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Melatonin analogue new indole hydrazide/hydrazone derivatives with antioxidant behavior: Synthesis and structure–activity relationships

GÖKCE GÜRKÖK[1], TULAY COBAN[2], & SIBEL SUZEN[1]

[1]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ankara University, 06100 Tandogan, Ankara, Turkey, and* [2]*Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Ankara University, 06100 Tandogan, Ankara, Turkey*

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Abstract
Melatonin (MLT) is a hormone produced in the brain by the pineal gland, from the amino acid tryptophan. It is also an antioxidant hormone with a particular role in the protection of nuclear and mitochondrial DNA. In recent years, many physiological properties of MLT have been described resulting in much attention in the development of synthetic compounds possessing the indole ring. Sixteen MLT analogue indole hydrazide/hydrazone derivatives were synthesized and *in vitro* antioxidant activity was investigated. Most of the compounds showed significantly higher activity than MLT at 10^{-3} M and 10^{-4} M concentrations.

Keywords: *Indole, hydrazone, hydrazide, melatonin, synthesis, antioxidant activity*

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have gained a lot of importance because of their active role in many diseases [1,2]. The damage to animal or plant cells and tissues caused by ROS is called oxidative stress. It is caused by an imbalance between the production of ROS and a biological system's ability to detoxify the reactive intermediates or repair the resulting damage. A particularly negative side of oxidative stress is the production of ROS, which include free radicals [3]. Human bodies are constantly exposed to ROS generated from endogenous and some exogenous sources. Antioxidants, both enzymatic and non-enzymatic, prevent oxidative damage to biological molecules by various mechanisms [4].

Melatonin (MLT), *N*-acetyl-5-methoxytryptamine, is the main secretory product of the pineal gland. Studies have established the role of MLT in many physiological processes, such as the regulation of circadian rhythm [5] and immune functions [6]. Also different therapeutic functions have been proposed for MLT and its derivatives [7–9]. It is a highly conserved molecule that it acts as a free radical scavenger and a broad-spectrum antioxidant [10–13]. The ability of MLT to react with free radicals was first shown in 1993, when Tan et al. [14] identified its interaction with hydroxyl radicals. MLT has some side effects, but much less so that pharmaceutical sleeping pills. Long-term safety is not known. People with the symptoms of severe mental illness, severe allergies, auto-immune diseases, or immune system cancers such as leukemia should not be taking MLT. Despite its possible contribution in the regulation of many physiological processes, two main problems limit its therapeutic use at present. The first is very short biological half life, due to its fast metabolism to 6-hydroxymelatonin and *N*(1)-acetyl-*N*(2)-formyl-5-methoxykynuramine (AFMK) and the second is the lack of selectivity of MLT at target sites [15,16].

Melatonin, at least in pharmacological concentrations, has the capability of increasing either mRNA

Correspondence: S. Suzen, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ankara University, 06100, Tandogan, Ankara, Turkey. Tel: 90 312 2033074. Fax: 90 312 2131081. E-mail: sibel@pharmacy.ankara.edu.tr

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levels or the activities of these major antioxidative enzymes. The effect of melatonin to neutralize the free radicals is receptor independent [17]. Based on the investigation of structure-activity relationships, the indole ring of the MLT molecule is the reactive center of dealings with oxidants due to its high resonance stability and very low activation energy barrier towards the free radical reactions [18–22]. In recent years, many physiological properties of MLT have been described resulting in much attention in the development of synthetic compounds possessing indole ring [16]. These compounds have structural similarity to MLT. However, the therapy of oxidative stress-related diseases has not found satisfactory application in clinical practice. This may be due to the insufficient efficacy of drugs available, their unsuitable pharmacokinetics, side effects and toxicity [16,23,24].

As a part of our ongoing study [19,20], sixteen MLT analogue indole hydrazide/hydrazone derivatives were synthesized and antioxidant activity was investigated *in vitro* by measuring DPPH, superoxide radical scavenging and lipid peroxidation (LP) inhibitory activities. The results were compared with MLT. All the analogue compounds except previously synthesized **1a** [25], **1b** [26], **1k** [27], **1l** [28], **1n** [29], **1o** [30] were characterized on the basis of ^{1}H and ^{13}C NMR, Mass, FT-IR spectra and elemental analysis.

Materials and methods

Chemistry

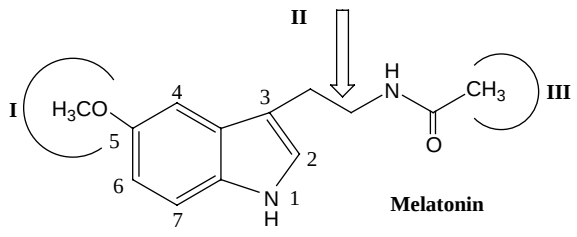
With this study based on MLT, *N*-acetyl-5-methoxytryptamine, a well-known antioxidant, free radical scavenger, and neuroprotectant, new indole imines were developed. Three parts of the MLT molecule were modified in order to find out the antioxidant behavior and structure activity relationship of the new indole analogue compounds.

Two sets of indole derivatives, with changes mainly in the 5-methoxy and acylamino groups showed as modifications I, II and III in Scheme 1. These chemically significant modulations of the lead structure were made at three different points: the methoxy group at the 5-position of the indole ring (modification I), 2-*N*-acetylaminoethyl side chain including formation of imine (modification II) and replacement acetyl with substituted aromatic rings (modification III). Particular attention was dedicated to the role of the 5-methoxy group, which was either eliminated (**1a-f, 1k, 1l**) or substituted with Br (**1g-j, 1m, 1n)**. These modifications resulted in a set of compounds having different physical property and different substitution at the indole nucleus. This helped to investigate the effect of substituents with different electronic properties on the antioxidant activity of new indole derivatives.

The target imines derived from 5-bromo-1*H*-indole-3-carboxaldehyde or 1*H*-indole-3-carboxaldehyde and appropriate hydrazine or hydrazide derivatives using simple reaction strategies. For the synthesis of compounds **1a-j** a similar methodology has been adopted from Kidwai et al. [25] Phenyl hydrazine derivatives and indole-3-carboxaldehydes were heated in the presence of ethanol. The hydrazones **1k–n** were also prepared [31] from the reaction of equimolar amounts of hydrazide with indole-3-carboxaldehydes in the presence of ethanol. Finally N,N'-bis-(1*H*-indole-3-ylmethylene)-hydrazine derivatives were synthesized using equimolar amounts of hydrazine hydrate with indole-3-carboxaldehydes in the presence of ethanol (Scheme 2). All the new compounds were characterized on the basis of spectral data.

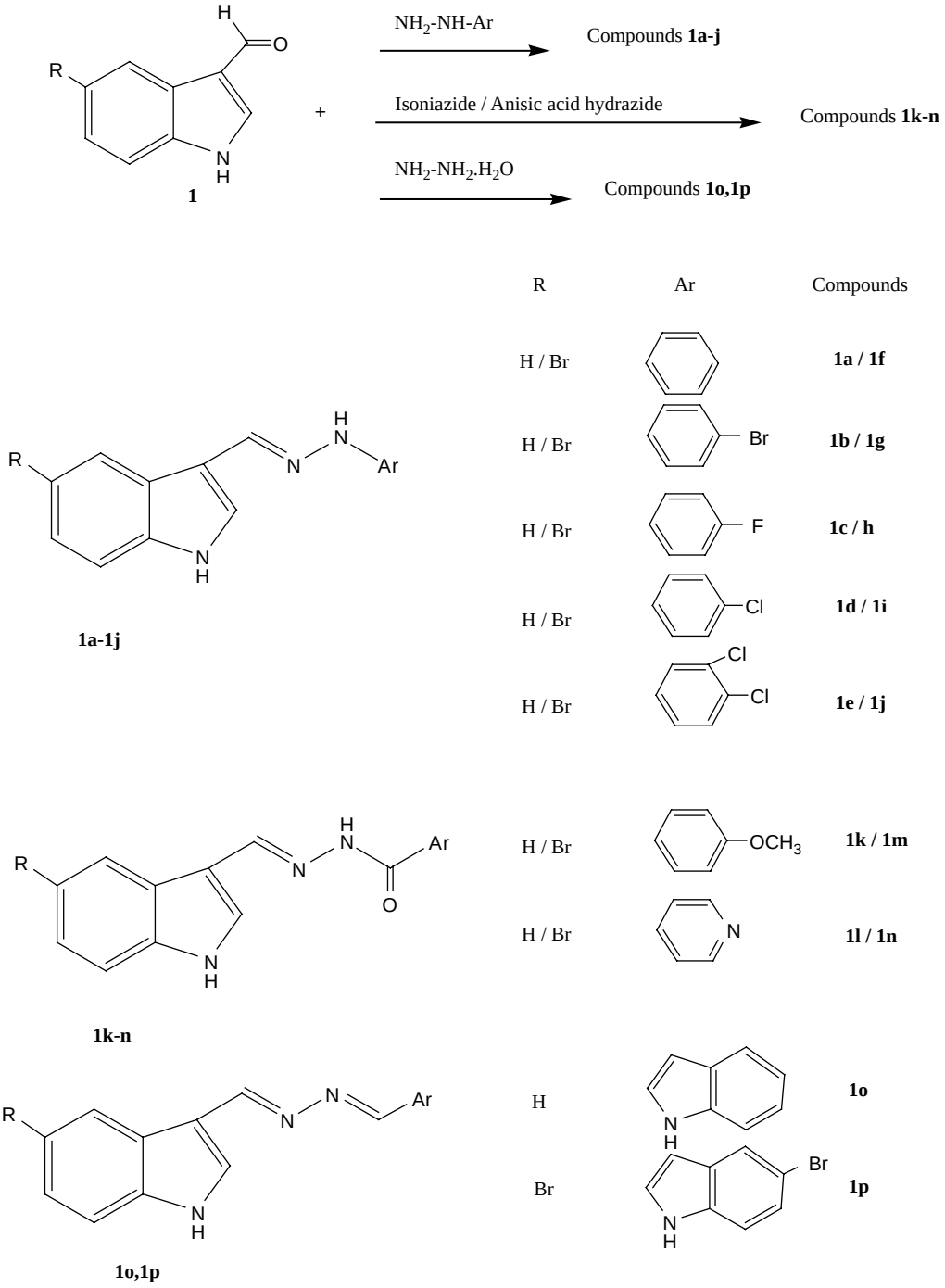
Experimental

Uncorrected melting points were determined with a Buchi SMP-20 apparatus. The ^{1}H and ^{13}C NMR spectra were measured with a Varian 400 MHz using TMS internal standard and DMSO-d_6 as solvent. ESI Mass spectra were determined on a Waters micromass ZQ. FT-IR spectra were recorded on Jasco 420 Fourier. Elemental analyses were performed using CHNS-932 (LECO). All spectral analysis was performed at Ankara University, Faculty of Pharmacy, Central Laboratory. Chromatography was carried out using Merck silica gel 60 (230-400 mesh ASTM). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), ascorbic acid (AA), xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), n-butanol were purchased from Sigma Chemical Co. (St Louis, MO, USA). The chemical reagents used in synthesis were purchased from Sigma (Germany) and Aldrich (USA).



Scheme 1. Modifications made on MLT molecule

*General procedure for the synthesis of compounds **1a-j**.* 1*H*-Indole-3-carboxaldehyde (for **1a-e**) or 5-bromo-1*H*-indole-3-carboxaldehyde (for **1f-j)** (0.1 mmol) were reacted with phenyl hydrazine and its derivatives (4-bromophenyl hydrazine, 4-flourophenyl hydrazine, 4-chlorophenyl hydrazine and 3,4-dichlorophenyl hydrazine) (0.13 mmol) in10 ml of EtOH in the presence of 0.5 g CH_3COONa for 30 min on the hot water bath. On cooling, the precipitate was collected



Scheme 2. Synthesised melatonin analogue indole hydrazide/hydrazone derivatives

washed with cold EtOH to give **1a-j** with 45-95% yield.

1H-Indole-3-carboxaldehyde (4-fluorophenyl)hydrazone ***(1c)***. Yield 58%, m.p 115-117°C; ^{1}H NMR (400 MHz): δ 6.90 (4H, m), 7.02 (2H, m), 7.27 (1H, d), 7.49 (1H, s), 7.96 (1H, s, azomethine-CH), 8.09 (1H, d), 9.69 (1H, s, s, hydrazine-NH) 11.22 (1H, brs, indole-NH); ^{13}C NMR: δ 112.40, 112.87, 113.37, 116.14, 116.35, 120.72, 122.25, 123.00, 124.85, 128.01,135.87, 137.67, 143.62 (azomethine-C), 154.75, 157.06; ESI mass m/z 254 (M + 1, %100), 255 (M + 2); Analysis for $C_{15}H_{12}N_3F$; Calcd: C; 71.13, H; 4.77, N; 16.59. Found: C; 70.72, H; 4.71, N; 15.98%. FT-IR (KBr) cm^{-1} 1615 C=N (azomethine) stretch band, 3373 N-H stretch band.

1H-Indole-3-carboxaldehyde (4-chlorophenyl)hydrazone ***(1d)***. Yield 89%, m.p 168-171°C; ^{1}H NMR (400 MHz): δ 7.02 (2H, d), 7.16 (2H, m), 7.24 (2H, d), 7.41 (1H, d), 7.62 (1H, s), 8.10 (1H, s,

azomethine-CH), 8.21 (1H, d), 9.79 (1H, s, hydrazine-NH) 11.36 (1H, brs, indole-NH); ^{13}C NMR: δ 112.42, 112.21, 113.42, 120.81, 121.31, 122.22, 123.05, 124.79, 128.31, 129.54, 136.54, 137.66, 145.68 (azomethine-C); ESI mass m/z 270 (M + 1), 272 (M + 2), 160 (%100); Analysis for $C_{15}H_{12}N_3Cl$; Calcd: C; 66.79, H; 4.48, N; 15.589. Found: C; 66.21, H; 4.42, N; 15.52%. FT-IR (KBr) cm^{-1} 1599 C=N (azomethine) stretch band, 3302 N-H stretch band.

1H-Indole-3-carboxaldehyde (3,4-chlorophenyl)hydrazone ***(1e)***. Yield 95%, m.p 193-196°C; ^{1}H NMR (400 MHz): δ 6.86 (1H, dd), 7.04 (3H, m), 7.29 (2H, d), 7.55 (1H, s), 8.01 (1H, s, azomethine-CH), 8.06 (1H, d,), 10.07 (1H, s, hydrazine-NH) 11.30 (1H, s, indole-NH); ^{13}C NMR: δ 112.31, 112.52, 112.75, 112.90, 118.66, 120.94, 122.05, 123.14, 124.75, 128.93, 131.57, 132.17, 137.70, 146.72 (azomethine-C): ESI mass m/z 304 (M^+, %100), 306 (M + 2); Analysis for $C_{15}H_{11}N_3Cl_2$; Calcd: C; 59.23, H; 3.64, N; 13.81. Found: C; 58.41, H; 3.66, N; 14.19%. FT-IR (KBr) cm^{-1} 1590 C=N (azomethine) stretch band, 3384 N-H stretch band.

5-Bromo-1H-indole-3-carboxaldehyde phenylhydrazone ***(1f)***. Yield 71%, m.p 114-116°C; ^{1}H NMR (400 MHz): δ 6.67 (1H, t), 7.01 (2H, d), 7.21 (2H, m), 7.28 (1H, m), 7.39 (1H, d), 7.68 (1H, s), 8.09 (1H, s, azomethine-CH), 8.39 (1H, s), 9.94 (1H, s, hydrazine-NH) 11.65 (1H, brs, indole-NH); ^{13}C NMR: δ 112.01, 113.08, 113.15, 114.46, 118.41, 124.46, 125.36, 126.63, 129.28, 129.81, 135.07, 136.40, 146.73 (azomethine-C); ESI mass m/z 314 (M^+, %100), 326 (M + 2); Analysis for $C_{15}H_{12}N_3Br$; Calcd: C; 57.34, H; 3.85, N; 13.37. Found: C; 57.10, H; 3.91, N; 13.01%. FT-IR (KBr) cm^{-1} 1598 C=N (azomethine) stretch band, 3385 N-H stretch band.

5-Bromo-1H-indole-3-carboxaldehyde (4-bromophenyl)hydrazone ***(1g)***. Yield 71%, m.p 114-116°C; ^{1}H NMR (400 MHz): δ 6.81 (2H, d), 7.17 (1H, dd), 7.25 (3H, m), 7.59 (1H, s), 7.96 (1H, s, azomethine-CH), 8.20 (1H, d), 9.97 (1H, s, hydrazine-NH) 11.55 (1H, brs, indole-NH); ^{13}C NMR: δ 108.99, 112.81, 113.27, 113.89, 114.53, 124.32, 125.48, 126.55, 129.78, 132.43, 136.11, 136.40, 145.95 (azomethine-C); ESI mass m/z 393 (M^+, %100), 394 (M + 1) 395 (M + 2) 396 (M + 3); Analysis for $C_{15}H_{11}N_3Br_2$; Calcd: C; 45.83, H; 2.82, N; 10.69. Found: C; 45.58, H; 2.70, N; 10.67%. FT-IR (KBr) cm^{-1} 1590 C=N (azomethine) stretch band, 3412 N-H stretch band.

5-Bromo-1H-indole-3-carboxaldehyde (4-flourophenyl)hydrazone ***(1h)***. Yield 45%, m.p 168-170°C; ^{1}H NMR (400 MHz): δ 6.84 (2H, m), 6.95 (2H, m), 7.15 (1H, d), 7.26 (1H, d), 7.57 (1H, s), 7.93 (1H, s, azomethine-CH), 8.20 (1H, s), 9.77 (1H, s, hydrazine-NH) 11.43 (1H, brs, indole-NH); ^{13}C NMR: δ 112.0, 112.29, 113.67, 115.51, 123.56, 124.61, 125.79, 128.58, 134.51, 135.59, 142,72 (azomethine-C), 154,07, 156.38, 168.66; ESI mass m/z 332 (M^+, %100), 333 (M + 1); Analysis for $C_{15}H_{11}N_3BrF$; C: 54.23, H; 3.34, N; 12.65. Found: C; 54.11, H; 3.28, N; 12.75%. FT-IR (KBr) cm^{-1} 1617 C=N (azomethine) stretch band, 3456 N-H stretch band.

5-Bromo-1H-indole-3-carboxaldehyde (4-chlorophenyl)hydrazone ***(1i)***. Yield 46%, m.p 165-167°C; ^{1}H NMR (400 MHz): δ 6.84 (2H, d), 7.12 (1H, d), 7.16 (1H, d), 7.18 (1H, d), 7.26 (1H, d), 7.59 (1H, d), 7.94 (1H, s, azomethine-CH), 8.19 (1H, d), 9.93 (1H, s, hydrazine-NH) 11.45 (1H, brs, indole-NH); ^{13}C NMR: δ 112.50, 112.93, 113.02, 114.18, 121.21, 123.99, 125.15, 126.22, 129.29, 129.38, 135.67, 136.06, 145.26 (azomethine-C); ESI mass m/z 348 (M^+, %100), 350 (M + 2) 352 (M + 4); Analysis for $C_{15}H_{11}N_3BrCl$; Calcd: C; 51.68, H; 3.18, N; 12.05. Found: C; 51.66, H; 3.17, N; 12.08%. FT-IR (KBr) cm^{-1} 1599 C=N (azomethine) stretch band, 3411 N-H stretch band.

5-Bromo-1H-indole-3-carboxaldehyde (3,4-dichlorophenyl)hydrazone ***(1j)***. Yield 56%, m.p 135-137°C; ^{1}H NMR (400 MHz): δ 6.90 (1H, dd), 7.18 (1H,d), 7.29 (1H, d), 7.40 (2H, d), 7.47 (1H, s), 8.11 (1H, s, azomethine-CH), 8.32 (1H, s), 10.30 (1H, brs, hydrazine-NH), indole-NH not observed; ^{13}C NMR: δ 112.32, 112.56, 112.81, 113.37, 114.58, 118.85, 124.33, 125.49, 126.53, 130.22, 131,58, 132.28, 136.41, 137.11, 146.66 (azomethine-C); ESI mass m/z 383 (M^+, %100), 386 (M + 3) 387, 388, 389; Analysis for $C_{15}H_{10}N_3BrCl_2$; Calcd: C; 47.03, H; 2.63, N; 10.97. Found: C; 46.44, H; 2.67, N; 11.17%. FT-IR (KBr) cm^{-1} 1591 C=N (azomethine) stretch band, 3420 N-H stretch band.

General procedure for the synthesis of compounds ***1k–n***. A solution of 1*H*-indole-3-carboxaldehyde or 5-bromo-1*H*-indole-3-carboxaldehyde (0.05 mmol) and anisic acid hydrazide (for **1k**, **1m**) or izonicotinic acid hydrazide (for **1l**, **1n**) (0.05 mmol) in 50 mL of EtOH was heated for 2.5 h on the hot water bath. On cooling, the precipitate was collected washed with cold EtOH to give **1k-n** with 46-80% yield.

N-*(4-Methoxybenzoyl)*-N′-*(5-bromo-indolyl-3-methylene)-hydrazine* ***(1m)***. Yield 68%, m.p 266-269°C; ^{1}H NMR (400 MHz): δ 3.85 (3H, s, OCH_3), 7.08 (2H, d), 7.34 (1H, dd), 7.44 (1H, d), 7.93 (3H, t), 8.50 (1H, s,) 8.61 (1H, s, azomethine-CH), 11.51 (1H, brs, hydrazine-NH), 11.79 (1H, brs, indole-NH); ^{13}C NMR: δ 55.30 (CH_3), 111.38, 112.94, 113.56, 113.75, 124.09, 125.0, 125.88, 129.21, 131.31, 135.67, 143.81 (azomethine-C), 161.65 (C=O), 161.92, 167.63; ESI mass m/z 371 (M^+),

372 (M + 1), 374 (M + 3, %100); Analysis for $C_{17}H_{14}N_3O_2Br$; Calcd: C; 54.86, H; 3.79, N; 11.29. Found: C; 54.23, H; 4.00, N; 11.33%. FT-IR (KBr) cm^{-1} 1617 C=N (azomethine) stretch band, 1653 NH-CO stretch band.

*General procedure for the synthesis of compounds **1o** and **1p***. A solution of 1*H*-indole-3-carboxaldehyde (for **1o**) or 5-bromo-1*H*-indole-3-carboxaldehyde (for **1p)** (0.1 mmol) and hydrazine hydrate (0.1 mmol) in 25 mL of EtOH was heated for 4 h on the hot water bath. On cooling, the precipitate was collected washed with cold EtOH to give **1k-n** with 12-24% yield.

N,N′*-bis-(5-bromo-1H-indole-3-ylmethylene)-hydrazine (**1p**)*. Yield 12%, m.p 315-318°C; ^{1}H NMR (400 MHz): δ 7.36 (2H, dd), 7.47 (2H, d), 8.00 (2H, s), 8.50 (2H, d), 8.93 (2H, s, azomethine-CH), 11.90 (2H, brs, indole-NH); ^{13}C NMR: δ 112.26, 113.93, 114.72, 124.89, 125.85, 127.07, 134.05, 136.61, 155.92 (azomethine-C); ESI mass m/z 445 (M + 1, %100), 447 (M + 2); Analysis for $C_{18}H_{12}N_4Br_2$; Calcd: C; 48.68, H; 2.72, N; 12.62. Found: C; 48.50, H; 2.76, N; 13.11%. FT-IR (KBr) cm^{-1} 1634 C=N (azomethine) stretch band.

In vitro *antioxidant activities*

All the MLT analogue indole hydrazide/hydrazone derivatives were subjected to test DPPH, superoxide radical scavenging and anti LP activities. All the results were compared with MLT.

DPPH free radical scavenging activity. The free radical scavenging activities of MLT analogues were tested by their ability to bleach the stable radical 2,2,diphenyl-1-picrylhydrazyl (DPPH) [32]. This assay has often used to estimate the antiradical activity of antioxidants. When DPPH$^{\cdot}$ reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in color (from violet to yellow) were measured at 517 nm on a visible spectrophotometer. Reaction mixture contained 100μM DPPH in methanol and different concentrations of synthesized compounds. Absorbance at 517 nm was determined after 30 min at room temperature and the scavenging activity were calculated as a percentage of the radical reduction. Each experiment was performed in triplicate. BHT was used as a reference compound. IC_{50} values were determined from a calibration curve for each compound. MLT was used as reference compound. The radical scavenging activity was obtained from the equation:

$$\text{Radical scavenging activity \%} = \{(OD_{control} - OD_{sample})/OD_{control}\} \times 100$$

where: $OD_{control}$: absorption of blank sample; OD_{sample}: absorption of tested solution

Superoxide radical scavenging activity. The capacity of the examined MLT analogues to scavenge superoxide anion formation was determined spectrophotometrically. Superoxide was generated by xanthine/xanthine oxidase and measured by the nitroblue tetrazolium (NBT) reduction method [33,34]. 50 μL of 4 mM xanthine, 50 mL of 225 mM NBT, 50 mL of 50 mM phosphate buffer (pH 7.8, 1 mM EDTA) and 10 mL of the test compounds were prepared in a 96 well plate, and 40 μL of xanthine oxidase was added to each mixture. The absorbance of each reaction mixture was monitored at 550 nm. Superoxide radical scavenging activity (%) was expressed as the degree of NBT reduction decrease of the test group *versus* the control group after 3 min. Superoxide radical scavenging activity was calculated as follows: Superoxide radical scavenging activity (%) = $(A_{control} - A_{sample})/(A_{control} - A_{blank}) \times 100$ Where, $A_{control}$ is the absorbance of the control, in which the sample was not treated, A_{sample} is the absorbance of test sample which the sample was treated, and A_{blank} is the absorbance of blank, to which the sample and the NBT solution were not added. Each experiment was triplicated. MLT was used as positive controls. IC_{50} values were calculated from the concentration of sample required to reduce 50% of the NBT.

Assay of lipid peroxidation. The effect of crude extract on rat liver homogenate induced with $FeCl_2$-ascorbic acid and LP was determined by the method of modified Mihara et al. [35] Wistar rats (200-225 g) were fed with standard laboratory rat chow and tap water. The animals were starved for 24 h prior to sacrifice and then killed by decapitation under anesthesia. The study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals. The livers were removed immediately and washed in ice-cold distilled water, and homogenized straight away with teflon homogenizer in ice chilled. LP was measured spectrophotometrically by estimation of thiobarbituric acid reactive substances (TBARS). Amounts of TBARS were expressed in terms of μmol malondialdehyde (MDA)/ g tissue. A typical optimized assay mixture contained 0.5 mL of liver homogenate, 0.1 mL of Tris-HCl buffer (pH 7.2), 0.05 mL of 0.1 mM ascorbic acid, 0.05 mL

of 4 mM $FeCl_2$ and 0.05 mL of various concentration of synthesized compounds, or MLT, were incubated for 1 h at 37°C. After incubation, 3.0 mL of H_3PO_4 and 1 mL of 0.6% TBA were added and shaken vigorously. The mixture was boiled for 30 min. After cooling, n-butanol was added and the mixture was shaken vigorously. The n-butanol phase was separated by centrifugation at 3000 rpm for 10 min. The absorbance of the supernatant was read at 532 nm against a blank, which contained all reagents except liver homogenate. MLT was used as positive control. Lipid peroxidation inhibitory activity (%) is expressed as follows: lipid peroxidation inhibitory activity (%):$(A_{control} - A_{sample})/(A_{control} - A_{blank}) \times 100$ Where, $A_{control}$ is the absorbance of the control, A_{sample} is the absorbance of the sample and A_{blank} is the absorbance of the blank, to which the sample and the free radical generating system (Fe^{+2}/ascorbate) were not added.

Statistical analysis

All data are the average of duplicate analyses. The data were recorded as mean ± standard deviation and analyzed by SPSS (version 11.0 for Windows 98 SPSS Inc.). One-way analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests. Values of $p < 0.05$ were regarded as significant.

Results

Sixteen MLT analogue indole hydrazide/hydrazone derivatives were synthesized and tested for their antioxidant activities using DPPH and superoxide radical scavenging and LP inhibitory activity tests. All the results were compared with standard antioxidant MLT. The results are shown in Tables I, I.1, II, II.1 and III.

All the tested compounds possessed strong scavenging activity against the DPPH radical scavenging activity with IC_{50} values (19.22 − 40.51 μM) (Tables I and I.1). Compounds, that have anisic acid hydrazide **1k**, **1m** and izonicotinic acid hydrazide **1n** in the 3rd position of indole ring showed no free radical scavenging effect. Rest of the compounds showed very strong antioxidant activity (approximately 20 times higher) compare to MLT in DPPH assay.

In addition, all the compounds also showed strong inhibitory effect on the superoxide radical scavenging assay with IC_{50} values (0.59–0.98 mM), with the exception of compounds **1k-1n** and **1o** (Table II and II.1). The results were found very similar to the DPPH radical scavenging assay. With a few exceptions all the compounds showed significantly higher antioxidant then that of MLT in superoxide dismutase assay.

Table I. DPPH Radical scavenging effect of synthesized compounds[a].

Compounds[b]	Concentration (μM)	% Inhibition	IC_{50} (μM)
1a	12.5	19 ± 2.8	47
	25	37 ± 3.5	
	50	56 ± 2.8	
	100	78 ± 3.5	
1b	12.5	42 ± 2.8	24
	25	57 ± 0.7	
	50	71 ± 0.7	
	100	85 ± 2.1	
1c	12.5	25 ± 1.4	37
	25	41 ± 0.7	
	50	56 ± 2.8	
	100	65 ± 4.2	
1d	12.5	34 ± 0.7	20
	25	73 ± 0.7	
	50	84 ± 3.5	
	100	84 ± 3.5	
1e	12.5	28 ± 1.4	36
	25	32 ± 0.7	
	50	63 ± 2.1	
	100	80 ± 2.8	
1f	12.5	25 ± 2.4	31
	25	46 ± 1.2	
	50	65 ± 0.7	
	100	74 ± 0.7	
1g	12.5	33 ± 2.4	26
	25	51 ± 0.7	
	50	70 ± 3.5	
	100	80 ± 2.8	
1h	12.5	21 ± 2.1	34
	25	41 ± 0.7	
	50	65 ± 3.5	
	100	85 ± 2.8	
1i	12.5	34 ± 2.1	22
	25	61 ± 2.1	
	50	80 ± 2.8	
	100	86 ± 2.8	
1j	12.5	41 ± 4.9	20
	25	69 ± 4.9	
	50	82 ± 1.4	
	100	84 ± 0.7	
MLT	250	19 ± 2.8	800
	500	33 ± 2.1	
	1000	61 ± 2.8	
Vitamin E	0.009	31 ± 0.7	13
	0.018	65 ± 1.4	
	0.036	86 ± 0.7	

[a]The values represent the average of 2-4 determinations ± standard deviations; [b]Compounds were diluted with *DMSO* (solvent showed no antioxidant activity).

Lastly almost all tested compounds revealed potent inhibitory activity against the LP inhibitory assay at 0.1 mM concentration (74–91%), with the exception of **1l** and **1n** that showed 24 and 37% inhibition at the same concentration (Table III). At a concentration of 0.05 mM almost all compounds exhibited 47–81% inhibition, with the exception of **1k-1n.**

Table I.I. DPPH Radical scavenging effect of synthesized compounds[a].

Compounds[b]	Concentration (mM)	% Inhibition	IC_{50} (mM)
1l	0.125	30 ± 0.7	0.24
	0.25	60 ± 2.8	
	0.5	73 ± 2.1	
	1	81 ± 2.8	
1o	0.125	12 ± 1.4	0.37
	0.25	41 ± 0.7	
	0.5	62 ± 2.1	
	1	81 ± 2.8	
1p	0.125	NE	0.78
	0.25	NE	
	0.5	31 ± 1.4	
	1	67 ± 2.8	
MLT	0.25	19 ± 2.8	0.8
	0.5	33 ± 2.1	
	1	61 ± 2.8	
Vitamin E	0.009	31 ± 0.7	0.013
	0.018	65 ± 1.4	
	0.036	86 ± 0.7	

[a]The values represent the average of 2–4 determinations ± standard deviations; [b]Compounds were diluted with *DMSO* (solvent showed no antioxidant activity). NE: No effect.

Table II. Superoxide radical scavenging effect of synthesized compounds[a].

Compounds[b]	Concentration (mM)	% Inhibition	IC_{50} (mM)
1a	0.25	15 ± 1.4	0.83
	0.5	25 ± 2.1	
	1	63 ± 2.8	
1b	0.25	9 ± 2.1	0.75
	0.5	32 ± 4.2	
	1	51 ± 2.8	
1c	0.25	12 ± 2.8	0.69
	0.5	34 ± 6.4	
	1	76 ± 3.5	
1d	0.25	EY	0.66
	0.5	39 ± 4.5	
	1	83 ± 6.0	
1e	0.25	15 ± 4.5	0.86
	0.5	25 ± 4.2	
	1	56 ± 6.0	
1f	0.25	35 ± 1.4	0.68
	0.5	53 ± 0.7	
	1	80 ± 3.5	
1g	0.25	20 ± 1.4	0.59
	0.5	36 ± 5.0	
	1	89 ± 2.8	
1h	0.25	19 ± 4.2	0.82
	0.5	39 ± 4.0	
	1	72 ± 6.0	
1i	0.25	21 ± 3.5	0.58
	0.5	38 ± 4.9	
	1	90 ± 2.8	
1j	0.25	4 ± 1.4	0.98
	0.5	22 ± 2.8	
	1	53 ± 7.0	
MLT	0.25	24 ± 1.4	0.64
	0.5	48 ± 4.0	
	1	80 ± 3.5	

[a]The values represent the average of 2-4 determinations ± standard deviations; [b]Compounds was diluted with *DMSO* (solvent expressed no antioxidant activity). NE: No effect.

According to DPPH, superoxide radical scavenging and anti lipid peroxidation activity results of the new MLT analogue indole derivatives, majority of the compounds were found as very potent antioxidant comparing with MLT.

Discussion

The present work was designed to investigate the potential antioxidant effect of MLT analogue indole hydrazide/hydrazone derivatives by *in vitro* well known antioxidant activity tests. Two sets of indole derivatives, with changes in the 5-methoxy and 2-acylaminoethyl groups of MLT were synthesized and tested for their *in vitro* antioxidant potency in the DPPH, superoxide dismutase and LP assays.

Most of the compounds tested showed significant antioxidant activity at concentrations comparable with or much higher than that of MLT. The only exception was in DPPH assay, compounds that have anisic acid hydrazide **1k**, **1m** and isoniazide **1n** in the 3-position of indole ring. These compounds and **1l** and **1o** were also found inactive in superoxide radical scavenging activity assay. Similarly compounds that embrace anisic acid hydrazide **1k, 1m** and izonicotinic acid hydrazide **1l, 1n** were found with lower activity while the rest of the compound having considerable LP inhibitory activity.

Replacement the 5-methoxy group of MLT by H and Br which have different electronic and lipophilic properties helped to investigate the role of structure-antioxidant activity relationships of the synthesized compounds. The real function of methoxy group in 5-position of the indole ring of MLT is still not clear. Although by replacement of the methoxy group, the antioxidant capacity of the molecule may be enhanced [18] interestingly there was no significant difference was observed between compounds bearing Br or H in the 5-position of the indole ring. This result is very similar to our earlier findings[14] and the data in literature [2,3]. Also Poeggeler et al. [36,37] showed that the MLT derivatives bearing Cl in the 5-position performed similar activity to MLT. According to the *in vitro* results, generally synthesized MLT analogue compounds that have substituted phenyl hydrazone side chain in the 3-position (**1a-1j**) showed substantial antioxidant activity. Among those compounds that have p-Br and p-Cl on the phenyl ring showed significantly higher antioxidant activity then MLT.

Like other indole derivatives and tryptophan metabolites, MLT has redox properties because of the

Table II.1. Superoxide radical scavenging effect of synthesized compounds[a].

Compounds[b]	Concentration (mM)	% Inhibition	IC_{50} (mM)
1k	0.25	NE	
	0.5	NE	
	1	NE	
1l	0.25	NE	
	0.5	6 ± 0.7	
	1	41 ± 2.8	
1m	0.25	NE	
	0.5	22 ± 3.5	
	1	46 ± 4.0	
1n	0.25	NE	
	0.5	NE	
	1	25 ± 2.4	
1o	0.25	NE	
	0.5	NE	
	1	39 ± 4.2	
1p	0.25	12 ± 0.7	0.93
	0.5	31 ± 1.4	
	1	52 ± 2.8	
MLT	0.25	24 ± 1.4	0.64
	0.5	48 ± 4.0	
	1	80 ± 3.5	

[a]The values represent the average of 2–4 determinations ± standard deviations; [b]Compounds were diluted with *DMSO* (solvent showed no antioxidant activity). NE: No effect.

presence of an electron-rich aromatic ring system, which allows the indoleamine to easily function as an electron donor [20–22]. A number of oxygen-centered radicals and other reactive species have been shown capable of oxidizing MLT in various experimental systems [38]. It is possible that making the indole ring more stable electronically helped to act as a better electro donor. Introduction of an imine group in to the side chain increased the stability of the indole molecule by helping the delocalization of the electrons. This might help to have high free radical scavenging activity. Also according to Reiter [39] MLT scavenges the radicals most likely via electron donation, thereby neutralizing the radicals and generating nitrogen centered radical, the indolyl (or melatonyl) cation radical. Most likely the synthesized MLT analogues that have substituted phenyl hydrazone side chain in the 3-position (**1a-1j**) that have high antioxidant activity help the formation of the indolyl cation radical unlike to compounds bearing anisic and isonicotinic acid hydrazides (**1k-1n**).

Recently diindolylmethane derivatives were found as anticarcinogenic and antioxidant molecules by Benabadji et al. [40]. We observed the lowest activity (in DPPH and LP assay) or no activity (in superoxide assay) with N,N′-bis-indole-3-il-methylene hydrazine derivatives (**1o-1p**). Introduction of imine groups in to the bridge between two indole rings clearly caused a negative effect on the antioxidant activity. Between the bis indole derivatives, 5-Br substituted indole derivative (**1p**) was found to be more active than non substituted derivative (**1o**).

Table III. Inhibitory effect of synthesized compounds on lipid peroxidation[a].

Compounds[b]	Concentrations (mM)	% Inhibition
Control	–	NE
1a	0.05	70 ± 2.1⋆
	0.1	88 ± 0.7⋆
1b	0.05	75 ± 0.7⋆
	0.1	91 ± 2.8⋆
1c	0.05	69 ± 1.4⋆
	0.1	90 ± 3.5⋆
1d	0.05	74 ± 2.8⋆
	0.1	88 ± 2.8⋆
1e	0.05	74 ± 1.4⋆
	0.1	88 ± 2.1⋆
1f	0.05	75 ± 1.4⋆
	0.1	87 ± 2.1⋆
1g	0.05	81 ± 2.1⋆
	0.1	88 ± 4.2⋆
1h	0.05	76 ± 2.1⋆
	0.1	88 ± 1.4⋆
1i	0.05	72 ± 1.4⋆
	0.1	89 ± 2.8⋆
1j	0.05	70 ± 3.5⋆
	0.1	91 ± 4.2⋆
1k	0.05	8 ± 0.7
	0.1	74 ± 3.5⋆
1l	0.05	8 ± 1.4
	0.1	24 ± 0.7⋆
1m	0.05	17 ± 2.8
	0.1	80 ± 3.5⋆
1n	0.05	13 ± 2.1
	0.1	37 ± 2.8⋆
1o	0.05	59 ± 4.2⋆
	0.1	88 ± 3.5⋆
1p	0.05	47 ± 1.4⋆
	0.1	84 ± 2.8⋆
MLT	0.05	22 ± 0.7⋆
	0.1	46 ± 1.4⋆
	1	90 ± 1.4⋆

⋆Significant differences vs. control at $p < 0.05$. NE: No effect; [a]The values represent the average of 2–4 determinations ± standard deviations; [b]Compounds were diluted with *DMSO* (solvent showed no antioxidant activity.

Conclusions

These results suggest a new approach for the *in vitro* antioxidant activity properties and structure activity relationship of 3 and 5 substituted indole ring. In conclusion, majority of synthesized indole derivatives related to MLT showed very high antioxidant activity in three *in vitro* assays, revealing differences in their relative potencies probably related to electronic

distribution. Lack of methoxy group or introduction of Br in the 5-position did not effect the antioxidant capacity of the new indole derivatives infect the *in vitro* assays showed that most of the compounds were much more active than MLT itself. Lastly it can be certainly said that introduction of hydrazide or hydrazone side chain containing aromatic halogenated ring increased the antioxidant activity of indoles comparing to MLT. This may be due to increased stability of the indole ring and delocalization of the electrons to help to scavenge free radicals by forming stable indolyl cation radical. These results confirmed that for antioxidant activity, not only the indole type aromatic ring is important, but so is the side chain containing the amide group.

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