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Design, synthesis of novel peptidomimetic derivatives of 4-HPR for rhabdoid tumors

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ABSTRACT

Rhabdoid tumors (RTs) are an extremely aggressive pediatric malignancy that results from loss of the *INI1*/hSNF5 tumor suppressor gene. Loss of *INI1* results in aberrant expression of Cyclin D1, which supports rhabdoid tumorigenesis and survival. 4-HPR, a synthetic retinoid that down-modulates Cyclin D1, has shown promise in treating various tumors including RTs. In this study, we have generated a chemical library of peptidomimetic derivatives of 4-HPR in an attempt to create a more biologically active compound for use as a therapeutic agent against RTs and other tumors. We have synthesized novel peptidomimetic compound by substituting alkene backbone with a ring structure that retains the biological activity in cell culture models of rhabdoid tumors. We further identified derivative of peptidomimetic compound (11d, $IC_{50} \sim 3$ μM) with approximately five times higher potency than 4-HPR (1, $IC_{50} \sim 15$ μM) based on a survival assay against rhabdoid tumor cells. These studies indicate that peptidomimetic derivatives that retain the cytotoxic activity are promising novel chemotherapeutic agents against RTs and other tumors.

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Rhabdoid tumors of the kidney (Malignant Rhabdoid Tumors, MRT), central nervous system (Atypical Teratoid and Rhabdoid Tumors, AT/RT), and soft tissues (Extra-renal Rhabdoid Tumors) are rare pediatric malignancies.[1–4] These tumors are aggressive and mostly incurable as there are no effective or standard treatment strategies currently available. There is a need to develop novel therapies preferably based on the understanding of the molecular basis of genesis of RTs. We previously demonstrated that Cyclin D1 is essential for the genesis and survival of RTs and that therapeutic targeting of Cyclin D1, with agents such as 4-HPR, is effective in inhibiting RT growth in preclinical models.[5,6]

4-HPR [*N*-(4-hydroxyphenyl) retinamide, or fenretinide] is a synthetic retinoid that has a low potential for toxicity and suppresses tumor cell growth in vitro at clinically achievable concentrations (IC_{50}'s ranging from 1 to 15 μM).[7] It is under phase III clinical trials for many cancers including neuroblastomas (National Cancer Institute Ref: 06-C-0227).[7] It has also been studied as a chemopreventive agent in animal models of carcinogen-induced epithelial tumors and in patients at risk for breast cancer. Compiled data from a series of phase III trials showed that 4-HPR acts as a potent chemopreventive agent, significantly reducing the risk of second breast cancer in pre-menopausal women.[8]

In vitro studies indicate that 4-HPR induces apoptosis in tumor cell lines by several mechanisms, including, activation of retinoid receptors RAR β and γ, ceremide-dependent cytotoxicity, generation of free radical oxygen species, increasing NOS expression resulting in NO-dependent cell cytotoxicity, and increases mitochondrial permeability transition resulting in both caspase-dependent and -independent cell death.[9–14] 4-HPR also induces cell cycle arrest likely correlated to its ability to down-modulate the expression or activity of proliferation related targets including c-myc, telomerase, p34/cdc2 and Cyclin D1.[15,16] Since RT cells over-express and are dependent on Cyclin D1, we demonstrated that 4-HPR exhibits anti-tumor effects on RT cells in vitro and in vivo, with low toxicity in mouse models.[5,6]

There are several reports indicating that synthetic analogs of 4-HPR are more active as anti-tumor agents or display reduced toxicity in vitro. For example, a non-hydrolyzable carbon-linked analog (*N*-benzyl hydroxyl retinamide, or 4-HBR) no longer suppresses plasma vitamin A levels as 4-HPR does, and hence is safer.[17] The sulfur-containing heteroretinoids induce apoptosis and reactive oxygen species more specifically in malignant cells versus normal cells therefore reducing unwanted toxicities.[18] Conjugations of 4-HPR have also displayed increased anti-tumor

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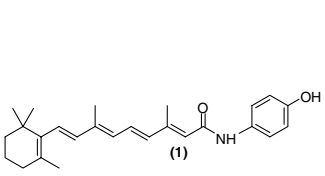
activity. Specifically, glycosyl conjugated mannosyl with 4-HPR had increased activity in the HL60 promyelocytic leukemia cell line.[19] Notably, 4-oxo-fenretinide has been shown to induce G2-M cell cycle arrest and apoptosis in both 4-HPR-sensitive and -resistant cell lines.[20]

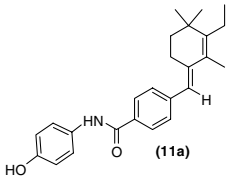
Since substituted or conjugated derivatives of 4-HPR show increased activity or similar activity with more favorable toxicity profiles, we wanted to further explore modification of this promising therapeutic agent. We have a long term goal of developing novel therapeutics for RTs within the brain (AT/RTs). We hypothesize that, structural modification of existing 4-HPR compounds to create peptidomimetic derivatives may increase the potency of these compounds against RTs, may allow for the delivery of these compounds across the blood–brain barrier. We report here design synthesis and biological activity of a novel peptidomimetic compound of 4-HPR against RT cells in culture.

The generation of peptidomimetics is based on the conformational, topochemical and electronic properties of the lead peptide backbone as possible with non-peptide fragments while still maintaining the pharmacophoric groups of the peptides.[21] This makes the compound more lipophilic, which increases its bioavailability and possibly facilitates crossing through the blood–brain barrier.[22] Replacement of the amide bond with alternative groups also prevents proteolysis and promotes metabolic stability.[23] Conformational flexibility is often required to allow for the pharmacophoric groups to find their binding sites, but further lead refinement should favor the formation of more conformationally restricted analogs that hold appropriate pharmacophoric groups in the bioactive conformation for binding to the target proteins.[24] Based on these principles, we have attempted to synthesized peptidomimetic derivatives of 4-HPR with substitution of the alkene backbone with a phenyl ring structure. During these studies, we obtained compounds (**11a**, Structure 1), with altered structure compared to 4-HPR, but some of these compounds remarkably retained biological activity in cell culture models.



(1)

4-HPR = 4-hydroxyphenyl retinamide



(11a)

(*E*)-4-((3-ethyl-2,4,4-trimethylcyclohex-2-enylidene) methyl)-*N*-(4-hydroxyphenyl)benzamide

We synthesized further derivatives by additional modifications in the 4-hydroxy phenylamide groups (compounds **11a**–**11g** in Scheme 1). These compounds were tested alongside parent compound, 4-HPR, in a biological survival assay to determine their anti-tumor activity against RT cells.

Novel peptidomimetic derivatives of 4-HPR were synthesized by modifying the existing organic reactions. After synthesizing the acid **8** from compound **3** by Witting reaction, we carried out amide coupling reaction with different aminophenol derivatives. The synthesis of **8** involves the reaction with methyl magnesium bromide with β-cyclocitral in THF that yields alcohol **4** as yellow oil (Scheme 1).[25] The alcohol gave satisfactory spectral data and was directly converted to **5** by treatment with triphenylphosphine hydrobromide in methanol. Recrystallization of **5** from methanol/ether (1:6) gave a yellow crystalline solid.[26] Formation of the Witting reagent from **5** in ether was accomplished with *n*-butyllithium in hexane at room temperature (dark-red color), then the Witting reagent was treated with methyl 4-formybenoate **6** in ether at −78 °C for 10–15 min and then stirred at room temperature under a nitrogen atmosphere for 30 h. After this analysis, crude ester **7** was purified by flash column chromatography (hexane/ethyl acetate 98:2) to obtain a brown oil with 85% yield.[27] The ester was saponified to generate a white solid which was filtered, washed with water, and dried. The product was recrystallized from hot ethanol and washed with dry hexane to obtain acid **8** as white crystals (87% yield). The structure of he compound was confirmed by ^{1}H, ^{13}C NMR, NOE, HMBC, and by HRMS. The acid **8** was first converted to acid chloride and coupled with aniline derivatives to obtain compounds **11a**–**11g** with 70–90% yield.

The detailed reaction procedure for the synthesis of lead compound **11d**-(*E*)-*4*-((3-ethyl-2,4,4-trimethyl-cyclohex-2-enylidene)methyl)-*N*-(4-hydroxy-3-iodophenyl)benzamide is provided in Ref. 28.

Cell culture: The MON RT cell line[29] was used for in vitro cytotoxicity studies. MON cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C with 5% CO_2 and 95% humidified air.

Compound preparation and storage: Stock solutions of 4-HPR and its peptidomimetic derivatives were prepared at 10 mM in 100% ethanol and stored frozen at −80 °C protected from light. Working solutions (50 μM) and serial dilutions were prepared by diluting the stock solutions in cell culture medium such that the concentration of ethanol was <2% in all dilutions.

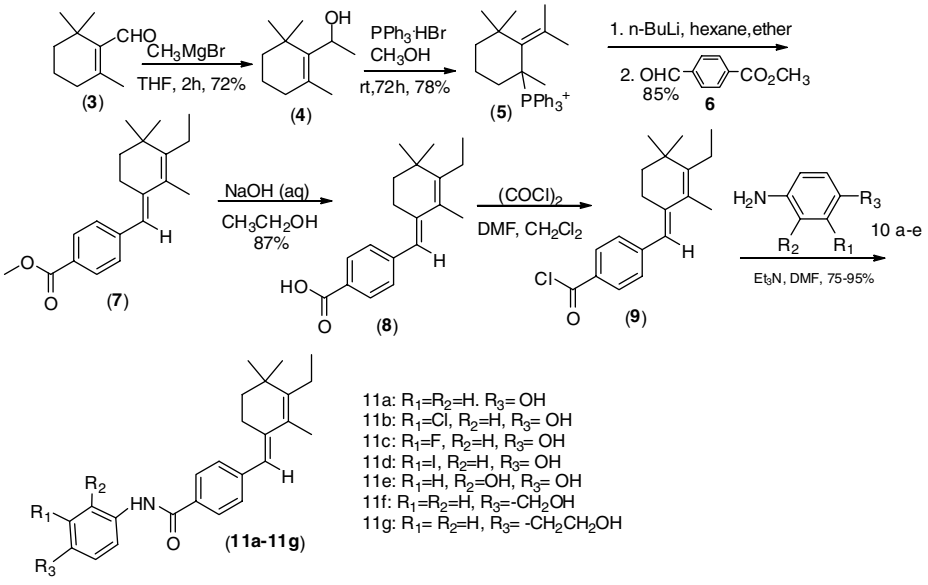
In vitro cell proliferation assay: Aliquots of 8×10^3 cells were plated in 96-well microtiter plates. Twenty-four hours after seeding, the cells were treated with serial dilutions of each drug and incubated for 3 days. Cell survival was determined using an MTS assay kit (CellTiter 96® Aqueous Non-Radioactive 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 (Eppenddorff, Westbury, NY).

Statistical analysis and determination of IC_{50} values: Statistical analysis of the data was carried out using GraphPad Prism (San Diego, CA). Relative IC_{50} values were calculated using the nonlinear regression curve fit with sigmoidal dose–response (variable slope) function. Relative IC_{50} is defined as the concentration giving a response exactly half way between the fitted top and bottom of the survival curve when graphed as percent inhibition versus the log of the concentration of compound. The fitting error, or standard error describes the error involved in fitting the curve.

Results and discussion: In a parallel study we have reported that the 4OH group at para-position of the phenyl ring of 4-HPR is necessary for cytotoxic activity against RT cells.[30] We wanted to test if this were to be true if we substitute the para-position in the new peptidomimetic compound as well. We synthesized several compounds substituting the alkene backbone with a ring structure and in addition further modifying the 4OH-phenyl moiety. We obtained compound (**11a**) and its derivatives (**11b–11g**), as represented in Scheme 1. These compounds were tested in a biological survival assay.

Previously, we have demonstrated that 4-HPR exhibits cytotoxic activity against many RT cells including MON, G401 and STM.[5] The cytotoxicity profiles of 4-HPR against these cells were very similar with IC_{50}s ranging from 5 to 12 μM.[5] MON cells were least sensitive with an IC_{50} of ~12 μM.[5] Therefore, we surmised that screening the activity of novel peptidomimetic compounds against MON cells will be most effective. If the novel compounds exhibit improved activity, then we will be encouraged to test these compounds against other RT cells and other cancer cells in the future. Therefore, as a first test of the activity of novel 4-HPR derivative compounds we chose MON cells to carry out the biological assay.

Results of the biological survival assay are illustrated in the Figure 1 and Table 1. The peptidomimetic compound **11a** exhibited



Scheme 1.

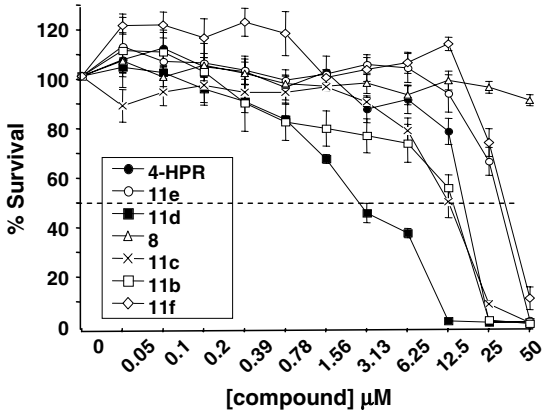


Figure 1. Effect of peptidomimetic derivatives of 4-HPR on the survival of rhabdoid cells. MON (INI1−/−) cells were treated with serial dilutions of the 4-HPR and 4-HPR derivatives for three days. Survival assay was carried out as described using MTS assay kit. Percentage of cell survival plotted against concentration of drugs (mean ± SEM).

Table 1
Relative IC_{50} values for each drug

Compound	Relative IC_{50} in μM	Fitting error (±SEM)
4-HPR	14.68	.221
8	>50	NA[a]
11(**b**)	10.44	.227
11(**c**)	12.88	.077
11(d)	3.149	.190
11(**e**)	47.18	.652
11(**f**)	29.06	.083

[a] IC_{50} greater than the highest concentration tested.

similar cytotoxicity profile as compared to compound **1** at lower concentrations of the drugs tested. However, the compounds **11a** and **11g** precipitated in cell culture medium and therefore, the exact IC_{50} value for these compounds could not be determined. Compounds **11e**, **11f**, and **8**, exhibited IC_{50} values of 47.18 μM, 29.06 μM, and >50 μM, respectively, which are much greater than the parent compounds. Two compounds with chloro- and fluoro-substitutions at the meta-position of the phenyl group (compounds **11b** and **11c**, respectively), exhibited similar level of activities to that of the parent 4-HPR compound (IC_{50} values of 10–13 μM, respectively). Interestingly, compound **11d**, with substitution of an iodo-moiety at the meta-position of the phenyl ring demonstrated improved efficacy with IC_{50}'s reduced to 3 μM. This compound did not show any precipitation in the culture conditions. These results indicated that compound **11d** is a promising derivative of 4-HPR that shows improved activity in vitro.

We have attempted to generate novel derivatives of 4-HPR, changing the alkene backbone of retinoic acid, substituting it with a rigid ring structure, and have tested their cytotoxic activity in cell culture models of RT. We found that substituting the alkene backbone of retinoic acid with rigid phenyl group does not change the activity of the parent compound. Furthermore, substitution of the para-hydroxy group of the novel compound with ethyl hydroxy group (**11f**) resulted in reduced activity, consistent with our parallel findings that 4-OH group at the para-position of the phenyl group is required for cytotoxic activity. Compounds **11b** and **11c** (chloro- and fluoro-substitutions at meta-position of phenyl ring, respectively) exhibited activity similar to that of 4-HPR. Interestingly, we found that iodo-derivative (compound **11d**) was more active than the parent compound, with a IC_{50} of 3 μM.

The reason for the improved activity of the compound **11d** as compared to 4-HPR and to compounds **11b** and **11c** is not completely clear. We propose that perhaps, the bulkiness of the iodo group (as compared to that of chloro and fluoro groups) may make it more active. We obtained similar improved results of substituting the 4-HPR with iodo group reported in a parallel study.[30] We propose that the peptidomimetic derivatives with substitution of alkene backbone with rigid ring structure is likely to be more stable in vivo, and hence represents a novel 4-HPR derivative with improved efficacy. Combining with their increased potency, these compounds are candidates for developing more stable and active anti-cancer drugs.

Our observation that a derivative of 4-HPR with substitution of the alkene backbone by a ring structure retains the biological activity is intriguing and suggests that the functional receptor or target of 4-HPR could be different from that of RA in rhabdoid cells. We are currently synthesizing chemical libraries of 4-HPR peptidomimetic compounds by changing the amide bonds with different amide isosteres groups to develop anti-cancer drugs with better

activity and are using functional genomic approaches to determine the exact target of active 4-HPR derivatives in rhabdoid 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.2008.05.097.

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28. *Synthesis of* ***11d***. A mixture of acid 8 (100 mg, 0.352 mmol) in dry DMF (1 mL) and dry CH_2Cl_2 (4 mL) was stirred at room temperature under nitrogen atmosphere for 15 min. Oxalyl chloride (1.31 mmol, 120 μL) was added drop by drop at 0 °C. The reaction mixture was stirred for another 1.5 h at room temperature under nitrogen atmosphere. Very carefully removed the solvent, then added dry DMF (2 mL) at 0 °C under nitrogen atmosphere. Then acid chloride solution (**9**) was added dropwise to a solution of 4-amino-2-iodophenol (0.704 mmol, 165 mg) and triethylamine (2.11 mmol, 300 μL) in dry DMF (2 mL). The reaction mixture was stirred at room temperature and progress of the reaction was monitored using TLC. After the reaction completed 3 h, the reaction was quenched with saturated NH_4Cl (8 mL) and extracted with ethyl acetate (2× 15 mL). The extracts were washed with (2× 10 mL) and brine (2× 10 mL), then dried over Na_2SO_4, and evaporated. The residue was purified by flash column chromatography using hexane/ethyl acetate (4:1) as the eluent to give a pale yellow solid 151.36 mg (86%), mp: 170–171 °C. ^{1}H NMR(300 MHz, DMSO): δ10.12 (s, 1H), 10.02 (s, 1H), 8.14 (d, J = 2.3 Hz, 1H), 7.9 (d, J = 8.3 Hz, 2H), 7.58 (dd, J = 8.7 Hz, 2.3 Hz, 1H), 7.42 (d, J = 8.3 Hz, 2H), 6.86 (d, J = 8.7 Hz, 1H), 6.47 (s, 1H), 2.60 (m, 2H), 2.20 (q, J = 7.5 Hz, 2H), 1.88 (s, 3H), 1.44 (m, 2H), and 1.09 (s, 6H) and 1.04 (t, J = 7.5 Hz, 3H). ^{13}C NMR $CDCl_3$: δ 167.4, 149.1, 144.3, 144.2, 142.6, 142.5, 192.6, 129.6, 129.5, 129.5, 127.8, 127.4, 120.9, 120.9, 85.2, 61.1, 52.3, 39.2, 36.2, 28.03, 24.7, 23.3, 15.5, 15.1, and 14.7 ESI MS: Calcd for $C_{25}H_{28}INO_2$ ($[M+H]^+$) 502.12. Found: 502.08.
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