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Synthesis and Ribonucleotide reductase inhibitory activity of thiosemicarbazones

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ABSTRACT

Ribonucleotide reductase (RR) is an important therapeutic target for anticancer drugs. The structure of human RR features a 1:1 complex of two homodimeric subunits, hRRM1 and hRRM2. Prokaryotically expressed and highly purified recombinant human RR subunits, hRRM1 and hRRM2, were used for holoenzyme-based [^{3}H]CDP reduction in vitro assay. Ten new thiosemicarbazones (**7**–**16**) were synthesized and screened for their RR inhibitory activity. Two thiosemicarbazones derived from *p*-hydroxy benzaldehyde (**9** and **10**) were found to be active but less potent than the standard, Hydroxyurea (HU). Guided by the activity of compounds **9** and **10**, 11 new thiosemicarbazones (**17**–**27**) derived from *p*-hydroxy benzaldehyde were prepared and screened for their RR inhibitory activity. All the 11 compounds were more potent than HU.

Ribonucleotide reductase (RR) catalyzes the reduction of ribonucleotides to their corresponding deoxyribonucleotides, which are the building blocks for DNA in almost all the living cells.[1] They transform ribonucleotides to deoxyribonucleotides by catalyzing the substitution of the 2′-OH group of ribonucleotide with 2′-H. Human RR belongs to Class Ia. Since the reduction of ribonucleotides is the rate-limiting step of DNA synthesis, inactivation of RR stops DNA synthesis, which inhibits cell proliferation. This along with the fact that RR has low activity in resting cells, high activity in rapidly growing normal cells, and very high activity in cancer cells has made it an important target for cancer therapy.[2] Also studies have indicated the critical role of RR in tumor promotion. Therefore, RR is considered as a relevant molecular target for the design and development of antitumor agents.[3]

Several potentially useful class of RR inhibitors were reported which includes: (1) *Free-radical* scavengers: Hydroxy urea, Trimidox, Didox, etc., (2) Iron chelators: Triapine, PIH, 311, etc., and (3) Substrate analogs (Gemcitabin, Azido CDP, etc.). [2] A class of α-(*N*)-Heterocyclic carboxaldehyde thiosemicarbazones (HCT) were reported to be carcinostatic [4] possibly due to their iron chelation property.[5] HCTs were reported to inhibit RR and are suggested to interact with the iron in the R2 subunit of RR.[6] Iron chelating property and carcinostatic activity of acylhydrazones and thiosemicarbazones derived from salicylaldehyde and 1-naphthol-2-carboxaldehyde (Fig. 1) have been well documented, but their inhibitory activity of RR has to be explored. In this paper, we report the synthesis and RR inhibitory activity of 21 new aromatic/heteroaromatic aldehyde thiosemicarbazones. These compounds were designed and synthesized to test the concept of iron chelation. In this work, those compounds which satisfy the functional group requirements for iron chelation and also those that do not satisfy these requirements but are structurally similarity to trimidox and didox were synthesized in order establish a structure–activity relationship (Scheme 1).

The synthesis of thiosemicarbazones was carried out by following the procedures reported earlier.[7,8] Potassium hydrazine carbodithioate was prepared by the reaction of hydrazine hydrate

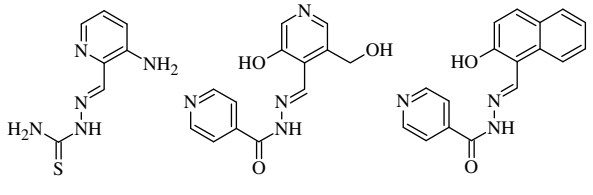

Figure 1. Iron chelators with RR inhibitory activity and carcinostatic property.

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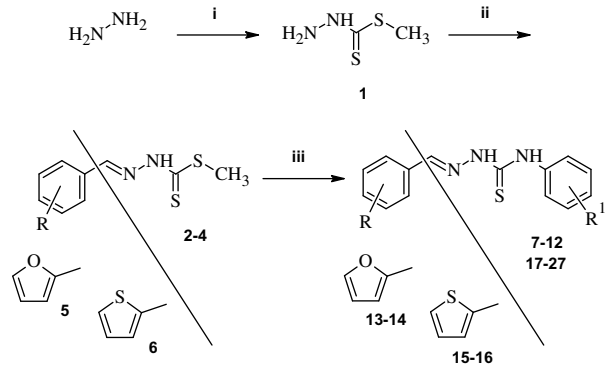
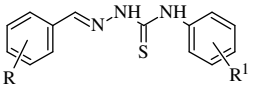


Scheme 1. Synthetic route for thiosemicarbazones. Reagents and conditions: (i) CS_2, KOH, <10 °C, stirring, 15–30 min; CH_3I, <10 °C, stirring, 30–45 min, (ii) R–C_6H_4–CHO, *i*-PrOH, rt, stirring, 30 min, (iii) R^1–C_6H_4–NH_2, EtOH, reflux, 8–12 h.

(85%), carbon disulfide and potassium hydroxide below 10 °C with constant stirring for a period of 15–30 min. This was then converted in to methylhydrazine carbodithioate (**1**) by the action of methyl iodide, added dropwise with stirring maintaining the temperature below 10 °C for a period of 30–45 min. Substituted benzaldehyde hydrazones of methylhydrazine carbodithioate (**2–6**) were prepared by the reaction of **1** with the respective substituted benzaldehydes in isopropanol, stirring at room temperature for a period of 30–45 min. Ten thiosemicarbazones (**7–16**) were prepared by the reaction of **2–6** with their respective aniline derivative in ethanol and by refluxing for a period of 8–12 h till the evolution of methyl mercaptan ceased.. Later another 11 thiosemicarbazones (**17–27**) were prepared by the reaction of and **3**, with respective anilines in a similar manner described above. All the intermediates were characterized by IR spectroscopy and by elemental analysis for CHNS. In the elemental analysis, the percentage variations between observed and calculated values were within ±0.4%. Final compounds were characterized by ^{1}H NMR and FAB-MS (Table 3, Supplemental material). The structure and physico-chemical characterization data of compounds **7–27** were presented in Table 1.

Table 1
Structure and physico-chemical characterization of compounds **7–27**



Compound	R	R_1	Mp[a] (°C)	Yield[b] (%)
7	2-OH	–H	170–171	75
8	2-OH	4-Cl	180–183	70
9	4-OH	–H	188–190	70
10	4-OH	4-Cl	165–166	62
11	3-OMe-4-OH	–H	158–160	55
12	3-OMe-4-OH	4-Cl	178–181	50
13*	2-Furyl	–H	170–175	76
14*	2-Furyl	4-Cl	168–170	70
15*	2-Thiophenyl	–H	180–182	80
16*	2-Thiophenyl	4-Cl	190–192	85
17	4-OH	2-Cl	132–133	60
18	4-OH	3-Cl	120–122	70
19	4-OH	2-Me	141–143	74
20	4-OH	3-Me	92–93	61
21	4-OH	4-Me	73–75	57
22	4-OH	2-OMe	138–141	54
23	4-OH	2-NO_2	106–108	49
24	4-OH	4-NO_2	166–169	62
25	4-OH	2-OH	195–198	57
26	4-OH	3-OH	132–135	47
27	4-OH	4-OH	158–161	65

* Furyl and thiophene ring replaces phenyl ring (R–C_6H_4–).
[a] Melting point determined by capillary method and are uncorrected.
[b] Percentage yield of final step.

RR inhibitory activity was carried out using recombinant proteins, that is, prepared by following the procedure reported earlier.[9–11] The coding sequences of hRRM2 and hRRM1 were obtained from human oropharyngeal carcinoma KB cells and cloned in-frame with an *N*-terminal 6×His-tag into the prokaryotic expression vector pET28 (Novagen, Madison, WI). The proteins were expressed in BL21 (DE3) bacteria (Stratagene, La Jolla, CA) and purified using Ni(II) affinity chromatography. In vitro RR inhibition assays were based on Steeper and Stuart CDP reductase activity method.[12] Each compound was dissolved in neat DMSO and diluted with 50 mM Tris–HCl (pH 7.5). The final concentration of DMSO in the reaction mixtures was 1% v/v. To perform the assay to test the potency of RR inhibitors using purified recombinant proteins, the method was modified and standardized as follows:

Step 1: A mixture of purified hRRM1 and hRRM2 was incubated at room temperature for 30 min with various concentrations of each compound. HU 20 mM was used as a positive control. *Step 2:* To initiate enzymatic reduction, a reaction buffer (0.125 μM [^{3}H]CDP, 50 mM HEPES (pH 7.2), 6 mM DTT, 4 mM MgOAc, 2 mM ATP, 0.05 mM CDP, 100 mM KCl, and 0.24 mM NADPH) was added to the protein/inhibitor mixture from Step 1 up to a final volume of 100 μl. The reaction mixture was incubated at 37 °C for 30 min. The enzyme substrate [^{3}H]CDP and the resulting product [^{3}H]dCDP in the reaction mixture were dephosphorylated by phosphodiesterase. Step 3: The [^{3}H]cytidine and [^{3}H]deoxycytidine in the reaction mixture were separated by HPLC using a C18 reversed phase column connected to a Model 2 β-RAM Radio Flow-Through detector (IN/US Systems, Tampa, FL). Negative control samples, which were run with each experiment, contained only 1% v/v DMSO. The inhibition of RR was expressed as percent of the negative control (relative activity). The relative enzyme activity dependence on inhibitor concentration was fitted using a non-linear regression equation ($f(x) = (a - d)/[1 + (x/c)b] + d$, where a = asymptotic maximum, b = slope parameter, c = value at the inflection point, and d = asymptotic minimum). The IC_{50} values, namely the compound's concentration that produces 50% inhibition, were calculated by setting $f(x) = 50$. The inhibitory potency is reported as the mean of three separate tests, each performed in duplicate.

The first 10 thiosemicarbazones (**7–16**, Table 1) synthesized were screened for RR inhibitory activity. Interestingly those thiosemicarbazones that can complex iron (**7 and 8**) were inactive.

Table 2
Ribonucleotide reductase inhibitory activity of compounds **7–27**

Compound	IC_{50}[a] (μM)
7	>500
8	>500
9	242.0
10	287.0
11	>500
12	>500
13	>500
14	>500
15	>500
16	>500
17	46.8
18	29.3
19	36.5
20	30.4
21	14.2
22	41.6
23	39.3
24	45.1
25	29.3
26	28.6
27	43.3
HU	148.0
3-AP (Triapine)	2.25

[a] Values are means of three experiments.

Surprisingly, 4-hydroxybenzaldehyde derived thiosemicarbazones (**9 and 10**) were found to be active but around 100- and 2-fold less potent than the standard drugs used in the study, Triapine and Hydroxyurea, respectively (Table 2).

Guided by the activity of 4-hydroxybenzaldehyde thiosemicarbazones, we prepared 11 analogs (**17–27**) of these active compounds (**9 and 10**) with different substitution on the N4-phenyl ring (Table 1.). The compounds **17–27** were potent than compounds **9 and 10**, and were more potent than standard drug hydroxyurea (Table 2). Also these compounds are found to be more potent than most of the *N*-hydroxy semicarbazones of aromatic aldehydes reported earlier.[10]

Within this small set of active molecules, a concrete SAR discussion may not be feasible. But few guiding principles can be derived to proceed further in designing this class of molecules as RR inhibitors. Rational selection of substitution for N1- and N4-phenyl rings guided by the activity of compounds **17–27** and synthesizing the new analogs may be considered as a next suitable approach towards reaching a more potent molecule. Present work suggests *p*-hydroxy substitution in N1-phenyl ring and *m*-hydroxy or *p*-methyl substitution in N4-phenyl ring are favorable for RR inhibitory activity. Guided by this principle, synthesis of analogs of compounds **21** and **26** are currently underway.

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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.2008.09.097.

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