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Original article

Chemopreventive and antioxidant activity of 6-substituted imidazo[2,1-*b*]thiazoles

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ABSTRACT

The synthesis of new imidazo[2,1-*b*]thiazoles bearing phenolic groups is reported. These compounds and some previously described analogs were evaluated as antioxidant agents with three chemical model systems, and cancer chemopreventive potential was examined by inhibition of NO production, TNF-α activated NFκB activity, and aromatase activity, as well as induction of QR1 and RXRE binding. Two of the test compounds, **9** and **12**, displayed promising activity by inhibiting iNOS, NFκB and aromatase in dose −dependent manner, with IC_{50} values in low micromolar range. The same compounds activated QR1 in a bifunctional manner. When incubated with human liver microsomes, the active compounds were further hydroxylated on the parent ring system, suggesting the next logical step in the development of these promising leads will entail synthetic production of metabolites followed by additional assessment of biological activity.

1. Introduction

In recent years evidence has accumulated in support of the supposition that excess of reactive oxygen species (ROS) and nitrogen species (RNS) plays a key role in the onset and development of various diseases such as lung, liver and breast cancers through damage to DNA and other biomolecules [1–3]. Accordingly, antioxidants can be viewed as important factors for the treatment and prevention of cancer [4], and it is reasonable to assume that compounds functionalized with groups endowed with potential antioxidant properties could be useful as new drugs for chemoprevention and chemotherapy. Indeed, several substances characterized by antioxidant and chemopreventive properties have been identified, including polyphenols, with activity due to the presence of phenolic hydroxyl group(s) [5–7], and compounds containing heterocyclic systems such as imidazole [8,9], thiazole [10] and thiadiazole. [11,12].

Based on these considerations, we describe a series of previously reported [13–16] and new imidazo[2,1-*b*]thiazoles bearing phenolic hydroxyl group(s). These compounds were subjected to chemical and biological tests to determine antioxidant activity and inhibition of NO production.

In addition, the compounds were evaluated for potential to (a) inhibit NFκB, a transcription factor, since its activity is involved in cancer development and progression, (b) induce NAD(P)H:quinone reductase 1, (QR1), a cytoprotective enzyme, which protects against carcinogenesis by detoxifying and eliminating carcinogens [17], (c) inhibit aromatase, which, under some situations (e.g., postmenopause), is a key player in estrogen production, in fact inhibitors have been shown to function as chemopreventive agents [18], and (d)

Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; TNF, tumor necrosis factor; NFκB, nuclear factor kappa beta; QR1, NAD(P)H, quinone reductase 1; RXR, retinoid X receptor; TPCK, tosyl phenylalanyl chloromethyl ketone; BR, Briggs–Rauscher; TEAC, Trolox Equivalent Antioxidant Activity; DPPH, 2,2-diphenyl-1-picrylhydrazyl; RAC, Relative Antioxidant Capacity; BDE, Bond Dissociation Enthalpies; iNOS, inducible nitric oxide synthase; L-NMMA, L-N^G-monomethyl arginine citrate; IR, induction ratio; AhR, aryl hydrocarbon receptor; RXRE, retinoid X receptor response element; SRM, selected reaction monitoring; SRB, sulforhodamine B; MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide.

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activate retinoid X receptor (RXR), which is involved in cell proliferation, differentiation and apoptosis [19].

Finally the metabolism of the most active compounds was studied with model systems to provide information relevant to metabolic stability, and the most abundant metabolites were partially characterized.

2. Chemistry

The imidazo[2,1-*b*]thiazoles **4**–**16** reported in Scheme 1, were prepared from the appropriate 2-aminothiazoles **1** and haloketones **2**. The intermediate compounds **3** were isolated ($\nu_{C=O}$ absorption was confirmed around 1700 cm^{-1}) and used in the subsequent step without further purification. The IR and ^1H NMR spectra of the new compounds are in agreement with the assigned structures.

3. Antioxidant activity

The chemical antioxidant activity of compounds **4**–**16** was assessed with three procedures: the Briggs–Rauscher (BR) oscillating reaction method under acidic conditions [20], the Trolox Equivalent Antioxidant Activity (TEAC) assay at pH = 7.4 [21], and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test in organic medium (MeOH or EtOH) [22,23].

The results are reported in Table 1.

3.1. Antioxidant activity at acidic pH

In view of a possible development of these new compounds as chemopreventive drugs orally administered, it is of some interest to test antioxidant activity at an acidic pH value approximating that of gastric fluids, even though no chemical assay can completely mimic the human environments. Some evidence suggests the stomach acts as a bioreactor in which many drugs can interact [24]; moreover, *in vivo* studies demonstrated that some polyphenols are promptly absorbed in the stomach [25,26]. Inspection of the data in Table 1 reveals that the relative antioxidant activity in acidic conditions of compound **5** is quite high, similar to that found for (−) catechin (2.2) from green tea [27], one of the strongest antioxidants. Compounds **9** and **14** showed activity similar to that of the standard (resorcinol = 1.00). The activities of compounds **4**, **6**, **12** and **15** are similar to those obtained for polyphenols from *Polygala alpestris* (0.10–0.57) and for puerarin (0.192) [28,29]. However, there was no apparent correlation between the relative antioxidant activity and number of phenolic –OH groups in acidic medium. This may be due to interference between the imidazothiazole system and some of the components of the BR mixture, in particular acidic iodate and hydrogen peroxide [28] (see Experimental section and Supporting information). In any case, no activity was found for the negative control **16** and for compound **8** containing an $-OCH_3$ group that is a very poor free radical scavenger [30].

Table 1
Antioxidant activity of 6-substituted imidazo[2,1-*b*]thiazoles.

Compd	$(RAC)_m$[a] (μM equiv. Resorcinol)	$(TEAC)_m$ (mM equiv. Trolox)	$(DPPH)_m$ (mM equiv. Trolox)
4	0.158 ± 0.005	1.05 ± 0.06	0.26 ± 0.01
5	2.0 ± 0.1	0.96 ± 0.01	0.79 ± 0.03
6	0.12 ± 0.01	3.09 ± 0.09	1.51 ± 0.05
7	0.0062 ± 0.0003	1.31 ± 0.09	0.0061 ± 0.0005
8	0	0.034 ± 0.002	0
9	1.032 ± 0.002	1.29 ± 0.03	0.71 ± 0.02
10	0.0335 ± 0.0001	1.00 ± 0.03	n.d.[b]
11	0.0472 ± 0.0002	0.78 ± 0.04	n.d.[c]
12	0.14 ± 0.03	3.06 ± 0.05	1.38 ± 0.05
13	0.72 ± 0.02	0.717 ± 0.007	0.46 ± 0.02
14	0.84 ± 0.07	0.96 ± 0.01	0.62 ± 0.02
15	0.35 ± 0.04	1.57 ± 0.02	0.38 ± 0.02
16	0	0	0

[a] RAC (Relative Antioxidant Capacity, standard resorcinol) values are the average of at least three measurements at different concentrations ± SE.
[b] n.d. (not determined): the color of the solution of this compound is similar to that of the DPPH reagent.
[c] n.d. (not determined): the graph % inhibition *versus* concentration of this compound is not linear.

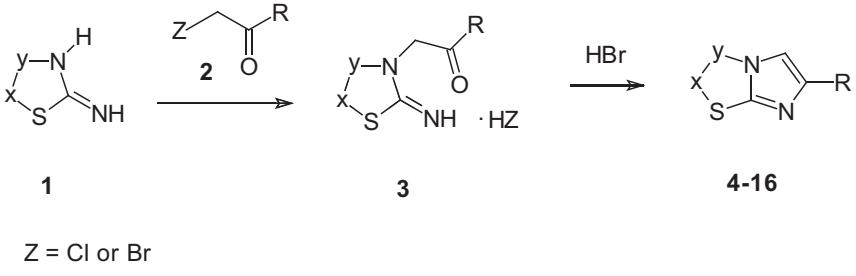
3.2. Antioxidant activity at pH = 7.4

TEAC measurements were conducted at the physiological pH of 7.4. Consistent results were obtained with this method. As expected, compounds **6** and **12**, differing only for the presence of a methyl group at the 2 position of imidazothiazole, showed the same activity within experimental error. The activity values for compounds bearing the same substituent at 6 position are very similar (derivatives **13** and **14**), whereas that of compound **9** is relatively higher. Similar results were also obtained for compounds **4**, **7** and **15**. The antioxidant capacity values at pH 7.4 of compounds **4**, **5**, **10** and **14** are similar to that of α-tocopherol (0.97) and ascorbic acid (1.05) [21]. Compounds **6** and **12** showed activity similar to that of quercetin (3.1), a powerful natural antioxidant [21]. As expected, compound **8** had negligible activity and the negative control **16** did not show any significant activity. In sum, the following order of relative activity was obtained:

6(3.09) ≅ **12**(3.06) > **15**(1.57) > **7**(1.31) > **9**(1.29) > **4**(1.05) > **10**(1.00) = **Trolox**(1.0, positive control) > **5**(0.96) = **14**(0.96) > **11**(0.78) > **13**(0.717) > **8** (0.034) > **16**(0.0, negative control).

3.3. Antioxidant activity in non-aqueous medium

The DPPH assay in non-aqueous medium is largely used for its (apparent) simplicity and rapidity. DPPH• is a solid, stable radical very soluble in MeOH or EtOH giving a violet solution. Free radical scavengers donor of hydrogen decolorize this solution. The entity of the decolorization was assessed spectrophotometrically at 515 nm, 15 min after mixing DPPH• and antioxidant solutions. Indeed, the



Scheme 1. Synthesis of imidazo[2,1-*b*]thiazole polyphenols.

kinetics of this reaction may be slow or very slow for many antioxidants, so measurement after 15 min can lead to an underestimate the antioxidant activity. As shown in Table 1, the DPPH values agree with the TEAC values for compounds **5**, **6**, **9**, and **12**–**14**. Data for other compounds are open to question [22] (see Experimental section and Supporting information).

4. Chemopreventive activity

Nuclear factor kappa beta (NFκB) is a transcription factor that regulates several biological responses such as cell survival, proliferation, expression of cytokines and inducible nitric oxide synthase (iNOS). The aberrant activation or over expression of this transcription factor is linked to several cancers. In principle, inhibition of NFκB can block tumor growth and cancer cellular proliferation, induce apoptosis of cancer cells, and modulate iNOS expression. Mounting evidence suggests that chronic inflammation mediates chronic diseases, including cancer. Many carcinogens and inflammatory agents have been shown to activate NFκB, and resulting tumors demonstrate its misregulation at stages development and progression. Inhibitors of NFκB mediate effects potentially leading to a greater sensitivity to the antitumor agents. Tools have been developed for the rapid assessment of NFκB activity, so in concert with a better understanding of NFκB activation mechanisms, many agents capable of suppressing NFκB activation have been identified [31]. On the basis of these considerations, the subject compounds were evaluated for their potential to inhibit TNF-α induced NFκB activity.

Table 2 summarizes inhibition of TNF-α induced NFκB activity in stable transfected 293/NFκB-Luc human embryonic kidney cells. Compounds **8**, **9**, **12** and **14** demonstrated significant inhibition with IC_{50} values of 0.36, 2.24, 0.53 and 4.5 μM, respectively. TPCK and BAY-11 were adapted as positive controls for NFκB assay. Compounds **8** and **12** showed better inhibition than positive controls. To avoid false positive results with this assay, cytotoxic potential was evaluated with the same cells. Compound **9** demonstrated cytotoxic effect, but the IC_{50} value for cytotoxicity was much higher than the IC_{50} value for NFκB inhibition. Therefore, the apparent inhibition of NFκB is not due to a cytotoxic response.

In addition, the compounds were tested in another indicative chemoprevention assay: inhibition of NO production. In normal physiology, a large amount of NO is produced by iNOS, but it can damage DNA and form nitrative or oxidative DNA species that eventually lead to carcinogenesis. Moreover, it has been reported that the aberrant and prolonged production of NO mainly due to the over expression of iNOS is involved in chronic inflammation which is frequently correlated with neoplastic transformation [32]. In view of this, the inhibitory activities of the compounds on NO production were evaluated using the RAW 264.7 cell line-based assay to determine cancer chemopreventive capacity.

As shown in Table 2, compounds **9**, **11**, **12**, and **14** exerted similar activities with IC_{50} values of 6.4, 6.8, 6.8, and 7.7 μM, respectively. Notably, these compounds showed more potent inhibition than a positive control, L-N^G-monomethyl arginine citrate (L-NMMA) (IC_{50}: 32.0 ± 2.2 μM). Compounds **9**, **11**, **12**, and 14, at the highest test concentration (50 μM), also inhibited the growth of RAW 264.7 cells, in comparison with LPS-treated control. However, the IC_{50} value for compound **9**, the most cytotoxic, was 11.6 μM, approximately two-times higher than the IC_{50} displayed for inhibition of NO production. Therefore, although these compounds displayed cytotoxicity at the high concentrations, they showed NO inhibitory effects at lower, non-toxic, concentrations. Also, compounds **5**, and **6** inhibited the production of NO with IC_{50} values of 19.9 μM, and 41.7 μM, without cytotoxic effects at tested concentrations. This inhibition is comparable to that of the positive control, L-NMMA.

At the highest concentration tested (50 μM) compounds **4**, **10**, **13** and **15** were not active in either assays.

NAD(P)H:quinone reductase 1 (QR1) is an important phase II cytoprotective enzyme which converts quinones to hydroquinones, reducing oxidative cycling [17]. It exhibits cancer protective activity mainly by inhibiting the formation of intracellular semiquinones radicals, and by generating α-tocopherolhydroquinone, which acts as a free radical scavenger. Induction of QR1 often coincides with induction of other phase II enzymes, and is therefore useful in the study of chemopreventive agents. Hepa 1c1c7 (mouse hepatoma) cells are used to assay for QR1 activity. These cells have been used previously to study phase II enzyme inducers and have been found to be reliable in high throughput assays. [33]

The results of QR1 induction are reported in Table 3. Compounds **9** and **12** demonstrated induction ratio (IR) greater than 2; concentrations required to doubles the activity of QR1 (CD) were 12.1 ± 1.9 μM and 24.6 ± 3.2 μM respectively.

Table 2
Inhibition of TNF-α induced NFκB activity and of NO production.

Compd	NFκB				Nitrite			
	% Inhib.[a]	% Survived	IC_{50} (μM)[b]	Cyt IC_{50} (μM)[c]	% Inhib.[a]	% Survived	IC_{50} (μM)[b]	Cyt IC_{50} (μM)[c]
4	49.5 ± 6.4	86.8 ± 3.9			25.3 ± 5.9	89.3 ± 6.8		
5	42.8 ± 5.7	69.2 ± 2.6			91.6 ± 3.2	75.0 ± 6.2	19.9 ± 2.1	
6	49.2 ± 3.9	78.1 ± 11.2			53.2 ± 3.9	91.8 ± 4.5	41.7 ± 3.0	
7	75.1 ± 3.0	74.9 ± 1.6	11 ± 2.94		20.2 ± 7.3	100.5 ± 6.9		
8	90.1 ± 2.3	75.6 ± 6.6	0.36 ± 0.2		32.8 ± 5.6	100.4 ± 6.6		
9	91.0 ± 1.0	43.6 ± 1.8	2.24 ± 0.14	46.5 ± 12	97.7 ± 1.4	48.2 ± 1.5	6.4 ± 0.4	11.6 ± 3.2
10	46.3 ± 4.9	78.2 ± 5.1			0.0 ± 6.2	100.6 ± 14.4		
11	40.8 ± 5.1	66.9 ± 2.4			90.9 ± 1.6	58.1 ± 1.1	6.8 ± 0.4	
12	97.5 ± 1.3	34.3 ± 8.9	0.53 ± 0.15	21.2 ± 5	98.8 ± 1.6	52.3 ± 3.0	6.8 ± 0.8	
13	39.8 ± 9.1	65.6 ± 3.0			25.2 ± 8.5	100.0 ± 11.4		
14	81.6 ± 3.8	78.6 ± 9.4	4.5 ± 0.42		98.5 ± 1.1	52.8 ± 3.4	7.7 ± 0.4	
15	29.9 ± 12.3	58.8 ± 12.2			9.2 ± 7.5	92.0 ± 5.8		
16	73.1 ± 3.4	84.8 ± 5.7	10.25 ± 1.2		18.0 ± 2.7	96.2 ± 6.0		
TPCK[d]			3.8 ± 1.1					
BAY-11[d]			2.0 ± 0.54					
L-NMMA[d]							32.0 ± 2.2	

Data are mean ± SD of two experiments in duplicates.
[a] Testing concentration: 50 μM.
[b] IC_{50} values are concentrations cause 50% inhibition activity (in μM).
[c] Cytotoxic IC_{50} values are concentrations cause 50% cell survival (in μM).
[d] TPCK and BAY-11 are two inhibitors of NFκB used as positive controls. L-NMMA was used as a positive control for the nitrite assay.

Table 3
Induction of QR1, inhibition of aromatase and induction of RXRE-luciferase activity.

Compd	Induction of QR1				Aromatase		RXRE, fold induction at 50 μM
	IR[a]	CD[b] (μM)	BP^rc1 cells, IR	TAOc1BP^rcI cells, IR	% inhibition at 50 μM	IC_{50}, μM	
4	0.71				80.0 ± 0.7	14.8 ± 1.2	0.9 ± 0.2
5	1.78				87.8 ± 1.3	8.6 ± 0.6	0.9 ± 0.3
6	1.45				75.0 ± 1.0	27.0 ± 2.6	0.9 ± 0.1
7	0.60				97.9 ± 1.7	2.9 ± 0.1	1.0 ± 0.4
8	0.91				99.1 ± 1.1	1.1 ± 0.1	0.6 ± 0.2
9	3.80	12.1 ± 1.9	0.90	0.30	98.6 ± 1.2	2.6 ± 0.2	0.5 ± 0.1
10	0.60				86.1 ± 2.6	3.9 ± 0.3	1.3 ± 0.3
11	1.81				52.8 ± 1.3	47.2 ± 1.3	0.5 ± 0.1
12	2.03	24.6 ± 3.2	1.20	0.20	99.6 ± 1.5	3.2 ± 0.0	0.3 ± 0.1
13	0.62				100.3 ± 1.7	2.8 ± 0.5	2.3 ± 0.4
14	1.96				95.8 ± 1.3	3.0 ± 0.4	0.6 ± 0.2
15	0.66				66.3 ± 2.4	21.1 ± 3.7	0.9 ± 0.1
16	1.40				81.0 ± 1.9	9.1 ± 0.5	0.6 ± 0.1
4′-Bromoflavone[c]	4.2	20 ± 12.8 nM					
Naringenin[c]						1.2 ± 0.2	
Bexarotene[c]							4.0 ± 0.3

Data presented are the result of two independent experiments run in triplicate.
[a] IR, induction ratio, represents the specific enzyme activity of compound-treated cells compared with DMSO-treated control.
[b] CD, concentration that doubles the activity. CD values were determined for compounds with IR >2.
[c] 4′-Bromoflavone, naringnin and bexarotene were used as a positive control for induction of QR1, inhibition of aromatase and induction of RXRE, respectively.

Inducers of anticarcinogenic enzymes are divided into two classes: bifunctional inducers, which induce phase I xenobiotic-metabolizing enzymes, which act through a process involving an aryl hydrocarbon receptor (AhR)-dependent mechanism, and subsequently generate intermediates which transcriptionally activate QR1 genes, and monofunctional inducers, which induce phase II enzymes directly without inducing the phase I enzymes, and operate independently of AhR. We tested both compounds with two mutant cell lines: BP^rc1 cells which lack the AhR nuclear translocator required for the transport of the AhR–inducer complex across the nuclear membrane [34], and TAOc1BP^rcI cells, defective in a functional AhR [35,36]. With these two cell lines, compounds **9** and **12** did not show IR greater than 2, implying they act through a bifunctional mechanism.

Aromatase converts androgens to aromatic estrogens through three consecutive hydroxylation reaction steps, and it is a proved target in breast cancer chemotherapy. Aromatase transcription is mediated by IKKβ, a kinase previously known for cancer-promoting activity [37]. Inhibitors of aromatase have been shown to function as chemopreventive agents.

Inhibitory capacity of compounds toward aromatase enzymatic activity was examined by measuring the fluorescent intensity of fluorescein, the hydrolysis product of dibenzylfluorescein by aromatase as previously described [38]. The results of aromatase inhibition are reported in Table 3. Most of the compounds showed IC_{50} values between 1.1–3.9 μM. Compound **16**, as a skeletal structure, showed an IC_{50} value of 9.1 ± 0.5 μM. The introduction of *para*-hydroxy substituent in the benzene ring decreased the inhibitory activity (compound **4**, IC_{50}: 14.8 ± 1.2 μM), while *meta*-hydroxy substitution improved the activity (compound **7**, IC_{50}: 2.9 ± 0.1 μM). However, *para*-hydroxy substitution with *meta*-nitro (compound **10**) or with *meta*-hydroxy substitution (compound **5**) resulted in recovery or improvement of aromatase inhibition compared with compound **16**. Compound **8**, which is substituted with *meta*-methoxy in the benzene ring, was the most active compound tested (IC_{50} 1.1 ± 0.1 μM), showing activity similar to that of Naringenin, a positive control, with an IC_{50} value of 1.2 ± 0.2 μM.

The retinoid X receptor (RXR) is a member of nuclear receptor family proteins. RXR is implicated in some pathological conditions as neoplastic formation and it is a potential target for cancer therapy. Similar to other nuclear receptors, heterodimerization with a ligand is required binding to DNA to operate adequately. Also, RXRs can form homodimers, the ligand-free RXR homodimers bind to DNA response elements (RXRE) and undergo conformational changes that lead to dissociation of corepressor proteins and binding of coactivator proteins, triggering transcription. This can lead to production of regulatory proteins of cell cycle and the induction of apoptosis. Consequently, compounds that bind to RXRs may function as chemopreventive agents.

For evaluation of chemopreventive agents capable of functioning as RXR agonists, we utilized a RXRE-luciferase reporter gene assay. Results are reported in Table 3. The compounds were found to be relatively weak inducers. Compound **13**, the most active, showed 2.3 fold induction, but was less effective than the positive control, bexarotene (4.0 ± 0.3 fold induction) [39].

5. Metabolism

Since compounds **9** and **12** showed the most promising antioxidant and chemopreventive profiles, they were further evaluated in preliminary metabolic studies. Human liver microsomes (1 mg/mL) were incubated with 1 μM compounds **9**, **12** or propranolol (as a reference compound that show medium metabolic stability) in 100 mM phosphate buffer at 37 °C. NADPH (1 mM) was added to initiate oxidative metabolism. At 0, 5, 10, 15, 20, 25, 30, 40, 50, or 60 min, reactions were terminated by the addition of acetonitrile/water/formic acid (86:10:4, v/v/v) containing 5 μM ketoconazole as an internal standard. After centrifugation, aliquots of the supernatant were analyzed using UHPLC-MS-MS to determine the amount of compound remaining or to characterize the most abundant metabolites.

5.1. Ultrahigh pressure-liquid chromatography-mass spectrometry

Quantitative analysis to determine metabolic stability of each compound was carried using UHPLC-MS–MS with positive ion electrospray, collision-induced dissociation and selected reaction monitoring (SRM) with a Shimadzu (Kyoto, Japan) LCMS8040 triple quadrupole mass spectrometer equipped electrospray and a Nexera UHPLC system. UHPLC separations were obtained using a Waters (Milford, MA) Acquity BEH Shield RP18, 2.1 × 50 mm, 1.7 μm UHPLC column. The mobile phase consisted of a linear gradient from 30% to 55% acetonitrile (containing aqueous 0.05% formic acid and

5 mM ammonium acetate) in 0.7 min and then to 100% acetonitrile (containing 0.05% formic acid) at 1.0 min. The UHPLC flow rate was 0.4 mL/min. The SRM transitions were *m/z* 233.0 to 123.0 (quantifier) and *m/z* 233.0 to 205.0 (qualifier) for compound **9**, *m/z* 263.0 to 139.0 (quantifier) and *m/z* 263.0 to 217.1 (qualifier) for compound **12**. The internal standard ketoconazole was monitored using the SRM transition of and *m/z* 531.2 to 489.3, and the SRM transition for propranolol was *m/z* 260.1 to 116.1.

For the characterization of metabolites of compounds **9** and **12**, the UHPLC-MS was carried out using data-dependent product ion tandem mass spectrometry. The UHPLC-MS–MS system was identical except that a 4.5 min gradient was used at a flow rate of 0.2 mL/min. Product ion scans were recorded from *m/z* 100 to 300.

5.2. Discussion of metabolism

Using a human liver microsomal drug metabolizing system, the half-life of propranolol was determined to be 20.8 min (Fig. 1). In this same system, the half-lives of compounds **9** and **12** were 5.9 min and 11.2 min, respectively (Fig. 1). Since propranolol is regarded as a drug that is metabolized at a medium to medium–high rate, the metabolic stabilities of compounds **9** and **12** appear to be low, indicating these compounds would be metabolized quickly *in vivo*.

UHPLC-MS analysis of the metabolite mixtures of compound **9** indicated that the primary metabolite was a monooxygenated form. In the chromatogram shown in Fig. 2, the monooxygenated metabolite of compound **9** eluted at 1.9 min. Data-dependent tandem mass spectrometry indicated that the site of oxygenation was on the ring containing the sulfur or nitrogen, since the fragment ion of *m/z* 123 containing these heteroatoms in the tandem mass spectrum of compound **9**, shifted to *m/z* 139 in the tandem mass spectrum of the metabolite (Fig. 2).

Similar UHPCL-MS and MS–MS analysis of compound **12** and its metabolites indicated that monooxygenation was also the primary route of metabolism of this compound. The most abundant metabolite of compound **12** produced a tandem mass spectrum that indicated the extra oxygen was added to the phenol ring (Fig. 3).

6. Conclusions

A series of imidazo[2,1-*b*]thiazoles has been synthesized as potential chemopreventive agents. By design, these molecules included phenolic OH groups in their structures, and compounds **4-15** were found to demonstrate good chemical antioxidant activity. The most consistent results were obtained at physiological pH (pH = 7.4, TEAC method). According to this test, compounds **6** and **12** were the most active, with an antioxidant activity similar to that of quercetin. In further tests, compounds **7**, **8**, **9**, **12**, **14** and **16** were found to inhibit NFκB, and compounds **5**, **6**, **9**, **11**, **12**, and **14** showed inhibitory effects on nitrite production. Compounds **9** and **12** also enhanced the activity of QR1. Most of the compounds could inhibit the activity of aromatase, but with an IC_{50} of 1.1 ± 0.1 μM, compound **8** appears most promising.

Since compounds **9** and **12** demonstrated inhibition of NFκB and NO production, good chemical antioxidant activity, and potential to induce QR1, preliminary metabolism studies were performed. The results suggest the parent ring structure undergoes further oxidation to a monohydroxylated form. The next logical step in the development of these promising agents is the elucidation of the structure followed by synthetic production and biological characterization.

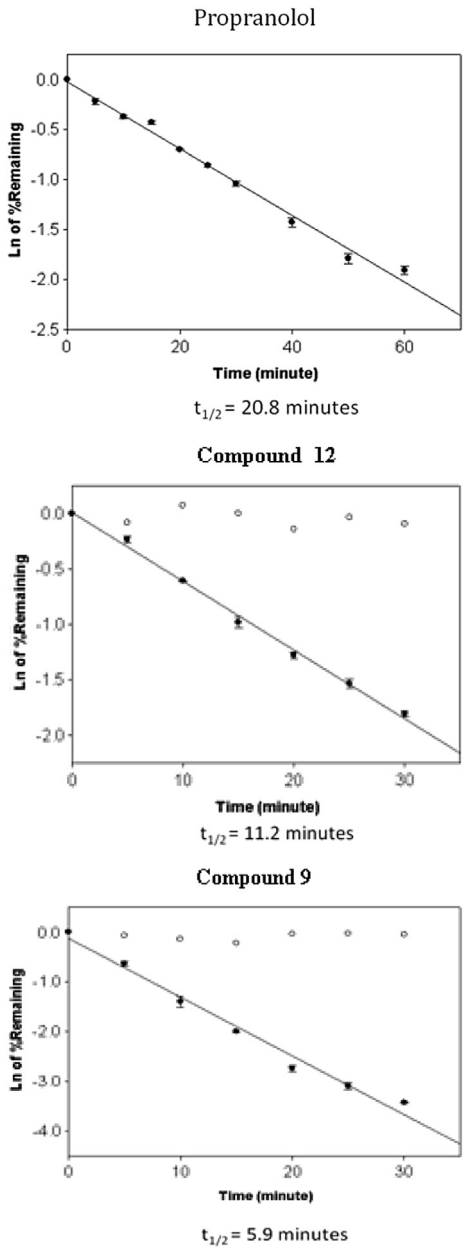


Fig. 1. Half-life determinations of propranolol, compounds **9** and **12**.

7. Experimental section

7.1. Chemistry

The melting points are uncorrected. Elemental analyses were performed with a Fisons Carlo Erba Instrument EA1108 and compounds used for tests were at least 95% pure. Bakerflex plates (silica gel IB2-F) were used for TLC: the eluent was petrol ether/acetone in various proportions. Kieselgel 60 was used for column chromatography. The IR spectra were recorded in nujol on a Nicolet Avatar 320 E.S.P.; ν_{max} is expressed in cm^{-1}. The ^1H NMR spectra were recorded in $(CD_3)_2SO$ on a Varian MR 400 MHz (ATB PFG probe); the chemical shift (referenced to solvent signal) is expressed in δ (ppm) and J in Hz (abbreviations: th = thiazole, im = imidazole, ar = aromatic). 2-Aminothiazole and 2-amino-5-methyl-thiazole are commercially available, regarding 2-halo-acetophenones **2**, although commercial, some were prepared according to the literature [40,41].

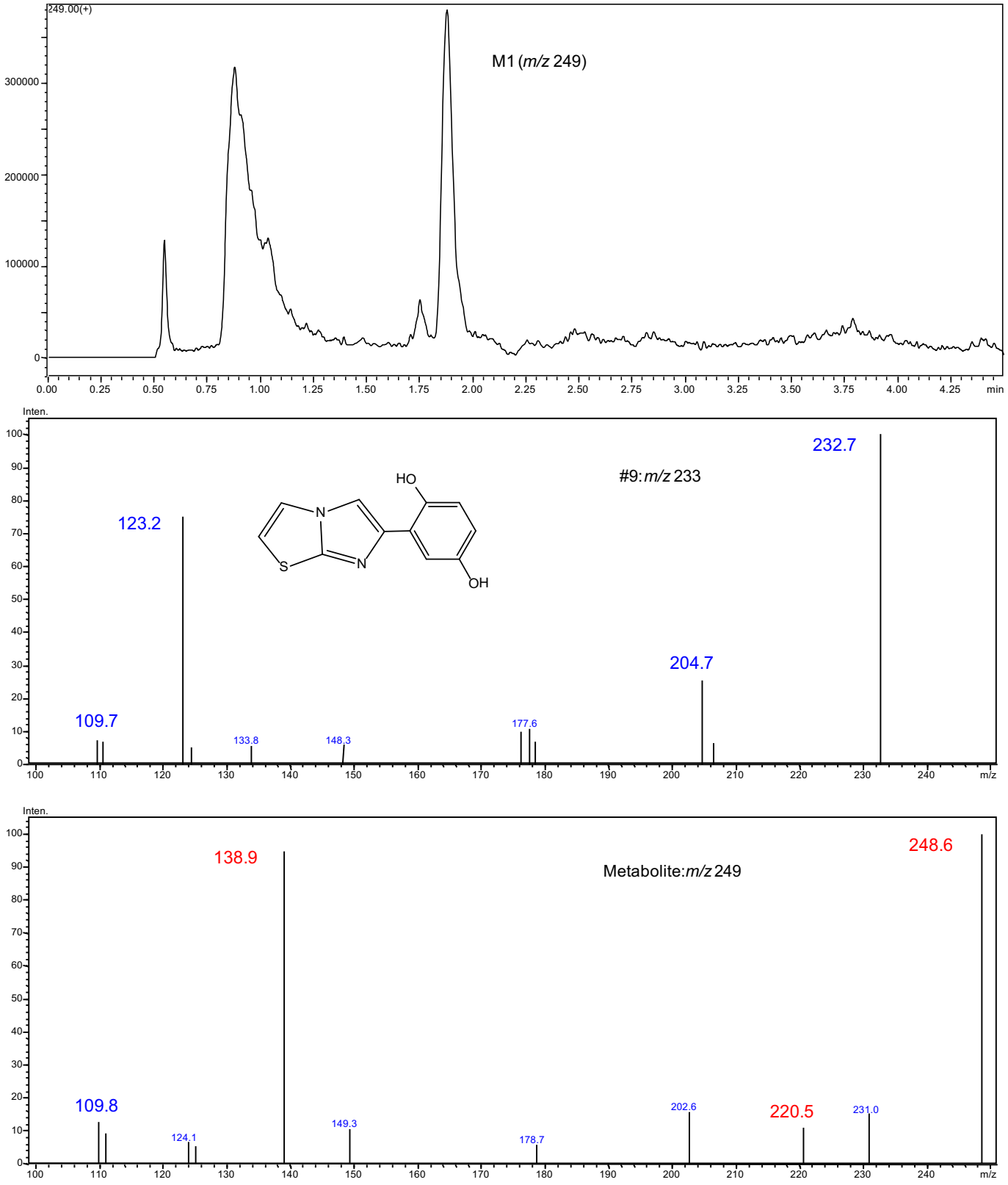


Fig. 2. LC-MS and LC-MS–MS analysis of metabolites of compound (#) **9** formed during incubation with human liver microsomes.

*7.1.1. Synthesis of compounds **6** and **10–12***

The appropriate 2-aminothiazole **1** (10 mmol) was dissolved in acetone (100 mL) and treated with the appropriate 2-halo-acetophenone **2** (10 mmol). The reaction mixture was refluxed for 5–20 h according to a TLC test. The resulting salt **3** was collected by filtration, and, without further purification, was treated with 150 mL of 2 N HCl. After 1 h reflux, the solution was alkalinized with 15% NH_4OH and the resulting base was collected by filtration.

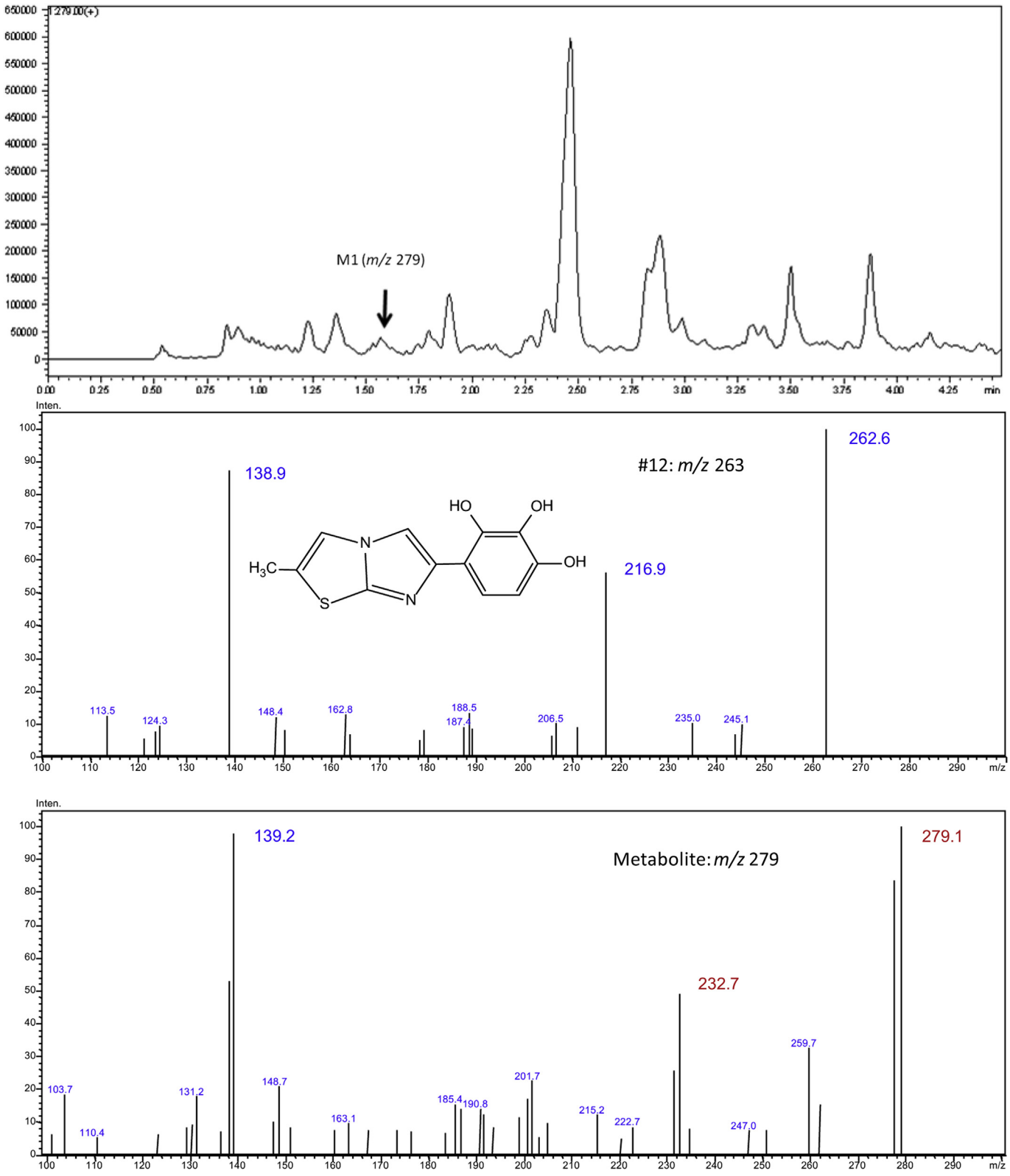


Fig. 3. LC-MS and LC-MS–MS analysis of metabolites of compound (#) **12** formed during incubation with human liver microsomes.

Compound **11** was purified by column chromatography with a yield of 35%; the eluent was petrol ether/acetone 9/1.

Yield was 65% for compounds **6** and **12**; 77% for compound **10**.

Data for **6**. mp 215–218 °C (from ethanol). I.R.: 3475, 3380, 1375, 1016, 720. ^{1}H NMR: 6.33 (1H, d, ar, $J = 8.4$), 7.06 (1H, d, ar, $J = 8.4$), 7.28 (1H, d, th, $J = 4.2$), 7.96 (1H, d, th, $J = 4.2$), 8.08 (1H, s, im), 8.19 (1H, s, OH), 8.91 (1H, s, OH), 10.87 (1H, s, OH). Anal. Calcd for $C_{11}H_8N_2O_3S$ (MW 248.26): C, 53.22; H, 3.25; N, 11.28. Found: C, 53.01; H, 3.38; N, 11.36.

Data for **10**. mp 272 °C (from ethanol). I.R.: 3426, 3252, 3134, 1552, 666. ^{1}H NMR: 7.17 (1H, d, ar-5, $J = 8.4$), 7.26 (1H, d, th, $J = 4.4$),

7.94 (1H, d, th, $J = 4.4$), 7.99 (1H, dd, ar-6, $J = 8.4$, $J = 2.4$), 8.24 (1H, s, im), 8.30 (1H, d, ar-2, $J = 2.4$), 10.98 (1H, broad, OH). Anal. Calcd for $C_{11}H_9N_3O_3S$ (MW 263.28): C, 50.18; H, 3.45; N, 15.96. Found: C, 49.98; H, 3.27; N, 16.02.

Data for **11**. mp 238 °C (from ethanol). I.R.: 3375, 3170, 3103, 1654, 819. ^{1}H NMR: 6.91 (1H, d, ar-3, $J = 8.8$), 7.24 (1H, d, th, $J = 4.4$), 7.87 (1H, dd, ar-4, $J = 8.8$, $J = 2$), 7.90 (1H, broad, OH), 7.97 (1H, d, th, $J = 4.4$), 8.09 (1H, s, im), 8.33 (1H, d, ar-6, $J = 2$), 8.54 (1H, broad, NH_2). Anal. Calcd for $C_{12}H_9N_3O_2S$ (MW 259.29): C, 55.59; H, 3.50; N, 16.21. Found: C, 55.78; H, 3.27; N, 16.43.

Data for **12**. mp 248–250 °C (from ethanol). I.R.: 3514; 3427; 3134; 1238; 891. ^{1}H NMR: 2.37 (1H, s, CH_3), 6.29 (1H, d, ar, $J = 8.4$), 6.99 (1H, d, ar, $J = 8.4$), 7.67 (1H, s, th), 7.95 (1H, s, im), 8.16 (1H, broad, OH), 8.88 (1H, broad, OH), 10.88 (1H, broad, OH). Anal. Calcd for $C_{12}H_{10}N_2O_3S$ (MW 262.29): C, 54.95; H, 3.84; N, 10.68. Found: C, 55.01; H, 3.99; N, 10.87.

7.1.2. Synthesis of compounds ***13*** and ***14***

Three mmol of 2-methyl-6-(2,5-dimethoxyphenyl)imidazo[2,1-*b*]thiazole [42] or 2,3-dimethyl-6-(2,5-dimethoxyphenyl)imidazo [2,1-*b*]thiazole [43] were treated with 30 mL of 48% HBr and refluxed for 5–7 h according to a TLC test. The mixture was cooled and the resulting precipitate was collected by filtration. The yield was 90%.

Data for **13**. mp 275–280 °C (from ethanol). I.R.: 3302, 3192, 1617, 1047, 781. ^{1}H NMR: 2.49 (3H, s, CH_3), 4.20 (2H, broad, OH), 6.71 (1H, dd, ar-4, $J = 8.6$, $J = 2.9$), 6.86 (1H, d, ar-3, $J = 8.6$), 7.08 (1H, d, ar-6, $J = 2.9$), 7.98 (1H, s, th), 8.42 (1H, s, im). Anal. Calcd for $C_{12}H_{10}N_2O_2S$ (MW 246.29): C, 58.52; H, 4.09; N, 11.37. Found: C, 58.28; H, 3.97; N, 11.67.

Data for **14**. mp 267–271 °C (from ethanol). I.R.: 3246, 1501, 845, 825, 778. ^{1}H NMR: 2.34 (3H, s, CH_3), 2.36 (3H, s, CH_3), 6.54 (1H, dd, ar-4, $J = 8.4$, $J = 2.8$), 6.69 (1H, d, ar-3, $J = 8.4$), 7.21 (1H, d, ar-6, $J = 2.8$), 8.10 (1H, s, OH), 8.73 (1H, s, OH), 10.40 (1H, s, OH). Anal. Calcd for $C_{13}H_{12}N_2O_2S$ (MW 260.31): C, 59.98; H, 4.65; N, 10.76. Found: C, 60.12; H, 4.75; N, 10.34.

7.2. Antioxidant activity

7.2.1. BR method

This method is based on the inhibition of oscillations produced by the presence of free-radical scavengers [20]. Oscillations are monitored potentiometrically. The Briggs–Rauscher system (BR) consists of hydrogen peroxide, acidic iodate, malonic acid and Mn(II) as catalyst and works at the physiological pH of the human stomach (pH ~ 2) [44]. Similar to other methods, the BR reaction method is based on the generation of free radicals in the reaction mixture. The generated hydroperoxyl radicals (HOO•) are among the main intermediates of the BR system. The mechanism of the action of antioxidants against HOO• radicals has been described in detail elsewhere [20,28]. When an antioxidant free-radical scavenger is added to an active oscillating BR mixture there is an immediate quenching of the oscillations, then, after a time (inhibition time, t_{inhib}) that depends on the linear relationship of the concentration and activity of the antioxidant, the oscillations resume. Relative antioxidant activity (RAC) is then obtained by comparison with a substance chosen as standard, usually resorcinol (1,3-benzenediol). Data are given as μM equivalent of resorcinol.

7.2.2. TEAC assay

We used the modified technique proposed by Re et al. [21] in which the reagent $ABTS^{\bullet+}$ is generated directly in a stable form prior to the addition of the antioxidants. The generation of the blue/green $ABTS^{\bullet+}$ chromophore resulted from the reaction between ABTS i.e. 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) as diammonium salt and potassium persulfate [21]. Addition of antioxidants to the preformed radical cation causes a decolorization of the solution that is followed spectrophotometrically at 734 nm. The extent of the decolorization is a function of concentration and time. Trolox, a Vitamin E analog soluble in neutral medium, was used as standard. Relative antioxidant activity is then obtained by comparison of straight lines ΔE6 = [Abs(blank) − Abs(Trolox or Sample)] vs concn. Data are given as mM equivalents of Trolox.

7.2.3. DPPH test

The principle of this method is the decolorization of the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) by antioxidants [45,22]. The DPPH• is intensely purple colored ($\lambda_{max} = 515$ nm). The extent of the decolorization is a function of concentration and time. Trolox, was used as the standard. Relative antioxidant activity is then obtained by comparison of straight lines percentage of inhibition (% inhib) vs concn. of sample and Trolox, respectively. Data are reported as mM equivalents of Trolox.

7.3. Chemopreventive activities

7.3.1. NFκB luciferase assay

Human embryonic kidney cells 293 Panomic (Fremont, CA, USA) were used for monitoring changes occurring along the NFκB pathway. Stable constructed cells were seeded into sterile 96-well plate at 20×10^3 cells per well. Cells maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Co. Carlsbad, CA, USA), supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine. After 48 h incubation, the medium was replaced and cells were treated with various concentration of test substances dissolved in DMSO. TNF-α (Human, Recombinant, *Escherichia coli*, Calbiochem, Gibbstown, NJ, USA) was used as an NFκB activator at a concentration of 2 ng/mL (0.14 nM). The plate was incubated for 6 h. Spent medium was discarded and the cells were washed once with PBS. Cells were lysed using 50 μL (for 96-well plate) Reporter Lysis Buffer from Promega (Madison, WI, USA), by incubating for 5 min on a shaker, and stored at −80 °C. The luciferase assay was performed using the Luc assay system from Promega [46]. The gene product, luciferase enzyme, reacts with luciferase substrate, emitting light which was detected using a luminometer (LUMIstar Galaxy BMG). Data for NFκB constructs are expressed as IC_{50} values (i.e., concentration required to inhibit TNF-α activated NFκB activity by 50%). As a positive control, two inhibitors were used: TPCK, $IC_{50} = 3.8 \pm 1.1$ μM and BAY-11, $IC_{50} = 2.0 \pm 0.54$ μM.

7.3.2. Nitric oxide (NO) assay

The level of NO in the cultured media was estimated by measuring the level of nitrite due to the instability of NO and its subsequent conversion to nitrite. The nitrite assay was performed as previously described [47]. RAW 264.7 cells (1×10^5 cells per well) were incubated in 96-well culture plates at 37 °C, 5% CO_2 in a humidified air incubator for 24 h. Then cells were treated with serially diluted compounds for 15 min, followed by treatment with or without LPS (1 μg/mL) for an additional 20 h. After the incubation, the amount of nitrite released in the cultured media was measured using Griess reagent [1:1 mixture (*v*/*v*) of 1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride solution], and absorbance was measured at 540 nm. The concentration of nitrite was calculated using a standard curve created with known concentrations of sodium nitrite. Under the same experimental conditions, sulforhodamine B (SRB) assays were performed to evaluate the cytotoxic effect of tested compounds on RAW 264.7 cells. After transferring 100 μL of the cultured media for Griess test, cells were fixed with 10% trichloroacetic acid, and stained with 0.4% SRB solution in 1% acetic acid. The protein-bound SRB was dissolved

in 10 mM Tris base solution, and the absorbance was measured at 515 nm. Percentage of cell survival was calculated in comparison with LPS-treated control [48].

7.3.3. QR1 assay

QR1 activity was assayed using Hepa 1c1c7 murine hepatoma cells, BP^{r}c1 mutant Hepa cells and TAOc1BP^{r}cI mutant Hepa cells. Cells were incubated in a 96-well plate with tested compounds at a maximum concentration of 50 μM. Digitonin was used to permeabilize cell membranes, and enzyme activity was measured by the reduction of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan, the production of which can be measured by absorption at 595 nm [49]. A total protein assay using crystal violet staining was run in parallel.

7.3.4. Aromatase assay

Compounds (3.5 μL) were preincubated with 30 μL of NADPH regenerating system (2.6 mM $NADP^+$, 7.6 mM glucose 6-phosphate, 0.8 U/mL glucose-6-phosphate dehydrogenase, 13.9 mM $MgCl_2$, and 1 mg/mL albumin in 50 mM potassium phosphate buffer, pH 7.4) in a 384-well plate for 10 min at 37 °C. Then 33 μL of enzyme and substrate mixture (1 μM CYP19 enzyme, BD Biosciences, 0.4 μM dibenzylfluorescein, 4 mg/mL albumin in 50 mM potassium phosphate, pH 7.4) was added, and further incubated for 30 min at 37 °C. The reaction was terminated by adding 25 μL of 2 N NaOH solution, and the plate was further incubated for 24 h at 37 °C to enhance the ratio of signal to background. Fluorescence was measured at 485 nm (excitation) and 530 nm (emission) [38].

7.3.5. RXRE-luciferase reporter gene assay (RXRE assay)

COS-1 cells (African green monkey kidney fibroblast-like cell line) were seeded in a 96-well culture plate and incubated for 24 h. Firefly luciferase reporter vector (100 ng) carrying retinoid X receptor response element RXRE (pRXRE; Panomics, Fremont, CA), 50 ng of pBABE-puro vector encoding the cDNA for human RXRα (phRXRα; Addgene Inc.,Cambridge, MA), and 3 ng of *Renilla reniformis* luciferase vector (pRL; Promega, Madison, WI) were transiently co-transfected into COS-1 cells in each well by using a transfection reagent Lipofectamine™ 2000. After 24 h of incubation, cells were treated with compounds and further incubated for 12 h. Cells were then washed with PBS and incubated with passive lysis buffer (Promega, Madison, WI) for 15 min. The RXRE transcriptional activities were determined by measuring the reporter luciferase activities using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) [39].

Author contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.07.052.

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