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Synthesis and evaluation of *in vitro* antioxidant capacities of some benzimidazole derivatives

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Abstract

New, except **1d,** melatonin analogue benzimidazole derivatives were synthesized and characterized in the present study. The potential role of melatonin as an antioxidant by scavenging and detoxifying ROS raised the possibility that compounds that are analogous to melatonin can also be used for their antioxidant properties. Therefore the antioxidant effects of the newly synthesized compounds were investigated *in vitro* by means of their inhibitory effect on hydrogen peroxide-induced erythrocyte membrane lipid peroxidation (EMLP) and on various erythrocyte antioxidant enzymes viz. superoxide dismutase (SOD), catalase (CAT) and glucose-6-phosphate dehydrogenase (G6PD). The synthesized benzimidazole derivatives showed remarkable antioxidant activity *in vitro* in the H_2O_2-induced EMLP system. Furthermore their effects on various antioxidant enzymes are discussed and evaluated from the perspective of structure- activity relationships.

Keywords: *Melatonin, benzimidazole, antioxidant activity, erythrocyte, in vitro*

Introduction

Increasing indirect evidence has suggested that oxidative damage of important cellular constituents, i.e. lipids, proteins and DNA, can be involved in aging [1,2] as well as may be playing a role in the pathogenesis of various diseases such as cancer, atherosclerosis, rheumatoid arthritis and ischemic injury [3–6]. It is known that lipid peroxidation is a chain reaction [7] initiated by reactive oxygen species (ROS), which causes the degradation of cell membranes. Most products of lipid peroxidation are known to have mutagenic and/or carcinogenic properties [8]. Melatonin plays a number of physiological roles. Recent interest has focused on its potential role as an antioxidant by scavenging and detoxifying ROS, particularly the highly cytotoxic hydroxyl radical ($HO^{\bullet}$) [9]. Benzimidazole derivatives show a wide variety of biological activities. It is present in naturally occurring cyanocobalamine and various drugs such as omeprazole, mebendazole, and acetamidazole [10]. In particular, recent antibacterial, antifungal and antioxidant activities of benzimidazoles have received much attention [11–14]. In the last decade, melatonin and related compounds have been shown to have effects on free radicals and lipid peroxidation [9,15]. Therefore, it is thought that compounds that are analogous to melatonin can be used for antioxidant purposes, either as drug or food supplements. Although compounds in which the carboxyl group is attached to the ring system have not received wide attention, previous studies showed significant results on the antioxidant activity [16]. Due to the similarity between the indole and benzimidazole rings there could be a possibility where the indole of melatonin could be replaced with a benzimidazole ring with or without the melatonin substitution pattern to give a relative likeness in antioxidant activity and this possibility

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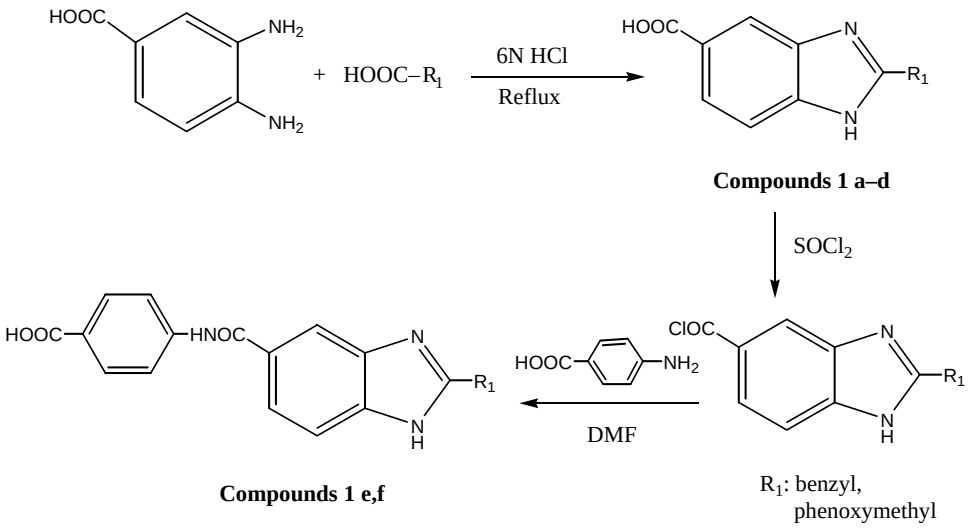


Scheme 1. Synthesis of compounds **1a–f**.

prompted us to synthesize new benzimidazole derivatives (Scheme 1) as a lead study. Some new melatonin analogue benzimidazole compounds have been synthesized (except **1d** [17]), characterized and their antioxidant efficiency evaluated by means of their inhibitory effect on hydrogen peroxide-induced erythrocyte membrane lipid peroxidation (EMLP) and on various erythrocyte antioxidant enzymes viz. superoxide dismutase (SOD), catalase (CAT) and glucose-6-phosphate dehydrogenase (G6PD). Compounds bearing several substituent groups were synthesized in order to investigate the structure-antioxidant activity relationships.

Materials and methods

All chemicals were purchased from Aldrich. Uncorrected melting points were determined with a Büchi SMP-20 apparatus. All the instrumental analyses were performed by TUBITAK (Instrumental Analysis Lab., Ankara) with a Bruker GmbH DPX-400, 400 MHz NMR spectrometer using TMS as an internal standard and mass spectra were recorded on a VG Platform II spectrometer using EI. Chromatography was carried out using Merck silica gel 60 (230–400 mesh ASTM).

Preparation of the benzimidazole derivatives

Compounds **1a–d** were synthesized as outlined in Scheme 1. 3,4-Diaminobenzoic acid (20 mmol) was treated with 30 mmol of the appropriate acid (phenylbutyric acid for **1a**, thioglycolic acid for **1b**, β-alanine for **1c** and 2-cyclohexylpropionic acid for **1d)** in 6N HCl [18]. The reaction mixture was refluxed 3 h for **1a**, 6 h for **1b**, 24 h for **1c**, and 8 h for **1d**. The mixture was then kept in the fridge overnight and the crystals filtered then recrystalized from ethanol.

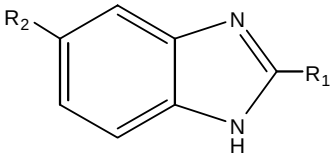
For the synthesis of compounds **1e** and **1f,** 2-benzyl-1H-benzimidazole-5-carboxylic acid (for **1e**) and 2-phenoxymethyl-1H-benzimidazole-5-carboxylic acid (for **1f**) were reacted with $SOCl_2$ (10 ml) for 6 h at 60°C. The acyl chloride derivative was treated with p-amino benzoic acid (4 mmol) in DMF for 12 h at 60°C. Then 20 g ice was added to the reaction and the precipitated crude product was filtered and purified by column chromatography (chloroform-isopropanol).

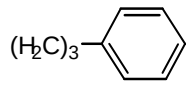
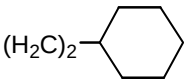
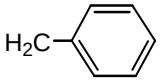
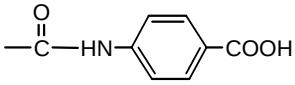
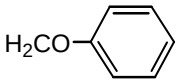
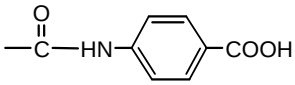
Compounds **1a–d** have a –COOH group on the fifth position of the aromatic ring, while compounds **1e–f** have a carboxamide derivative. Several aromatic and aliphatic groups were chosen for R_1 in order to compare their effect on antioxidant efficiency. The physical properties of the compounds are given in Table I.

2-(3-phenyl)propyl-1H-benzimidazole-5-carboxylic acid ***1a***. ^{1}H NMR (d_6-DMSO): δ = 2.18 (2H, m, CH_2-CH_2Ph), 2.67 (2H, t, CH_2-(CH_2)$_2$Ph), 2.13 (2H, t, CH_2Ph), 7.21 (5H, m, Ph), 7.76 (1H, d, Ar-H$_7$), 7.99 (1H, d, Ar-H$_6$), 8.19 (1H, d, Ar-H$_4$). MS (EI): m/z (%) = 235 (7.53) (M^+-COOH),189 (8.54),176 (100.00), 159 (8.04), 104 (14.73), 103 (21.53), 91 (73.27), 77 (19.065), 45 (15.97).

2-mercaptomethyl-1H-benzimidazole-5-carboxylic acid ***1b***. ^{1}H NMR (d_6-DMSO): δ = 4.23 (2H, d, CH$_2$), 4.62 (1H, s, SH), 7.80 (1H, d, Ar-H$_7$), 8.00 (1H, d, Ar-H$_6$), 8.26 (1H, s, Ar-H$_4$). MS (EI): m/z (%) = 208 (26.49) [M^+], 176 (100.00), 159 (72.39), 131 (52.24), 104 (14.18), 90 (24.63), 83 (27.24), 63 (54.85).

Table I. Physical properties of compounds **1a–f**.



Comp.	R_1	R_2	Yield (%)	Mp (°C)	Molecular Formula
1a		—COOH	20	315–317	$C_{17}H_{17}N_2O_2Cl$
1b	CH_2SH	—COOH	29	310	$C_9H_9N_2O_2ClS$
1c	$(CH_2)_2$—NH_2	—COOH	16	300–302	$C_{10}H_{12}N_3O_2Cl$
1d		—COOH	20	290	$C_{16}H_{21}N_2O_2Cl$
1e			43	295	$C_{22}H_{17}N_3O_3$
1f			14	297	$C_{22}H_{17}N_4O_4$

2-(2-amino)ethyl-1H-benzimidazole-5-carboxylic acid ***1c***. ^{1}H NMR (d_6-DMSO): δ = 3.48 (2H, d, C*H*$_2$-NH), 3.55 (2H, t, C*H*$_2$-CH$_2$), 7.78 (1H, d, Ar-H$_7$), 8.00 (1H, d, Ar-H$_6$), 8.24 (1H, s, Ar-H$_4$). MS (EI): m/z (%) = 189 (1.58) [M^+-NH_2], 158 (1.42), 132 (13.25), 105 (21.76), 91 (53.01), 85 (58.56), 83 (100.00), 77 (48.15), 63 (47.22), 52 (57.41).

2-(2-cyclohexyl)ethyl-1H-benzimidazole-5-carboxylic acid ***1d*** *[17]*

N-(p-carboxy)phenyl-2-benzyl-1H-benzimidazole-5-carboxamide ***1e***. ^{1}H NMR (d_6-DMSO): δ = 3.30 (2H, s, CH_2), 7.60-8.60 (12H, m, Ar-H), 10.50 (1H, s, N*H*-CO), 13.90 (1H, s, COO*H*). MS (EI): m/z (%) = 371 (5.13) [M^+], 280 (32.42), 149 (19.82), 104 (11.82), 83 (32.42), 57 (72.27), 43 (76.95), 35 (100.00).

N-(p-carboxy)phenyl-2-phenoxymethyl-1H-benzimidazole-5-carboxamide ***1f***. ^{1}H NMR (d_6-DMSO): δ = 5.40 (2H, s, CH_2), 7.00 (1H, m, H$_{4'}$), 7.12 (2H, d, H$_{2',6'}$), 7.35 (2H, m, H$_{3',5'}$), 7.60-8.40 (7H, m, H$_{4,6,7,2'',3'',5'',6''}$), 10.50 (1H, s, N*H*-CO). MS (EI): m/z (%) = 343 (4.39) [M^+-COOH], 298 (10.64), 269 (11.00), 120 (15.70), 119 (44.01), 104 (42.77), 91 (76.86), 77 (76.76), 43 (71.74), 41 (100.00).

Antioxidant activity studies

Blood collection and erythrocyte isolation. Blood samples obtained from healthy volunteers were collected into heparinized tubes and centrifuged at 2000 × g for 15 min. After removing the plasma and the buffy coats, the erythrocytes were washed with an equal volume of cold saline solution (0.155 mol/L) three times and packed erythrocytes were obtained.

Inhibitory effect on hydrogen peroxide-induced peroxidation of human erythrocytes. When erythrocytes are treated with hydrogen peroxide (H_2O_2) at pH 7.4 in the presence of sodium azide (to inhibit catalase), the lipid components of their membranes undergo peroxidation. The oxidant/antioxidant properties of compounds (**1a–1f**) were evaluated in the system where human erythrocytes were used as detailed by Quinlan et al. [21]. Following incubation of 100 µl

erythrocytes with the indicated concentrations of the compounds for 30 min, 800 µl 10 mM H_2O_2 was added to the tubes and they were incubated for an additional 90 min. The reaction was stopped by addition of 1 ml of 28% trichloroacetic acid. After centrifugation at 2000 × g for 15 min., the supernatant of each tube was transferred to a clean tube and derivatized with thiobarbituric acid (TBA). The absorbance of the TBA-MDA adduct was measured at 532 nm.

In vitro *effect on various antioxidant enzymes*

In vitro *incubations*. After washing erythrocytes with saline, they were lysed by adding 3 volumes of ice-cold nanopure water. Cellular debris was removed by centrifugation (3,000 × g for 30 min.) and portions of the obtained erythrocyte lysate were incubated with or without the test compounds for 90 min at 37°C in a shaking water bath. The compounds were dissolved and added to the incubation mixture in DMSO. The volume of DMSO never exceeded 1% (v/v) of the total incubation mixture. The test concentrations of the compounds were chosen according to their previously determined inhibitory concentrations on the hydrogen peroxide-induced peroxidation of human erythrocyte membranes, the concentration that gave the highest inhibition being used.

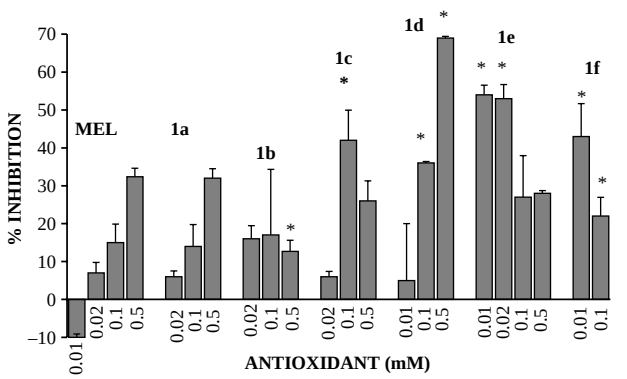
Measurement of enzyme activities. SOD activity was measured according to the method of Marklund et al [22], using the prevention of pyrogallol auto-oxidation at 420 nm. Catalase activity was determined spectrophotometrically [23] at 240 nm using H_2O_2 as substrate. Determination of G6PD activity was performed as detailed by Fairbanks [24], where glucose-6-phosphate and $NADP^+$ were used as substrates and the rate of increase in absorbance at 340 nm was determined spectrophotometrically.

Statistical analysis. The data are represented as mean value +S.E.M. (standard error of the mean). The statistical differences between groups were analyzed by the paired t-test. A probability value of $P \leq 0.05$ was considered to denote a statistically significant difference.

Results

Inhibitory effect on hydrogen peroxide-induced peroxidation of human erythrocyte membranes

Figure 1 shows the inhibitory effect of melatonin and the synthesized benzimidazole derivatives on H_2O_2-induced peroxidation of human erythrocytes *in vitro*. All the synthesized compounds decreased the peroxidation to some extent. Melatonin, a well-known antioxidant, was used as a reference control for comparative purposes. Compounds **1c**, **1d**, **1e** and **1f** were found to have a higher antioxidant activity than melatonin in the present system.

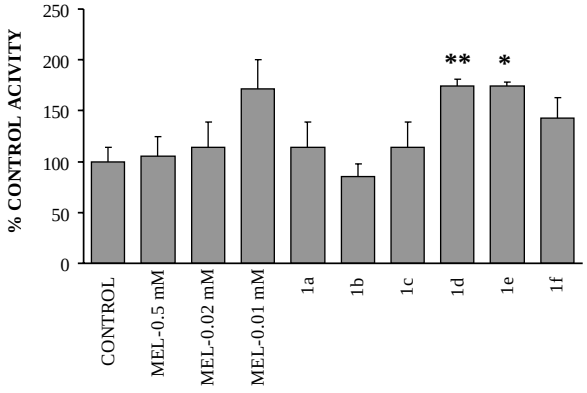


# p<0.05, compared to the values for corresponding concentrations of melatonin.

Figure 1. Dose-dependent inhibitory effect of benzimidazoles on H_2O_2-induced lipid peroxidation in erythrocyte membranes. Data are expressed as mean ± SE of 3–8 incubations.

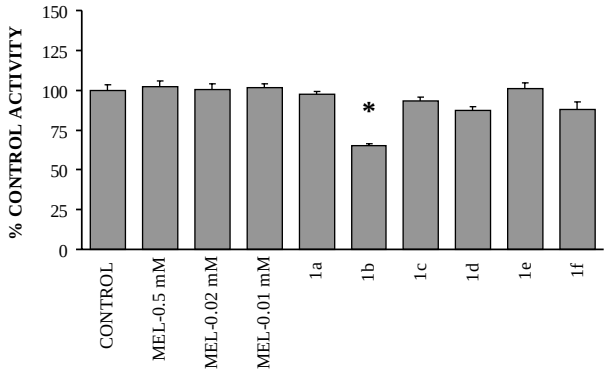
In vitro *effect on antioxidant enzymes*

The mean enzyme activities from control and experimental incubations are shown in Figures 2,3 and 4. The compounds were used at concentrations that caused the highest inhibitory effect on EMLP. The reference compound melatonin was incubated at 3 different concentrations: 0.01, 0.02, and 0.5 mM, compound **1a–d** at 0.5 mM, compound **1e** at 0.02 mM, and compound **1f** at 0.01 mM. Results showed that both **1d** and **1e** were found to activate SOD in human erythrocytes *in vitro* (Figure 2). The only compound causing an inhibition on erythrocyte



*p<0.05, compared to the control value.

Figure 2. *In vitro* effect of melatonin and benzimidazole derivatives on SOD activity from human erythrocytes. **1a-f** was added at 0.5mM concentration to the incubation media. Values represent means from at least 3 different incubations ± SEM.



*p<0.05, compared to the control value.

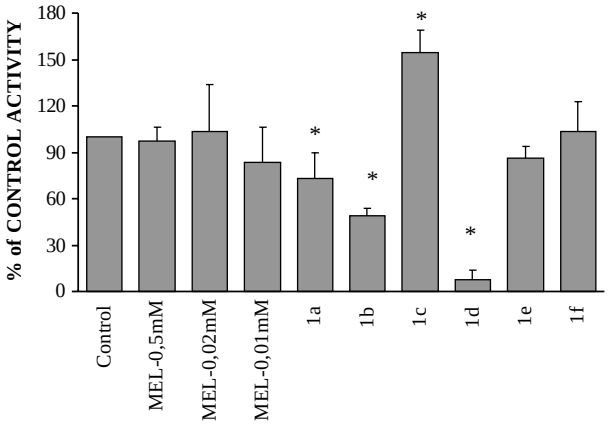
Figure 3. *In vitro* effect of melatonin and benzimidazole derivatives on catalase activity from human erythrocytes. **1a-d** was added at 0.5mM, **1e** at 0.02mM and **1f** at 0.01mM concentration to the incubation media. Values represent means from at least 3 different incubations ± SEM.

CAT activity was **1b** (Figure 3). Derivatives **1a**, **1b** and **1d** were found to inhibit G6PD activity, whereas **1c** caused a significant activation of G6PD (Figure 4).

Discussion

Six benzimidazole analogues of melatonin were synthesized and characterized in the present study and their effects on the antioxidant system of human erythrocytes investigated *in vitro*.

There are several reasons for choosing erythrocytes as an *in vitro* test system for studies on the biological effects of free radicals. First of all, they are both structurally simple and easily obtained. Secondly, erythrocytes offer an excellent model to evaluate the oxidant/antioxidant potency of chemicals; they are continually exposed to high oxygen tensions, they are unable to replace damaged components, the membrane lipids are composed partly of polyunsaturated fatty acid side chains which are vulnerable to peroxidation, and the hemoglobin itself can function as an oxidase and a peroxidase [19]. Lastly, chemicals get into the blood stream after systemic exposure so that erythrocytes may be considered as a primary target for such chemicals.

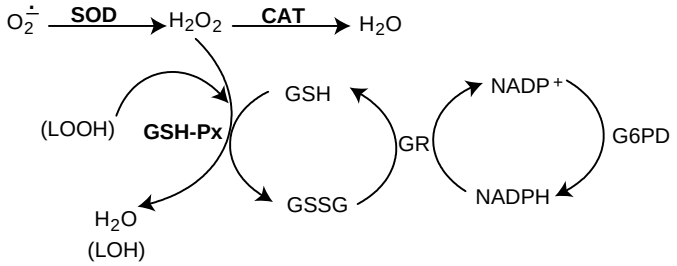


* p<0.05, compared to the control values.

Figure 4. *In vitro* effect of melatonin and benzimidazole derivatives on G6PD activity from human erythrocytes. **1a-d** was added at 0.5mM, **1e** at 0.02mM and **1f** at 0.01mM concentration to the incubation media. Values represent means from at least 3 different incubations ± SEM.

H_2O_2-induced EMLP was suggested as a simple and relevant test for evaluating the antioxidant/pro-oxidant potential of drugs and/or drug candidates [20]. The present data show that all the benzimidazole derivatives are capable of inhibiting the H_2O_2-induced lipid peroxidation of human erythrocyte membranes. Some of the benzimidazole derivatives were found to have a higher antioxidant capacity compared to melatonin, used as a reference and model antioxidant in the present assay. **1a** showed a dose-dependent antioxidant effect, which had a very similar pattern that of melatonin. Compound **1b**, containing a thiol side chain, was found to have an inhibitory effect on H_2O_2-induced EMLP, but interestingly the effect was lower and did not change with increasing concentration of the compound. Usually —SH groups are essential for the antioxidant activity. However, the -CH_2SH side chain of compound **1b** is not as bulky as the long side chain of melatonin so that the side chains from these two compounds are sterically nonequivalent which might play a role in the lower antioxidant activity of **1b** compared to melatonin. The antioxidant effects of **1c**, **1e** and **1f** were also concentration-independent; the effect was found to be reduced at higher concentrations. **1d** was found to have the highest inhibitory effect on H_2O_2-induced EMLP at 0.5 mM. This finding could be explained by the apolar side chain of the benzimidazole, which makes the compound interact easily with apolar components of the erythrocyte membrane such as phospholipids.

Besides to their effect on EMLP, the potential interaction of benzimidazole derivatives with erythrocyte antioxidant enzymes was also investigated. For this purpose, SOD, CAT and G6PD were chosen as important components of the antioxidant defense system (Figure 5). SOD catalyses dismutation of superoxide anion radical to H_2O_2. Two enzymes degrade H_2O_2: glutathione peroxidase and CAT. Degradation of H_2O_2 at physiological concentrations is catalyzed by glutathione peroxidase, while higher concentrations are detoxified by CAT. In the present study, **1d** and **1e** were found to activate SOD, which might improve the antioxidant defense capacity of the cells. Interestingly, **1b** was found to inhibit CAT activity *in vitro*, despite its nucleophilic thiol group. The thiol group of the compound might interact with carbonyl groups of the enzyme via a hydrogen bond and this may cause a decrease in enzyme activity. Further *in vitro* and *in vivo* studies can provide an explanation for this observation.



LOOH, lipid hydroperoxide; LOH, alcohol derivative of lipid; GR, glutathione reductase.

Figure 5. The interrelationships between antioxidant enzymes in detoxification of reactive oxygen species.

The intracellular glutathione redox system, with a high glutathione (GSH) concentration and lower level of glutathione disulfide (GSSG), is predominantly responsible for the protection of both hemoglobin and the erythrocyte membrane against oxidation and hemolysis. Interrelated enzyme systems function to achieve the efficient recycling of GSSG to GSH and to provide the reducing equivalent, NADPH. Although G6PD is not cited among antioxidant enzymes, it is indispensable for the defence against oxidative stress because erythrocytes lack mitochondria and acquire NADPH exclusively from the hexose monophosphate pathway (Figure 5). In the present study, increased G6PD activity was found with **1c** suggesting an adaptation to the oxidative stress where more NADPH is needed and consumed during reduction of peroxides and other reactive species. However we found inhibited G6PD activity in erythrocytes incubated with **1a**, **1b** and **1d** *in vitro*, which might be the consequence of redox-sensitive inactivation of G6PD.

In conclusion, the synthesized benzimidazole derivatives showed remarkable antioxidant activity *in vitro* on the H_2O_2-induced EMLP system and the present study also provided information on the antioxidant enzyme interaction of these compounds. The parameters investigated here are the initial toxicological evaluation and further studies are required to confirm similar effects *in vivo* as well as to illuminate the interaction of these new derivatives with other biochemical / physiological pathways.

Acknowledgements

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