR), potentially reduces suppression of plasma vitamin A levels.[23] The sulfur-containing heteroretinoids induce apoptosis and reactive oxygen species specifically in malignant, but not in benign cells allowing for anticancer activity with reduced toxicity.[24] Conjugations of 4-HPR have also been reported to have antitumor activity. The antitumor potency of 4-HPR increases when conjugated to glucoronides; glycosyl conjugated mannosyl-4-HPR showed increased activity on the HL60 promyelocytic leukemia cell line.[25] In addition, it was recently reported that 4-oxo-fenretinide induced marked G2-M cell cycle arrest and apoptosis in both fenretinide-sensitive and resistant cell lines.[26] Since substituted or conjugated compounds of 4-HPR are biologically active, compared to parent 4-HPR, we hypothesize that, by structural modification/manipulation of 4-HPR compounds, we may be able to derive more active compounds or compounds that are amenable for further conjugation such as linking to nanoparticles. These modifications may not only increase its potency but also may allow the compound to cross the blood brain barrier freely. Furthermore, we may be able to derive radioconjugates that may facilitate combining of radiotherapy and chemotherapy in a single chemotherapeutic agent. The dose required for achieving the desired cytocidal effect with 4-HPR alone in mice might not be obtainable in humans and further chemical modification may improve the bio-availability of this compound. These reasons prompted us to generate small libraries of synthetic derivatives of 4-HPR and screen for their ability to induce cytotoxicity in RT cell lines in vitro. The initial studies have allowed us to identify the active moiety on 4-HPR required for inhibiting RT cell growth and provided information for further development and improvement of 4-HPR.

Synthesis of 4-HPR derivatives. The amide derivatives of retinoic acids were synthesized by amidation reactions.[27] The 4-HPR (**1**) and the derivatives (**5a**–**5n**) were synthesized (Scheme 1) starting from retinoic acid. First, retinoic acid was converted to acid chloride derivatives, then in the presence of DMF, base, and aniline derivatives, the corresponding 4-hydroxy phenyl amine derivatives (**5a**–**5n**) were synthesized. This procedure gave 70–90% yield. After purification through Silica gel chromatography, eluting with ethylacetate and hexane, compounds **5a**–**5n** (Scheme 1) were obtained. To characterize these new compounds, analytical data of these compounds (NMR 1H, 13C) were compared with known compounds.

The typical reaction procedure for synthesis of **5J**-(2E,4E,6E,8E)-*N*-(4-hydroxy-3-Iodophenyl)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-nona-2,4,6,8-tetraenamide is as follows. A mixture of all-trans retinoic acid (ATRA) (57 mg, 0.19 mmol) in dry DMF (1 mL) and dry CH_2Cl_2 (2 mL) was stirred under nitrogen atmosphere for 1 h. Oxalyl chloride (0.72 mmol, 62 μL) was added drop by drop at 0 °C. The deep red reaction mixture was stirred for another 1.5 h at room temperature under nitrogen atmosphere. The solvent was removed very carefully and dry DMF (2 mL) was added for immediate use. At 0 °C under nitrogen atmosphere, retinoyl chloride solution was added dropwise to a solution of 4-amino-2-iodophenol (0.38 mmol, 90 mg) and triethylamine (0.57 mmol, 80 μL) in dry DMF (2 mL). The dark-colored reaction mixture was stirred at room temperature until TLC analysis indicated none remaining (about 2–3 h). The reaction was quenched with saturated NH_4Cl and extracted with ethyl acetate. The extracts were washed with H_2O and brine, then dried overage Na_2SO_4, and evaporated. The residue was purified by flash column chromatography using hexane/ethyl acetate (8/1) as the eluent to give 5j as a yellow solid (87.2.8 mg, 89%). 1H NMR (300 MHz, $CDCl_3$): δ 9.61 (s, 1H), 7.69 (s, 1H), 7.15 (d, J = 8.92, 1H), 6.96 (d, J = 9.2, 1H), 6.31 ~ 6.14



a: R^1= F, R^2= H, R^3= H .; b: R^1= Cl, R^2 = H, R^3 = H

c: R^1 = Br, R^2 = H, R^3= H.; d: R^1 = I, R^2 = H, R^3 = H

e: R^1 = NO_2, R^2 = H, R^3 = H.; f: R^1 = OCH_3, R^2 = H, R^3 = H

g: R^1 = OH, R^2 = H, R^3 = H.; h: R^1 = OH, R^2 = F, R^3 = H

i: R^1 = OH, R^2 = Cl, R^3 = H.; j: **R^1 = OH, R^2 = H, R^3 = I, R^4 = H**

k: R^1 = OH, R^2 = NO_2, R^3 = H.; l: R^1 = CH_2OH, R^2 = H, R^3 = H

m: R^1 = CH_2CH_2OH, R^2 = H, R^3 = H.; n : R^1 = OH, R^2 = H, R^3 = H, R^4 = OH

5a-5m R^4= H

Scheme 1.

(m, 4H), 5.77 (s, 1H), 5.12 (br s, 1H), 2.43 (s, 3H), 2.04 (br s, 5H), 1.74 (s, 3H), 1.62 (m, 2H), 1.49 (m, 2H), 1.05 (s, 6H). ESI MS:$[M+H]^+$ 518.05, calcd M 517.15 for $C_{26}H_{32}INO_2$.

Cell culture and drugs. The rhabdoid tumor cell line, MON (6) was maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37 °C with 5% CO_2 and 95% humidified air. Stock solutions of 4-HPR and its chemical derivatives were prepared by reconstituting in 100% ethanol as 10 mM solutions. The aliquots were stored frozen at −80 °C, protected from light. Working solutions (3 μM) and serial dilutions were prepared by diluting the stock solution with culture medium, such that the concentration of ethanol was <2% in all dilutions.

MTS assays to test the activity of 4-HPR and its derivatives. Aliquots of 8×10^3 MON cells were plated in 96-well microtitre plates. Twenty-four hours after seeding, the cells were treated with serial dilutions of each drug and incubated for 3 days. Cell survival was determined using an MTS assay kit (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay Kit, Promega, Madison, WI). Cell plating, drug treatment and survival assay were performed using the epMotion 5070 automated liquid handling robotic system (Eppendorff, Westbury, NY).

Results of survival assay. Survival assay was carried out testing the effect of first set of compounds (a–d) and comparing their effect on MON cells to that of the parent compounds 4-HPR and retinoic acid (ATRA). The results of survival assay indicated that while 4-HPR exhibited an IC_{50} of ~15 μM, retinoic acid (IC_{50} 100 μM) was largely ineffective in inhibiting the growth of rhabdoid cells (Fig. 1A). Substitution at the para position with any of the halogens, that is, fluoro- (**5a**), chloro- (**5b**), bromo- (**5c**), or iodo- (**5d**) moieties, greatly reduced the activity of 4-HPR in RT cells (IC_{50}'s increased to 150 μM or greater, Fig. 1A), though (**5d**) precipitated in cell culture medium, and therefore IC_{50} was not determined (Fig. 1A).

To determine if the reduction in the activity in 4-HPR compounds was due to the presence of halogen groups or other reasons, we substituted para-hydroxy group with alternatives including the methoxy (**5f**) and nitro (**5e**) moieties. The para-methoxy (**5f**) derivative was insoluble in cell culture medium and the para-nitro derivative (**5e**) was largely ineffective, with an IC_{50} greater than 200 μM (Fig. 1B).

We further synthesized 2,4 dihydroxy, or 4-methyl and 4-ethyl hydroxy phenyl derivatives, to determine if a fixed position is required for the hydroxyl group on the phenyl ring or extending the carbon chain is tolerated. We found that substitution of methyl (**5l**) or ethyl hydroxy (**5m**) derivatives at para-position resulted in lack of activity consistent with the idea that a fixed position for the para-hydroxy group is important for activity (Fig. 1C). Interestingly, we found that 2,4-hydroxy derivative (**5n**), has more activity compared to 4-HPR (IC_{50} 8 μM as opposed to 19 μM for 4-HPR in this particular experiment, Fig. 1C). These results suggested that substitutions at meta-position of phenyl group could enhance the activity.

The above results indicated that hydroxy group at para-position is important for 4-HPR activity in RT cells. Furthermore, 2,4-dihydroxy derivatives were more active indicating that addition at meta-position is tolerated. To further confirm these observations and to derive halogen substituted compounds of 4-HPR, we synthesized the next set of derivatives with substitutions at the meta positions, keeping the 4-OH group intact. We found that addition of fluoro- (**5h**) and chloro- (**5i**) halogen moieties to the meta-position resulted in compounds with similar or slightly more activity compared to 4-HPR in RT cells (Fig. 1D). Substitution of a nitro group at meta-position (**5k**), resulted in a compound that was insoluble in cell culture medium. Interestingly, substitution of iodo-moiety at meta-position (**5j**) substantially increased the efficacy ($IC_{50} \sim 3$ μM, Fig. 1D). These results indicate that the presence of the para-hydroxy group is important for maintaining the efficacy of 4-HPR and its derivatives against rhabdoid tumor cells, and that the substitution at meta-position is tolerated. The IC_{50} values for all the compounds tested are listed in the table (Table 1).

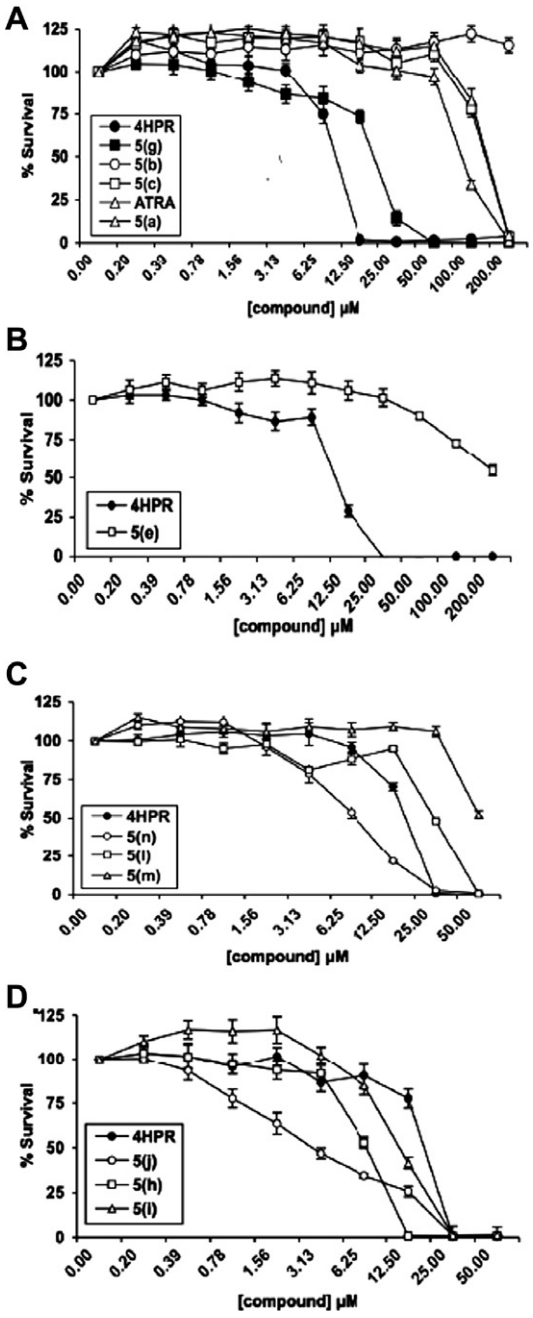


Figure 1. Effect of 4HPR derivatives on survival of rhabdoid tumors cells. MON (INI1−/−) cells were treated with serial dilutions of the 4HPR, ATRA, and 4HPR derivatives for three days. Survival assay was carried out as described using MTS assay kit. A–D Percentage of cell survival plotted against concentration of drugs (Mean ± SEM). (The original figure is included in the Supplemental data).

4-HPR is a synthetic retinamide that has promising anticancer activity and minimal toxicity in humans. Our attempt to obtain derivatives of 4-HPR that lend themselves to further modifications such as conjugation with nano-particles or radiochemicals led to the identification of active moiety required for its cytotoxic activity on rhabdoid cells. We have demonstrated that sub-

Table 1
Table indicating the IC_{50} values, derived from the survival curves, for each drug

Figure	Compound	IC_{50} in μM
1A	4HPR	9
	ATRA	100
	5(g)	19
	5(c)	150
	5(b)	>200
	5(a)	150
	5(d)	n/a
1B	4HPR	12
	5(f)	n/a
	5(e)	>200
1C	5(l)	25
	5(m)	>50
	5(n)	8
1D	4HPR	19
	5(h)	8
	5(k)	n/a
	5(j)	3
	5(i)	12.5

stitution of para-hydroxy group with halogens, nitro, methoxy, hydroxymethyl, or hydroxyethyl groups abolished its cytotoxic activity. However, ortho- and meta-positions can be substituted with halogens or hydroxy groups. We found that substitution of halogens at the meta-position retained the cytotoxic activity, with iodo-substitutions exhibiting better IC_{50} values than parent 4-HPR.

In summary, our analysis indicated that 4-hydroxy position of 4-HPR is important for its activity. This conclusion was not made in a previous study which indicated that side chain length of the functional group may correlate with activity. Our studies suggested that 4-hydroxy group may directly contact the target protein in the RT cells to mediate its activity. However, the exact target protein that binds to 4-HPR to mediate its activity is not completely understood. It has been demonstrated that 4-HPR binds to RAR-β and RAR-γ more tightly when compared to that of all trans retinoic acid (ATRA) but activates these receptors to a lesser extent. Furthermore, studies of 4-HPR derivatives indicate that the cytotoxic activity is not correlated to RAR activation. Consistent with this idea we found that ATRA did not have appreciable cytotoxic activity in our cell culture models. We propose that using the synthetic derivatives that retain activity in cell culture models, we will be able to not only derive clinically important halogen conjugates for radiotherapy, but may also be able to identify target proteins responsible for mediating 4-HPR activity in RT cells.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.05.021.

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