Journal of Enzyme Inhibition and Medicinal Chemistry, 2012; Early Online: 1–13

ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.3109/14756366.2012.717223

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RESEARCH ARTICLE

Antioxidant activity of indole-based melatonin analogues in erythrocytes and their voltammetric characterization

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Abstract
Melatonin (MLT) is a strong free-radical scavenger, which protects the body from the effects of oxidants. In recent years, MLT have been described resulting in much attention in the development of synthetic compounds possessing. As a part of our ongoing study a series of indole-based MLT analogue hydrazide/hydrazone derivatives were synthesized, characterized and *in vitro* antioxidant activity was investigated by evaluating their reducing effect against oxidation of a redox sensitive fluorescent probe. Membrane stabilizing effect of all compounds was also investigated by lactate dehydrogenase leakage assay. Furthermore voltammetric methods have been applied to the synthesized compounds to characterize oxidation potentials to get insight into their metabolism owing to the oxidation mechanisms taking place at the electrode and in the body share similar principles.

Keywords: indole, hydrazone/hydrazide, melatonin, synthesis, antioxidant activity, voltammetry

Introduction

Oxidants and antioxidants have a significant function in maintaining a balance between free radicals and the antioxidant system of the body[1]. Reactive oxygen species (ROS) and reactive nitrogen species are well known as both harmful and beneficial species. Overproduction of ROS results in oxidative stress that can cause fatal damage to cell structures, including lipids and membranes, proteins, and DNA[2]. It is known that antioxidants could be suitable for preventive in several diseases related with oxidative stress such as atherosclerosis, inflammatory injury, cancer, cardiovascular diseases, and neurodegenerative disorders[3,4].

It is experimentally and clinically well established that melatonin (MLT) and its metabolites can function as endogenous free-radical scavengers and broad-spectrum antioxidants[5]. Studies also showed the role of MLT and its derivatives in many physiological processes and therapeutic functions, such as the regulation of circadian rhythm and immune functions[6]. 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. Synthetic indole derivatives such as indole-3-propionic acid[7], indole amine-triazoles[8], and stobadine[9] have shown significant antioxidant activity. Our group identified the antioxidant activity of MLT analogue indole derivatives such as 2-phenylindoles[10], indole-3-propionamides[11], 5-bromoindole hydrazide/hydrazones derivatives[12], and *N*-methylindole hydrazide/hydrazones[13]. In these studies most of the compounds showed significant antioxidant activity at concentrations comparable with or much higher than that of MLT.

Hydrazone derivatives possess a range of pharmacological activities including antitumoral, antimicrobial, anti-malarial, anti-convulsant, and anti-inflammatory activity[13]. Recently hydrazones were found as potential antioxidants[14–17]. It was shown that oxidative

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(Received 28 July 2012; accepted 28 July 2012)

stress induces lipid peroxidation of cellular membranes resulting in the generation of reactive carbonyl compounds that involves in the "carbonyl stress"[18,19]. Hydrazine derivatives also exhibit carbonyl scavenger activity by the reaction of ketones/aldehydes[20].

The use of electrochemical techniques for the determination of compounds of pharmaceutical interest is continually gaining in importance[21]. The obtained results from the redox properties of drugs and biomolecules might have profound effects on our understanding of their *in vivo* redox behavior or pharmaceutical activity[22–24]. Voltammetric techniques are most suitable for investigating the redox properties of new drug candidates to obtain information for its metabolic fate[25]. Electrode mechanisms might mimic enzyme reactions; therefore, electrochemistry may be of value in the study of enzyme reactions in biological systems[24]. Due to the existence similarity between electrochemical and biological reactions it can be assumed that the oxidation mechanisms taking place at the electrode and in the body share similar principles. We have previously reported the electroanalytical evaluation and determination of indole-3-propionic acid derivatives[26], indolylthiohydantoin derivatives[27], 2-phenylindole derivatives[28], and indole-3-carboxaldehyde izonicotinoyl hydrazones[29] by voltammetric studies in order to enlighten the possible relevance to *in vitro* metabolism of these compounds.

In new drug development studies, combination of different pharmacophores in the same structure may lead to new compounds having higher biological activity. It was thought the combination of indole-and hydrazone-type compounds might provide new effective drugs against free radicals. As a part of our ongoing study 21 new indole-based MLT analogue hydrazide/hydrazone derivatives were synthesized and their antioxidant activity was investigated *in vitro* by evaluating their reducing effect against oxidation of a redox sensitive fluorescent probe, 2′,7′-dichlorofluorescin (DCFH)-diacetate (DA), in the presence or absence of H_2O_2. The results were compared with a well-known antioxidant ascorbic acid and MLT, as their parent compound. Membrane stabilizing effect of all compounds was also investigated via lactate dehydrogenase (LDH) leakage assay in Chinese Hamster Ovary (CHO) cells. All the analogue compounds were characterized on the basis of ^{1}H- and ^{13}C-NMR, mass, Fourier transform infrared spectroscopy (FT-IR) spectra and elemental analysis. Furthermore voltammetric studies were established for the electrochemical oxidation and determination of synthesized MLT analogues using a glassy carbon electrode and investigated the possible oxido-reduction mechanisms that gave ideas about *in vitro* metabolism of these compounds.

Material and methods

With this study based on MLT, series of 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. The main modifications of MLT molecule were shown in Figure 1. The target imines derived from 1-methylindole-2-carboxaldehyde and appropriate hydrazine or hydrazide derivatives using simple reaction strategies. For the synthesis of compounds **1a-p** a similar methodology has been adopted from Kidwai et al. [30]. Phenyl hydrazine derivatives and 1-methylindole-2-carboxaldehyde were heated in the presence of ethanol. The hydrazones 1t, 1u were prepared from the reaction of equimolar amounts of hydrazide with 1-methylindole-2-carboxaldehyde in the

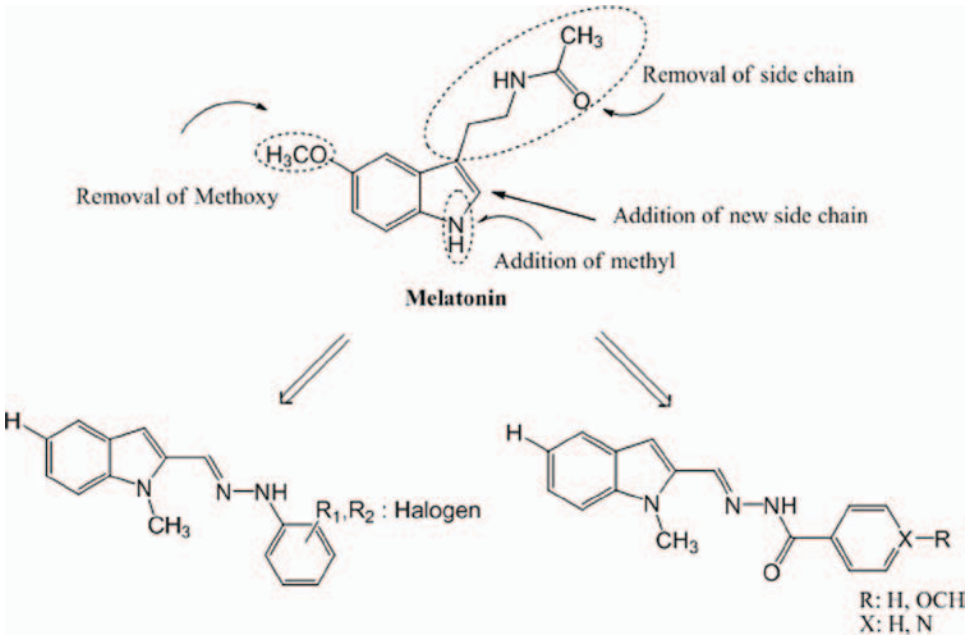

Figure 1. Parts of the MLT molecule modified to develop new indole-based MLT analogue compounds. MLT, melatonin.

presence of ethanol. Finally compound **1v** was synthesized using equimolar amounts of hydrazine hydrate with 1-methylindole-2-carboxaldehyde and compound **1y** was synthesized using one equimolar of hydrazine hydrate with two equimolars 1-methylindole-2-carboxaldehyde in the presence of ethanol (Figure 2). All the compounds were characterized on the basis of spectral data.

The chemical reagents used in synthesis were purchased from Sigma (Taufkirchen, Germany) and Aldrich (St. Louis, MO, USA). Uncorrected melting points were determined with a Buchi SMP-20 apparatus. The ^{1}H- and ^{13}C-NMR spectra were measured with a Varian 400MHz 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 420Fourier. Elemental analyses were performed using CHNS-932 (LECO). All spectral analysis was performed at Ankara University, Faculty of Pharmacy, Central Laboratory. Voltammetric measurements were performed using a Bioanalytical Systems (BAS 100 W, USA) electrochemical analyzer.

General procedure for the synthesis of compounds 1a-p.

1-Methylindole-2-carboxaldehyde (0.1 mmol) was reacted with phenyl hydrazine or its derivatives (0.13 mmol) in 10 ml of ethanol in the presence of 0.5 g CH_3COONa for 30 min on the hot water bath. On cooling, the precipitate was collected washed with cold ethanol to give **1a-p** with 22–91% yield.

1-Methylindole-2-carboxaldehyde phenylhydrazone **(1a)** Yield 41.1%; m.p. 185–186°C; ^{1}H-NMR: 4.06(3H, s), 6.70(1H, s), 6.76(1H, m), 7.04(3H, m), 7.21(3H, m), 7.50(2H, dd), 8.03(1H, s, azomethine CH), 1038(1H, s, hydrazine NH); ^{13}C-NMR: 32.55, 104.41, 110.31, 112.56, 119.50, 120.32, 120.94, 122.87, 127.91, 129.93, 131.03, 135.79, 139.58, 145.82 (azomethine CH); ESI MS *m/z* 250 (M + 1, %100), 251 (M + 2); Anal. Calcd. For $C_{16}H_{15}N_3$: C, 77.07%; H, 6.07%; N, 16.86%. Found: C, 76.11%; H, 6.35%; N, 16.28%. FT-IR (KBr) cm^{-1} 1603 (C=N azomethine stretch), 3302 (N–H stretch).

1-Methylindole-2-carboxaldehyde (2- fluorophenyl) hydrazone **(1b)** Yield 54.3%; m.p. 157–158°C; ^{1}H-NMR: 4.06(3H,s), 6.728(1H, s), 6.78(1H, m), 7.12(4H, m), 7.49(3H, m), 8.32(1H, s, azomethine), 10.29(1H, s, hydrazine); ^{13}C-NMR: 32.53, 104.94, 110.39, 114.33, 115.66, 115.84, 119.32, 120.36, 121.09, 123.09, 125.80, 127.82, 133.88, 134.04, 135.47, 139.63, 148.61 (azomethine C), 150.98; ESI MS *m/z* 268 (M + 1, %100), 269 (M + 2); Anal. Calcd. For $C_{16}H_{14}N_3F$: C, 71.88%; H, 5.28%; N, 15.73%. Found: C, 71.22%; H, 5.60%; N, 15.29%. FT-IR (KBr) cm^{-1} 1623 (C=N azomethine stretch), 3320 (N–H stretch).

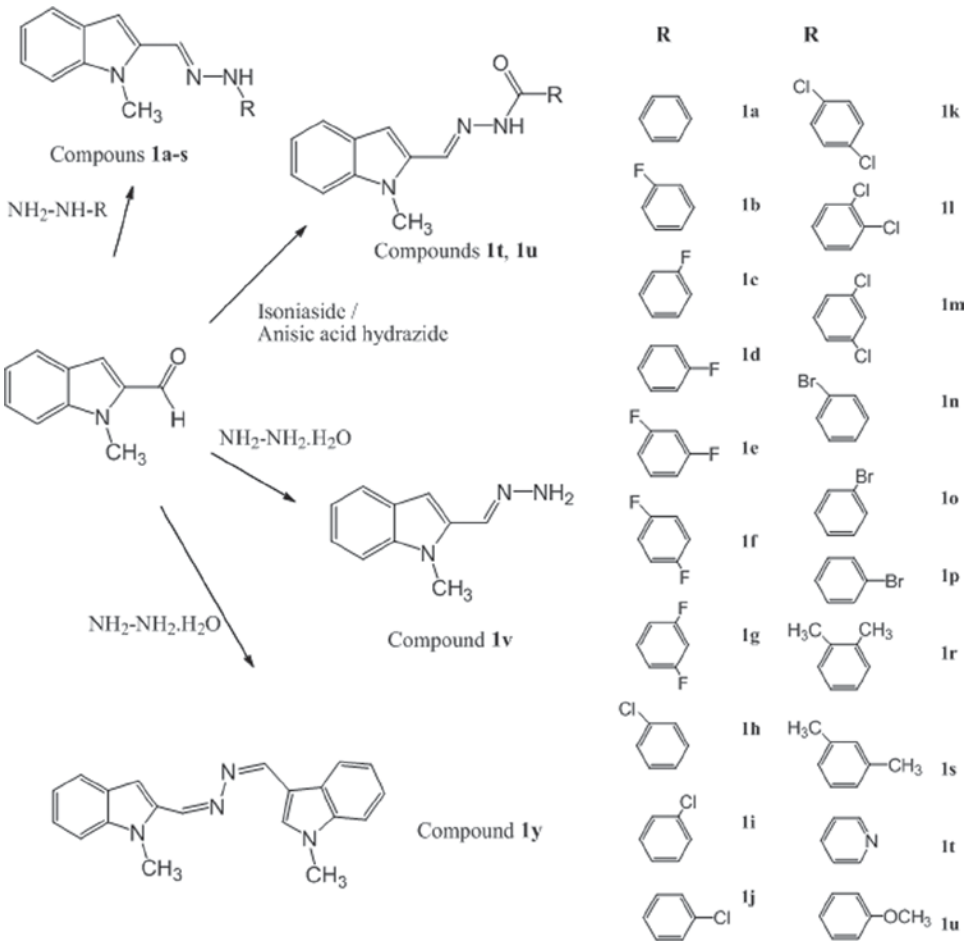

Figure 2. Synthetic pathways of new indole-based MLT analogue compounds. MLT, melatonin.

1-Methylindole-2-carboxaldehyde (3- fluorophenyl) hydrazone **(1c)** Yield 48.9%; m.p. 153–154°C; ^{1}H-NMR: 4.07(3H, s), 6.79(3H, m), 7.05(1H, t), 7.23(2H, m), 7.51(2H, dd), 8.05(1H, s, azomethine CH), 10.61(1H, s, hydrazine NH); ^{13}C-NMR: 31.75, 98.14, 98.41, 104.28, 104.65, 104.87, 107.98, 109.58, 119.57, 120.28, 122.30, 127.01, 130.73, 131.49, 134.59, 138.85, 147.01(azomethine C), 161.99, 162.09, 164.48; ESI MS *m/z* 268 (M + 1, %100), 269 (M + 2); Anal. Calcd. For $C_{16}H_{14}N_3F$: C, 71.88%; H, 5.28%; N, 15.73%. Found: C, 70.99%; H, 5.46%; N, 15.12%. FT-IR (KBr) cm^{-1} 1609 (C=N azomethine stretch), 3305 (N–H stretch).

1-Methylindole-2-carboxaldehyde (4- fluorophenyl) hydrazone **(1d)** Yield 51.1%; m.p. 174–175°C; ^{1}H-NMR: 4.05(3H, s), 6.70(1H, s), 7.11(6H, m), 7.50(2H, dd), 8.02(1H, s, azomethine CH), 10.39(1H, s, hydrazine NH); ^{13}C-NMR: 32.51, 104.41, 110.29, 113.51, 116.30, 116.53, 120.29, 120.92, 122.85, 127.86, 131.08, 135.70, 139.54, 142.51 (azomethine C), 155.39, 157.72; ESI MS *m/z* 268 (M + 1, %100), 269 (M + 2); Anal. Calcd. For $C_{16}H_{14}N_3F$: C, 71.88%; H, 5.28%; N, 15.73%. Found: C, 71.22%; H, 5.40%; N, 15.22%. FT-IR (KBr) cm^{-1} 1609 (C=N azomethine stretch), 3298 (N–H stretch).

1-Methylindole-2-carboxaldehyde (2,4-difluorophenyl) hydrazone **(1e)** Yield 27.4%; m.p. 159–160°C; ^{1}H-NMR: 4.05(3H, s), 6.73(1H, s), 7.04(2H, m), 7.23(2H, m), 7.47(3H, m), 8.30(1H, s, azomethine CH), 10.26(1H, s, hydrazine CH); ESI MS *m/z* 286 (M + 1, %100), 287 (M + 2); Anal. Calcd. For $C_{16}H_{13}N_3F_2$: C, 67.34%; H, 4.60%; N, 14.73%. Found: C, 66.62%; H, 4.74%; N, 14.40%. FT-IR (KBr) cm^{-1} 1602 (C=N azomethine stretch), 3317 (N–H stretch).

1-Methylindole-2-carboxaldehyde (2,5-difluorophenyl) hydrazone **(1f)** Yield 61.9%; m.p. 168–169°C; ^{1}H-NMR: 4.05(3H, s), 6.55(1H, m), 6.78(1H, s), 7.14(4H, m), 7.53(2H, dd), 8.35(1H, s, azomethine CH), 10.35(1H, s, hydrazine NH); ESI MS *m/z* 286 (M + 1, %100), 287 (M + 2); Anal. Calcd. For $C_{16}H_{13}N_3F_2$: C, 67.34%; H, 4.60%; N, 14.73%. Found: C, 67.10%; H, 4.58%; N, 14.30%. FT-IR (KBr) cm^{-1} 1631 (C=N azomethine stretch), 3325 (N–H stretch).

1-Methylindole-2-carboxaldehyde (3,5-difluorophenyl)hydrazone **(1g)** Yield 59.9%; m.p. 183–184°C; ^{1}H-NMR: 4.02(3H, s), 6.54(1H, m), 6.61(2H, dd), 6.77(1H, s), 7.03(1H,t), 7.18(1H, m), 7.49(2H, dd), 8.05(1H, s, azomethine CH), 10.78(1H, s, hydrazine NH); ESI MS *m/z* 286 (M + 1, %100), 287 (M + 2); Anal. Calcd. For $C_{16}H_{13}N_3F_2$: C, 67.34%; H, 4.60%; N, 14.73%. Found: C, 67.03%; H, 4.88%; N, 14.30%. FT-IR (KBr) cm^{-1} 1631 (C=N azomethine stretch), 3305 (N–H stretch).

1-Methylindole-2-carboxaldehyde (2-chlorophenyl) hydrazone **(1h)** Yield 56.3%, m.p. 136.5-137.5°C; ^{1}H-NMR: 4.03(3H, s), 6.77(2H, m), 7.03(1H,t), 7.25(3H, m), 7.47(3H, d. dd), 8.47(1H, s, azomethine-CH), 9.23(1H, s, hydrazine-NH); ^{13}C-NMR: 32.49, 105.04, 110.43, 114.44, 116.82, 120.29, 120.39, 121.14, 123.17, 127.78, 128.86, 130.09, 134.86, 135.37, 139.60, 141.91 (azomethine C); ESI MS *m/z* 284.7 (M + 1, %100), 286.7 (M + 3); Anal. Calcd. For $C_{16}H_{14}N_3Cl$: C, 67.71%; H, 4.98%; N, 14.81%. Found: C, 67.37%; H, 5.16%; N, 14.64. FT-IR (KBr) cm^{-1} 1591 (C=N azomethine stretch), 3316 (N–H stretch).

1-Methylindole-2-carboxaldehyde (3-chlorophenyl) hydrazone **(1i)** Yield 28.1%, m.p. 172–173°C; ^{1}H-NMR: 4.05(3H, s), 6.78(2H,m), 6.96(1H,m), 7.05(2H,m), 7.22(2H, m), 7.51(2H, dd), 8.05(1H, s, azomethine-CH), 10.59(1H, s, hydrazine-NH). ^{13}C-NMR: 32.49, 105.05, 110.37, 111.29, 111.82, 118.83, 120.36, 121.07, 123.09, 127.78, 131.54, 132.45, 134.56, 135.33, 139.62, 147.25 (azomethine C); ESI MS *m/z* 284.41(M + 1, %100), 286.56 (M + 3); Anal. Calcd. For $C_{16}H_{14}N_3Cl$: C, 67.71%; H, 4.98%; N, 14.81%. Found: C, 67.24%; H, 5.27%; N, 14.77%. FT-IR (KBr) cm^{-1} 1599 (C=N azomethine stretch), 3297 (N–H stretch).

1-Methylindole-2-carboxaldehyde (4-chlorophenyl) hydrazone **(1j)** Yield 91.5%, m.p. 198–199°C; ^{1}H-NMR: 4.05(3H, s), 6.73(1H, s), 7.04(3H, m), 7.23(3H, m), 7.51(2H, dd), 8.04(1H, s, azomethine-CH), 10.53(1H,s, hydrazine-NH); ^{13}C-NMR: 32.52, 104.80, 110.34, 113.99, 120.33, 121.00, 122.65, 127.82, 129.69, 131.86, 135.49, 139.60, 144.73 (azomethine C); ESI MS *m/z* 284.7(M + 1, %100), 286.8 (M + 3); Anal. Calcd. For $C_{16}H_{14}N_3Cl$: C, 67.71%; H, 4.98%; N, 14.81%. Found: C, 67.51%; H, 5.26%; N, 14.37%. FT-IR (KBr) cm^{-1} 1599 (C=N azomethine stretch), 3304 (N–H stretch).

1-Methylindole-2-carboxaldehyde (2,4-dichlorophenyl) hydrazone **(1k)** Yield 24.7%, m.p. 143–144°C; ^{1}H-NMR: 4.04(3H, s), 6.78(1H, s), 7.06(1H, m), 7.21(1H, m), 7.34(1H, dd), 7.25(4H, m), 8.51(1H, s, azomethine), 10.09(1H, s, hydrazine); ^{13}C-NMR: 32.49, 105.39, 110.48, 115.43, 117.28, 120.42, 121.22, 122.81, 123.30, 127.74, 128.87, 129.32, 135.15, 135.59, 139.64, 141.11 (azomethine C); ESI MS *m/z* 318(M + ,%100), 320 (M + 2); Anal. Calcd. For $C_{16}H_{13}N_3Cl_2$: C, 60.37%; H, 4.12%; N, 13.21%. Found: C, 60.20%; H, 4.16%; N, 13.00%. FT-IR (KBr) cm^{-1} 1593 (C=N azomethine stretch), 3309 (N–H stretch).

1-Methylindole-2-carboxaldehyde (3,4-dichlorophenyl)hydrazone **(1l)** Yield 40.9%; m.p. 220–221°C; ^{1}H-NMR: 4.04(3H, s), 6.79(1H, s), 7.02(2H, m), 7.21(2H, m), 7.50(3H, m), 8.06(1H, S, azomethine), 10.68(1H, s, hydrazine); ^{13}C-NMR: 31.70, 104.56, 109.64, 112.12, 112.64, 119.31, 119.61, 120.35, 122.42, 126.96, 130.94, 131.52, 132.34, 134.34, 138.88, 145.06 (azomethine); ESI MS *m/z* 318(M +, %100), 320 (M + 2); Anal. Calcd. For $C_{16}H_{13}N_3Cl_2$: C, 60.37%; H, 4.12%; N, 13.21%. Found: C, 60.05%; H, 4.19%; N, 12.84%. FT-IR (KBr) cm^{-1} 1589 (C=N azomethine stretch), 3300 (N–H stretch).

1-Methylindole-2-carboxaldehyde (3,5-dichlorophenyl)hydrazone **(1m)** Yield 22.0 %; m.p. 173.5–174.5°C; ^{1}H-NMR: 4.04(3H, s), 6.82(1H, s), 6.87(1H, t), 6.98(2H, d), 7.06(1H,t), 7.21(1H, m), 7.52(2H, dd), 8.07(1H, s, azomethine), 10.76(1H, s, hydrazine); ^{13}C-NMR: 32.46, 105.67, 110.45, 110.75, 118.02, 120.43, 121.22, 123.34, 127.71, 133.83, 134.93, 139.70, 147.97(azomethine); ESI MS *m/z* 318(M+, %100), 320 (M + 2); Anal. Calcd. For $C_{16}H_{13}N_3Cl_2$: C, 60.37%; H, 4.12%; N, 13.21%. Found: C, 60.34%; H, 4.38%; N, 13.01%. FT-IR (KBr) cm^{-1} 1580 (C=N azomethine stretch), 3306 (N–H stretch).

1-Methylindole-2-carboxaldehyde (2-bromophenyl) hydrazone **(1n)** Yield 34.8%; m.p. 134–135°C; ^{1}H-NMR: 4.05(3H, s), 6.75(2H, m), 7.05(1H, t), 7.21(1H, m), 7.32

(1H, m), 7.52(4H, m), 8.53(1H, s, azomethine CH), 9.72(1H, s, hydrazine NH); ^{13}C-NMR: 32.46, 105.00, 106.68, 110.44, 114.91, 120.39, 121.00, 121.16, 123.17, 127.78, 129.40, 133.32, 135.04, 135.38, 139.60, 142.96(azomethine C); ESI MS *m/z* 328 (M+, %100), 287 (M + 2), 330 (M + 2), 331 (M + 3); Anal. Calcd. For $C_{16}H_{14}N_3Br$: C, 58.53%; H, 4.30%; N, 12.80%. Found: C, 58.36%; H, 4.51%, N, 12.69%. FT-IR (KBr) cm^{-1} 1602 (C=N azomethine stretch), 3305 (N–H stretch).

1-Methylindole-2-carboxaldehyde (3-bromophenyl) hydrazone **(1o)** Yield 44.5%; m.p. 177–178°C; ^{1}H-NMR: 4.05(3H, s), 6.76(1H, s), 6.91(1H, t), 7.03(2H, m), 7.19(3H, m), 7.51(2H, dd), 8.05(1H, s, azomethine CH), 10.56(1H, s, hydrazine NH); ^{13}C-NMR: 32.48, 105.04, 110.38, 111.65, 114.72, 120.36, 121.73, 123.10, 123.15, 127.79, 131.85, 132.48, 135.33, 139.63, 147.37 (azomethine C); ESI MS *m/z* 328 (M+, %100), 287 (M + 2), 330 (M + 2), 331 (M + 3); Anal. Calcd. For $C_{16}H_{14}N_3Br$: C, 58.53%; H, 4.30%; N, 12.80%. Found: C, 57.88%; H, 4.49%; N, 12.33%. FT-IR (KBr) cm^{-1} 1590 (C=N azomethine stretch), 3296 (N–H stretch).

1-Methylindole-2-carboxaldehyde (4-bromophenyl) hydrazone **(1p)** Yield 87.2%; m.p. 207–208°C; ^{1}H-NMR: 4.05(3H, s), 6.73(1H, s), 6.91(1H, t), 7.02(2H, m), 7.19(3H, m), 7.46(4H, m), 8.04(1H, s, azomethine CH), 10.53(1H, s, hydrazine NH); ^{13}C-NMR: 32.52, 104.83, 110.22, 110.34, 114.51, 120.34, 121.01, 123.01, 127.82, 131.95, 132.51, 135.48, 139.61, 145.10 (azomethine C); ESI MS *m/z* 328 (M + ,%100), 287 (M + 2), 330 (M + 2); Anal. Calcd. For $C_{16}H_{14}N_3Br$: C, 58.53%; H, 4.30%; N, 12.80%. Found: C, 58.49%; H, 4.51%; N, 12.62%. FT-IR (KBr) cm^{-1} 1590 (C=N azomethine stretch), 3306 (N–H stretch).

1-Methylindole-2-carboxaldehyde (2,3-dimethylphenyl)hydrazone **(1r)** Yield 73.9%, m.p. 151–152°C; ^{1}H-NMR: 2.14(3H, s), 2.24(3H, s), 4.06(3H, s), 6.67(2H, t), 7.03(2H, m), 7.50(2H,dd), 8.34(1H, s, azomethine-CH), 9.59(1H, s, hydrazine-NH); ^{13}C-NMR: 12.62, 20.11, 31.68, 103.36, 109.50, 110.17, 119.04, 119.50, 120.12, 120.85, 122.02, 125.93, 127.13, 131.58, 135.174, 136.28, 138.73, 142.92 (azomethine-C); ESI MS *m/z* 278 (M + 1, %100), 279 (M + 2); Anal. Calcd. For $C_{18}H_{19}N_3$:C, 77.39%; H, 6.91%; N, 15.16%. Found: C, 76.83%; H, 6.78%; N, 13.98. FT-IR (KBr) cm^{-1} 1586 (C=N azomethine stretch), 3338 (N–H stretch).

1-Methylindole-2-carboxaldehyde (2,4- dimethylphenyl)hydrazone **(1s)** Yield 50.7%, m.p. 146–147°C; ^{1}H-NMR: 2.20(3H, s), 3.35(3H, s), 4.05(3H, s), 6.69(1H,s), 6.92(2H,t), 7.04(1H, m), 7.20(2H, m), 7.50(2H, dd), 8.30(1H, s, azomethine-CH), 9.52(1H, s, hydrazine-NH); ^{13}C-NMR: 18.12, 20.87, 32.43, 103.96, 110.26, 112.62, 120.27, 120.87, 121.32, 122.74, 127.91, 128.00, 131.67, 131.92, 135.99, 139.48, 141.46 (azomethine-C); ESI MS *m/z* 278 (M + 1, %100), 279 (M + 2); Anal. Calcd. For $C_{18}H_{19}N_3$:C, 77.39%; H, 6.91%; N, 15.16%. Found: C, 77.50%; H, 7.13%; N, 14.59%. FT-IR (KBr) cm^{-1} 1615 (C=N azomethine stretch), 3307 (N–H stretch).

General procedure for the synthesis of compounds 1t,1u

A solution of 1-methylindole-2-carboxaldehyde (0.05 mmol) and izonicotinic acid hydrazide (for **1t**) or anisic acid hydrazide (for **1u**) (0.05 mmol) in 50 mL of ethanol was heated for 2.5 h on the hot water bath. On cooling, the precipitate was collected washed with cold ethanol to give **1t** and **1u** with 12–16% yield.

1-Methylindole-2-carboxaldehyde izonicotynoilhydrazone **(1t)** Yield 16.5%; m.p. 190–191°C; ^{1}H-NMR: 4.08(3H, s), 6.95(1H, s), 7.07(1H, t), 7.25(1H, t), 7.55(2H, m), 7.82(2H, d), 8.59(1H, s, azomethine CH), 8.75(2H, d), 12.06(1H, s, hydrazine NH); ESI MS *m/z* 279 (M + 1, %100), 280 (M + 2); Anal. Calcd. For $C_{16}H_{14}N_4O$: C, 69.04%; H, 5.07%; N, 20.14%. Found: C, 66.80%; H, 5.65%; N, 17.84%. FT-IR (KBr) cm^{-1} 1649 (C=N azomethine stretch), 3320 (N–H stretch).

N-(4-methoxybenzoyl)-N′-(1-methylindolyl-2-methylene)-hydrazine **(1u)** Yield 12.4%; m.p. 199–200°C; ^{1}H-NMR: 3.82(3H, s), 4.07(3H, s), 6.88(1H, s), 7.06(3H, m), 7.23(1H, t), 7.53(2H, dd), 7.91(2H, d), 8.57(1H, s, azomethine CH), 11.71(1H, s, hydrazine NH); ESI MS *m/z* 308 (M + 1, %100), 309 (M + 2); Anal. Calcd. For $C_{18}H_{17}N_3O_2$: C, 70.33%; H, 5.58%; N, 13.68%. Found: C, 68.40%; H, 6.42%, N, 12.42%. FT-IR (KBr) cm^{-1} 1647 (C=N azomethine stretch), 3327 (N–H stretch).

General procedure for the synthesis of compounds 1v,1y

1-Methylindole-2-carboxaldehyde (0.1 mmol) and hydrazine hydrate (0.1 mmol for **1v**, 0.2 mmol for **1y**) in 25 mL of ethanol was heated for 4 h on the hot water bath. On cooling, the precipitate was collected washed with cold ethanol to give **1v** and **1y** with 56–57% yield.

N,N′-bis-(1-methylindole-2-ylmethylene)hydrazine **(1v)** Yield 56.0%; m.p. 297–298°C; ^{1}H-NMR: 4.16(6H, s), 7.13(4H, m), 7.31(2H, t), 7.62(2H, dd), 8.90(2H, s, azomethine CH); ESI MS *m/z* 315 (M + 1, %100), 316 (M + 2), 317 (M + 3); Anal. Calcd. For $C_{20}H_{18}N_4$: C, 76.4%; H, 5.58%; N, 13.68%. Found: C, 68.40%; H, 6.42%; 12.42%. FT-IR (KBr) cm^{-1} 1627 (C=N azomethine stretch).

1-Methylindole-2-carboxaldehyde hydrazone **(1y)** Yield 57.1%; m.p. 124–125°C; ^{1}H-NMR: 4.69(3H, s), 7.30(1H, s), 7.66(2H, s, hydrazine NH), 7.90(1H, t), 7.92(1H, s), 8.19(1H, d), 8.27(1H, d), 8.67(1H, s, azomethine CH); ^{13}C-NMR: 32.80, 103.11, 110.95, 120.91,121.49,123.10, 128.71, 133.43, 137.31, 139.89(azomethine C); ESI MS *m/z* 174 (M + 1, %100); Anal. Calcd. For $C_{10}H_{11}N_3$: C, 69.33%; H, 6.40%; N, 24.27%. Found: C, 69.34%; H, 6.79%; N, 23.34%. FT-IR (KBr) cm^{-1} 1463 (C=N azomethine stretch), 3355 (N–H stretch).

Biological activity studies

Antioxidant activity on ROS-induced DCFH-DA oxidation

For estimation of ROS inside cells DCFH-DA was used as a fluorescent probe. In cellular systems non fluorescent DCFH-DA readily crosses the cell membrane and undergoes hydrolysis by intracellular esterases to nonfluorescent DCFH. DCFH is then rapidly oxidized in the presence of reactive oxygen species to highly fluorescent DCFH[31]. In the present study, human erythrocytes were used to evaluate the potential antioxidant effect of test compounds.

The assay is performed as detailed in Shirinzadeh et al.[13]. Effects of the compounds (100 μM) on DCFH oxidation were investigated after incubation of the cells with test compounds alone or after co-incubation of the cells with test compounds and H_2O_2 (10 μM) as a known reactive oxygen species. Antioxidant effects of the compounds were compared to the effect of ascorbic acid (100 μM) as a well-known antioxidant and melatonin (MLT) (100 μM) as their parent compound.

Membrane stabilizing effect; LDH leakage

CHO-K1 cells were cultured in FC-1: Dulbecco's Modified Eagles Medium (1:1), supplemented with 10% foetal bovine serum, 1% of a 100 U/ml penicillin-streptomycin solution, 2 mM (final concentration) L-glutamine and 1 mM (final concentration) sodium pyruvate at 37°C, 5% CO_2. This medium was used in all cell incubations. Cytotoxic effects of the compounds were evaluated via LDH activity assay according to the method of Hassoun et al.[32] with a minor modification. The activity of LDH in 100 μL of media was determined by direct calculation based on the decrease in absorbance[33].

Electrochemical measurements

The cyclic, linear sweep, differential pulse voltammetric (DPV) and square wave voltammetric (SWV) measurements at a glassy carbon electrode were performed using a BAS 100W (Bioanalytical System, Los Angeles, California, USA) electrochemical analyzer. The three electrode system a glassy carbon working electrode (BAS; Ø: 3 mm diameter), with a platinum wire counter electrode and Ag/AgCl reference electrode (KCl 3M, BAS) and a standard one-compartment three-electrode cell of 10 mL capacity. For analytical application, the following parameters were employed: SWV amplitude, 25 mV; frequency, 15 Hz; scan increment, 4 mV. DPV parameters were: pulse amplitude, 50 mV; pulse width 50 ms; scan rate, 20 mV s^{-1}. The pH values were measured using a pH meter Model 538 (WTW, Wien, Austria) using a combined electrode (glass electrode reference electrode) with an accuracy of ±0.05 pH. A stock solution of the selected synthesized indoles was prepared daily by direct dissolution in methanol. The working solutions were prepared by the dilution of the stock solution with selected supporting electrolytes contained different percent of methanol. Four different supporting electrolytes, namely sulfuric acid (0.1 M), phosphate buffer (0.2 M, pH 2.0–8.0), acetate buffer (0.2 M, pH 3.7–5.7), and Britton–Robinson buffer (0.04 M, pH 2–12) were prepared in double distilled water. All solutions were protected from light and were used within 24 h to avoid decomposition.

Results and discussion

Antioxidant effects on ROS-induced DCFH-DA oxidation

The protective effect of newly synthesized MLT analogues against DCFH-DA oxidation was determined in human erythrocytes that were preloaded with the fluorescent probe. Oxidation of the probe was screened at 10 min intervals up to 60 min. DCFH oxidation at 60 min incubation is given for cells exposed to the test compound alone (Figure 3) and for cells co-exposed to H_2O_2 and the test compounds (Figure 4). The aim of the second model (Figure 4) is to evaluate the antioxidant capacity of the synthesized MLT analogs in a ROS challenge situation. Almost all compounds were found to exhibit higher antioxidant activity when they are challenged with H_2O_2. Comparing to the results from our previous study[13], *N*-methylation of the indole ring decreased the antioxidant capacity of all MLT derivatives. Similar to our previous results[13] among mono-halogenated derivatives p-halogenation was found to decrease antioxidant activity compared to o- and m-substitution of the same halogen atom. **1t**, **1u**, and **1y** had the weakest antioxidant activity in both cases (with or without H_2O_2). This can be explained by having no aromatic halogenations or a carbonyl group on the side chain.

Membrane stabilizing effect; LDH leakage

Lipid peroxidation is known to change membrane fragility, motility and permeability[34]. LDH is a cytoplasmic

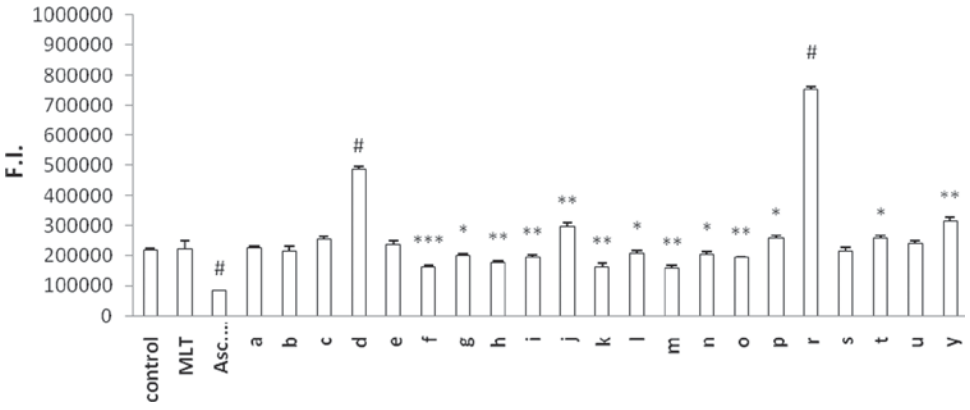

Figure 3. Oxidation of DCFH via reactive oxygen species in erythrocytes after incubation with ascorbic acid, MLT and newly synthesized analogs for 60 min. Control group represents cells incubated with PBS without any compound addition. Values are mean ± SD of three individual experiments. *$p < 0.05$, compared to control group. **$p < 0.005$, compared to control group. #$p < 0.0005$, compared to control group. MLT, melatonin.

enzyme and its leakage from injured cells into the culture medium has been shown to be useful as an indicator of cellular membrane damage. In the present study, we determined LDH activity in the culture medium in order to assess the membrane stabilizing effect of MLT analogues. No increases in LDH activity were observed with any of the tested compounds. Even several compounds (**1b, 1g**, and **1t**) were found to decrease LDH leakage indicating membrane stabilizing effect. This assay is also an indicator of a possible cytotoxic effect of the test compounds by means of inducing membrane damage. From this point of view, as possible drug candidates, we can conclude that none of the synthesized MLT analogs are found to have cytotoxic effect. In accordance with the antioxidant assay (Figures 3 and 4) **1d** and **1r** were found to have the highest, but statistically not significant, LDH activity in media (Figure 5). This finding can be explained by the increased ROS formation with **1d** and **1r** analogs resulted in membrane damage and leakage of the LDH enzyme into the media.

Electrochemical measurements

In this study the measurements from the selected indole derivatives are two-dimensional, with the potential being related to qualitative properties and current related properties. In order to characterize the electrochemical process that occurs on the glassy carbon electrode, both pH, nature of the buffer and scan rate studies were carried out in the potential range from –0.25 to 1.6 V. Cyclic voltammetry (CV) experiments were achieved over a range from acidic (0.1M H_2SO_4) to alkaline (pH 12.00) in acidic solutions and different buffer aqueous media. For the quantitative determination, DPV and SWV methods were applied to the selected synthesized derivatives [35–37]. For the synthesized compounds, the peak currents and peak potentials were determined in different supporting electrolytes such as H_2SO_4, phosphate, acetate, and Britton-Robison buffers containing 40, 50, 20, and 30% methanol (v/v) to maintain solubility for **1f**, **1g**, **1y**, and **1u**, respectively. The compounds were selected as two of the most active and structurally similar compounds **1f**, **1g**, and structurally different compounds **1y** and **1u**. Therefore, several electrochemical measurements using cyclic, linear sweep, differential pulse, and square wave voltammetric methods were performed with various supporting electrolytes and buffers in order to obtain such information.

As first step, compounds **1f**, **1g**, **1y,** and **1u** were subjected to a cyclic and linear sweep voltammetric studies with the aim to characterize of their electrochemical oxidation behavior in details on the glassy carbon electrode over a wide pH range (1.5–12.0). Voltammograms exhibited one distinct and well defined main anodic peak (Ox_1) and/or one ill-defined anodic wave at different potential values depending on pH values and supporting electrolyte composition (Figures 6 and 7). In addition to this first oxidation step, the second oxidation response was obtained when working on compound **1u** depending on the methoxy benzene oxidation (Ox_2). CV measurements showed an irreversible nature of the oxidation process. In acidic media, the anodic oxidation did not occur until about +0.50 V for all selected compounds on glassy

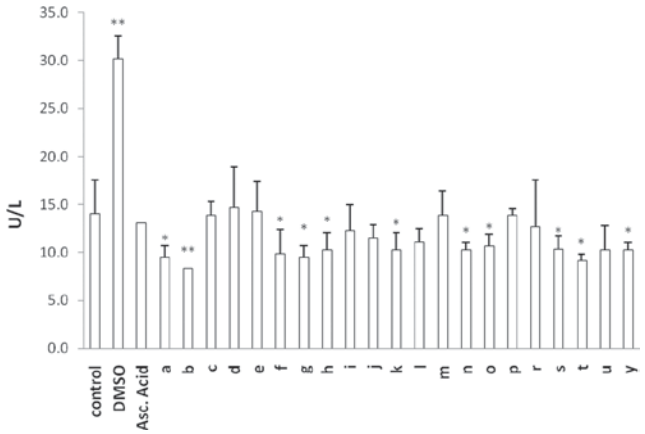

Figure 5. Effect of MLT analogs on LDH leakage from CHO cells into the culture media. Control group represents cells incubated in regular media without any compound addition. % 2.5 DMSO is used to cause membrane damage where ascorbic acid is used as an antioxidant. $^{*}p < 0.05$, $^{**}p < 0.005$ compared with control.

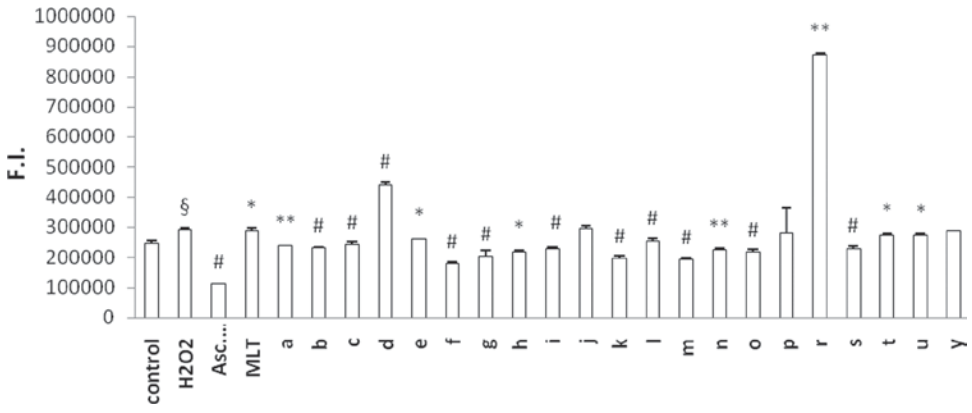

Figure 4. Antioxidant effect of indole-analogs on H_2O_2-induced oxidation of DCFH in erythrocytes. Control group represents cells incubated with PBS without any compound addition. Values are mean ± SD of three individual experiments. $^{\S}p < 0.005$, compared with control. $^{*}p < 0.05$, compared with H_2O_2 group. $^{**}p < 0.005$, compared with H_2O_2 group. $^{\#}p < 0.0005$, compared with H_2O_2 group.

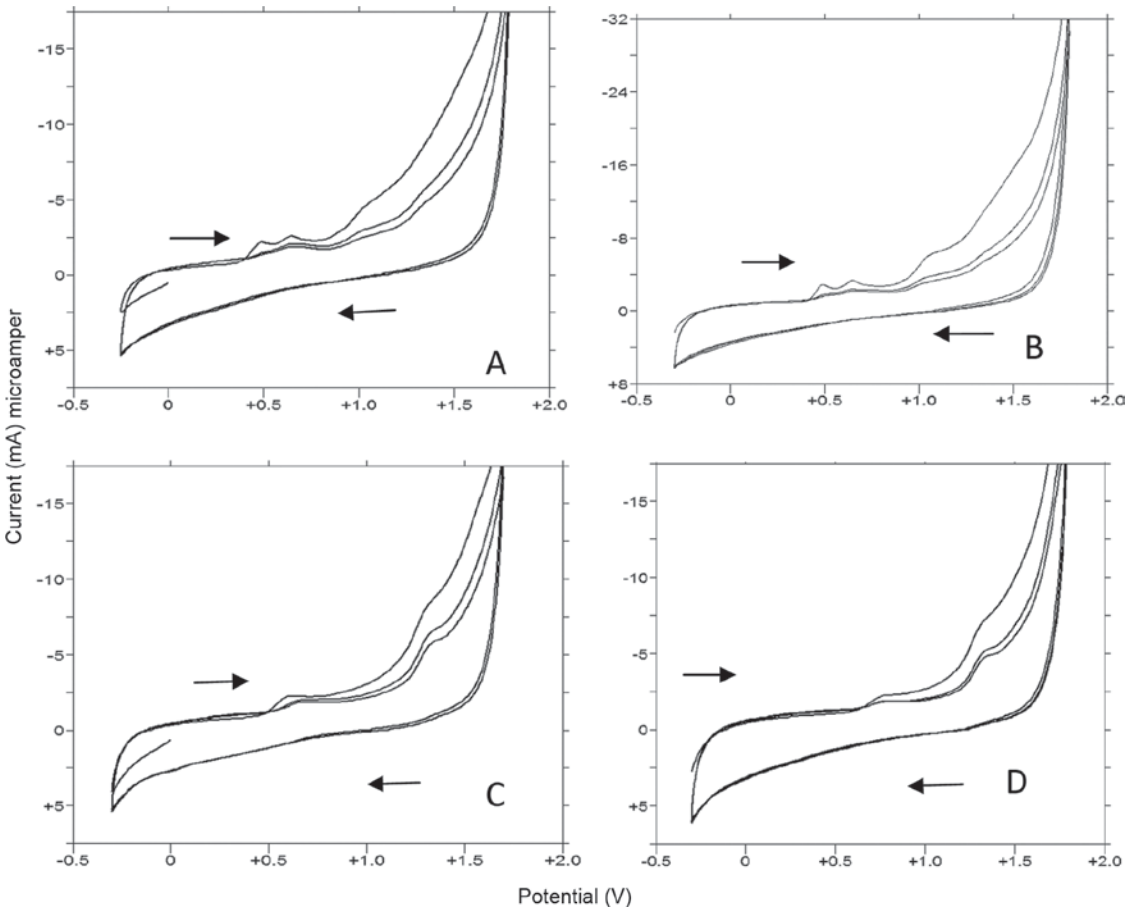

Figure 6. CV voltammograms of 20 μg/mL **1f** and 10 μg/mL **1g**, **1y**, and **1u** in 0.1 M H_2SO_4. (A,B,C, and D): (A) 20 μg/mL **1f**, 40% MeOH (B) 10 μg/mL **1g**, 50% MeOH(C) 10 μg/mL **1y** 20% MeOH (D) 10 μg/mL **1u** 30% MeOH.

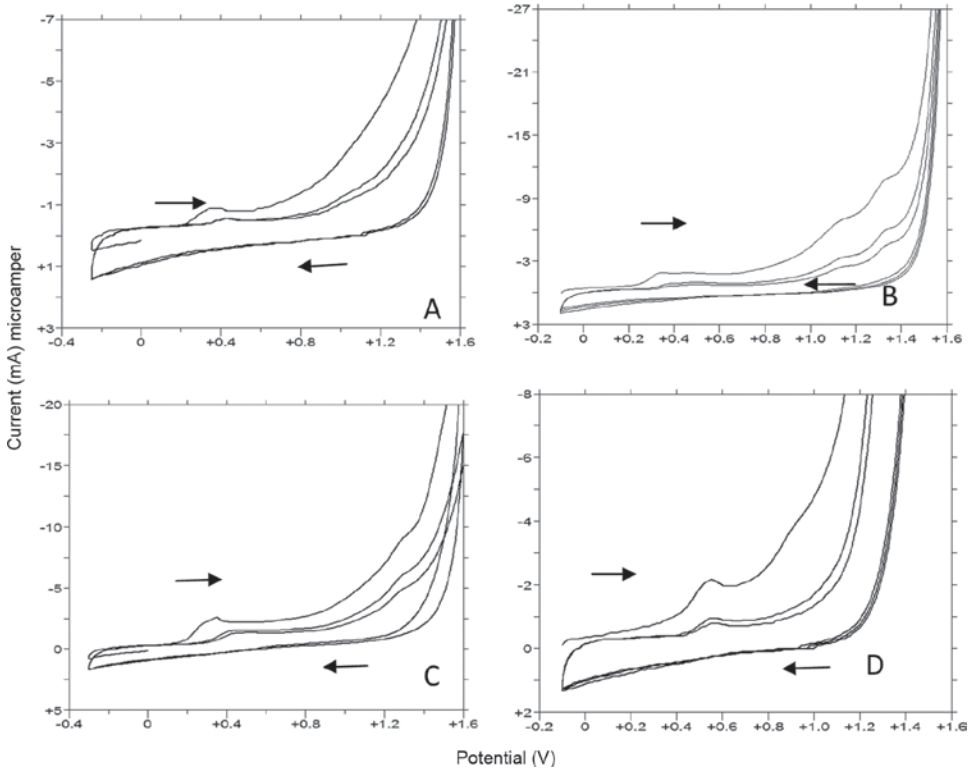

Figure 7. CV voltammograms of **1f**, **1g**, **1y**, and **1u**. (A,B,C, and D): (A) 20 μg/mL **1f**, pH 6 FT (B) 10 μg/mL **1g**, pH 4.7 AT (C) 10 μg/mL **1y**, pH 7.0 BRT (D) 10 μg/mL **1u**, pH 10 BRT.

carbon electrode (Figure 6). By reversing at +1.80 V no reduction signal corresponding to the anodic response was observed on the cathodic branch in all pH values (Figure 7). The anodic oxidation peaks of the selected indolic compounds decreased to the second or higher cycles (Figures 6 and 7). This phenomenon may be partly attributed to the consumption of adsorbed indolic compounds on the electrode surface.

The effect of the potential scan rate varied between 5 and 1000 mV s^{-1} on the peak current and potential of the selected indolic compounds were evaluated. The different amounts shifts in the peak potentials confirmed the irreversibility of the oxidation processes of these compounds (Table 1). Scan rate studies were carried out to assess whether the processes at the glassy carbon electrode was under diffusion or adsorption control for the first and/or second peaks. When the scan rate was varied between 5 and 1000 mV s^{-1} in 20 μg/mL solution of compound **1f**, and 10 μg/mL solution of compounds **1g**, **1y**, and **1u** a linear dependence of the peak intensity ip (μA) upon the square root of the scan rate $\nu^{1/2}$ (mV s^{-1}) was found in all studied supporting electrolytes, demonstrating a diffusional behavior. The equation is noted in Table 1 for all compounds and all studied media. All the equations gave a straight line with a slope values between 0.36 and 0.66, very close to the theoretical value of 0.5, which is expressed for an ideal reaction the diffusion controlled electrode process[38]. Nonetheless, the peak potentials shifted to the more positive potentials to the anodic direction when the scan rate was increased (Table 1) in all supporting electrolyte.

The effect of pH on the peak potential and intensity were investigated between pH 2.0 and 12.0 for all selected indole compounds using CV, DPV, and SWV techniques. All obtained results from CV, DPV, and SWV were found similar. For this reason, only DPV results for the obtained oxidation steps were given as below for *E*p–pH equations:

Ep (mV) = 664.25 – 55.67 pH; $r = 0.998$, $n = 11$ (for **1f**, between pH 2 and 12)
Ep (mV) = 497.02 – 50.78 pH; $r = 0.997$, $n = 10$ (for **1g**, between pH 2 and 12)
Ep (mV) = 632.68 – 42.40 pH; $r = 0.999$, $n = 6$ (for **1y**, between pH 1 and 12)
Ep (mV) = 915.82 – 45.03 pH; $r = 0.981$, $n = 8$ (for **1u**, Ox_1, between pH 2 and 12)
Ep (mV) = 1293.90 – 18.63 pH; $r = 0.994$, $n = 5$ (for **1u**, Ox_2, between pH 1 and 6)

The peak potential of the anodic process moved to less positive potential values and an ill-defined oxidation wave disappeared by increasing pH. In all instances and techniques, the indole compounds undergoes one main irreversible oxidation process and/or one additional ill-defined wave, which shifted towards less positive potentials as the pH was increased. When working on compound **1u**, one additional oxidation step was obtained depending on the apart from indole oxidation, which is occurred by the methoxy benzene oxidation (Ox_2).

According to the slopes of the above equations are found between 42.40 and 55.67 mV/pH for the main oxidation process (Ox_1) of all studied compounds. These slopes are found close to the expected theoretical value of 59 mV/pH. For the second oxidation step of compound **1u**, the slope value was obtained as 18.63 mV/pH. These slopes are found close to the expected theoretical value of 30 mV/pH. According to the obtained slope values of these equations, same amount of electrons and protons are involved in the rate-determining steps. The experimental results showed that shapes of the curves and maximum peak current were obtained in different supporting electrolytes and pH values with constant amount of methanol, which indicated in Table 2. The main peak

Table 1. The results of the scan rates experiments on **1f**, **1g**, **1y**, and **1u**.

	Step	Medium	Scan rate range (mV s^{-1})	ΔE_p/ (mV)	Equation of $\upsilon^{1/2}$ vs. Ip	Correlation coefficient.	Equation of log υ vs. log ip	Correlation coefficient
1f	Ox_1	0.1M H_2SO_4 %40 methanol	10–750	53.0	y = 0.0355x – 0.0595	0.999	y = 0.619x – 1.8005	0.998
	Ox_1	pH 10 BR buffer %40 methanol	5–100	54.0	y = 0.0261x – 0.0417	0.952	y = 0.5504x – 1.9032	0.960
1g	Ox_1	0.1M H_2SO_4 %50 methanol	5–1000	110.0	y = 0.0783x – 0.0806	0.999	y = 0.5344x – 1.2369	0.996
		pH 4.7 acetate buffer %50 methanol	5–500	101.0	y = 0.0637x + 0.0003	0.996	y = 0.5634x – 1.3248	0.990
	Ox_1	pH 7 phosphate buffer %50 methanol	5–500	152.0	y = 0.0234x + 0.0722	0.990	y = 0.3603x – 1.2067	0.994
		pH 10 BR buffer %50 methanol	10–750	90.0	y = 0.098x – 0.2867	0.993	y = 0.6541x – 1.5226	0.990
1y	Ox_1	0.1M H_2SO_4 %20 Methanol	10–1000	27.0	y = 0.0379x – 0.0542	0.998	y = 0.664x – 1.8913	0.981
	Ox_1	pH 3 phosphate buffer %20 methanol	25–750	72.0	y = 0.0627x – 0.1948	0.989	y = 0.7281x – 1.874	0.980
	Ox_1	pH 3.7 acetate buffer %20 methanol	10–1000	75.0	y = 0.094x – 0.2216	0.999	y = 0.6608x – 1.5158	0.998
	Ox_1	pH 7 BR buffer %20 methanol	10–750	52.0	y = 0.2081x – 0.2692	0.996	y = 0.5393x – 0.827	0.998
1u	Ox_1	pH 5.7 acetate buffer %30 methanol	5–1000	99.0	y = 0.1354x – 0.351	0.995	y = 0.6651x – 1.4151	0.990
	Ox_1	pH 2 BR buffer %30 methanol	10–100	113.0	y = 0.0576x – 0.0197	0.994	y = 0.4864x – 1.2225	0.994
	Ox_2	0.1M H_2SO_4 %30 methanol	10–500	17.0	y = 0.1282x – 0.3656	0.986	y = 0.6284x – 1.3465	0.992

that appeared with less positive potential was the best developed and became sharper and occurred as without shoulder in these supporting electrolytes. For this reason, the indicated supporting electrolytes in Table 2 were chosen with respect to have sharp response and better peak shape for the calibration equation.

Although the exact oxidation mechanism was not determined, some conclusions about the potentially electroactive centers under working conditions could be reached. From the CV curves, the main voltammetric behavior of indole and methoxy benzene derivatives, which are structurally related to the mechanism of oxidation of synthesized indole compounds, may be postulated by the oxidation of these groups[26–29,35–41]. Our results on model compounds show similar behavior that the electroactive center corresponding to the main anodic response was the nitrogen atom on the indole ring. In addition to, the methoxy benzene anodic response in compound **1u** was obtained as the second oxidation step. Indole and its derivatives are extensively metabolized *in vivo*[26–29,36,37]. To support the working hypothesis that indole moiety (similar oxidation pathway with indol-3-substituted derivative) and methoxy benzene moiety (similar to phenolic group oxidation step) of compounds **1f**, **1g**, **1y**, and **1u** that undergoes oxidation, behavior of Step I (related to indole moiety) and Step II (related to methoxy benzene moiety) of the compounds were compared with similar model compounds. Comparative study on indole, synthesized indole compounds, indole-3-acetic acid, etodolac, fluvastatine, and ziprasidon were realized by CV at a function of pH in order to identify the oxidation process of the synthesized compounds. It was assumed that oxidation occurred firstly on the nitrogen atom of indole ring, which is electroactive in both acidic and basic media, leading finally to hydroxylation of the benzene ring[36–39,42]. The electrochemical oxidation of indole derivatives with a substituent presented similar features to the indole electroactive centre. It is a well-established fact that the benzene ring of indoles is less reactive than the pyrrole ring. Since all indole derivatives are substituted, the oxidation reaction taking place at the main peak (Ox_1) corresponds to the oxidation at nitrogen on the pyrrole ring[43,44]. The cyclic voltammograms of the indole library and model compounds demonstrated that the oxidation potential peak values were close to those of the nonsubstituted indole nucleus, but higher than those described for the antioxidant melatonin. In addition, no reversibility was observed in the obtained voltammograms. The lack of reversibility is an advantage, meaning that once oxidized these species do not tend to receive electrons.

In addition to this step for **1u**, second oxidation step was obtained because of the methoxy group on the benzene moiety of the molecule. Nearly in all pH values, both indole and methoxy benzene rings responses of **1u** was clearly and separately obtained. The study on verapamil, tamsulosin, and other synthesized indole compounds was realized by CV at GC electrode as a function of pH in order to identify the responsible atom for the second oxidation process of **1u**. Our results revealed a good agreement with the redox mechanism postulated for similar compounds such as 4-methoxyphenol, tamsulosin and verapamil suggested that tamsulosin can be determined electrochemically by oxidation of methoxybenzene groups[40,41,45,46].

Analytical applications and methods validation

In order to develop a voltammetric methodology for determining compound **1f**, **g**, **y**, and **u**, DPV and SWV modes were selected. In this study SWV were used as an alternative technique. Various electrolytes such as sulphuric acid, Britton–Robinson, phosphate, and acetate

Table 2. Calibration parameters of **1f**, **1g**, **1y**, and **1u**.

	1f		**1g**		**1y**		**1u**			
Medium	pH:10 BR buffer (%40 methanol)		pH: 10 BR buffer (%50 methanol)		pH:3 phosphate buffer (%20 metanol)		0.1M H_2SO_4 buffer (%30 methanol)		pH:5.7 cetate uffer (%30 metanol)	
Technique	DPV	DPV	DPV	DPV	DPV	SWV	DPV	SWV	DPV	SWV
Potential/V	0.100	0.100	0.400	0.400	0.400	0.412	1.200	1.300	0.648	0.700
Linearity/range (μg/mL)	1–8	1–8	1–10	1–10	1–10	1–8	1–10	1–10	1–8	1–8
Slope	0.0814	0.0814	0.0988	0.0988	0.0988	0.0999	0.1033	0.1177	0.1156	0.1059
Intercept	0.0237	0.0237	0.0218	0.0218	0.0218	0.0113	0.0209	0.0073	0.0169	0.0261
Correlation coefficient	0.997	0.997	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999
SE of slope	3.180×10^{-3}	3.180×10^{-3}	1.914×10^{1}	1.914×10^{1}	1.914×10^{1}	2.425×10^{1}	1.354×10^{-2}	5.848×10^{-4}	1.340×10^{-3}	1.102×10^{-2}
SE of intercept	1.569×10^{-2}	1.569×10^{-2}	1.231×10^{2}	1.231×10^{2}	1.231×10^{2}	1.574×10^{2}	7.983×10^{-2}	1.832×10^{-3}	4.716×10^{-3}	6.369×10^{-2}
Limit of detection	0.125	0.125	0.038	0.038	0.038	0.110	0.061	0.033	0.083	0.135
Limit of quantification	0.381	0.381	0.115	0.115	0.115	0.334	0.186	0.100	0.254	0.410
Repeatability (RSD %)	0.555	0.555	0.5846	0.5846	0.5846	0.3472	0.545	0.412	0.246	0.372
Reproducibility (RSD %)	1.686	1.686	0.4925	0.4925	0.4925	1.5586	0.820	0.497	1.271	1.975

BR, Britton–Robinson; DPV, differential pulse voltammetric measurement; RSD, relative standard deviation; SWV, square wave voltammetric measurement.

buffer were examined as a supporting electrolyte. The best condition for analytical applications proved to be indifferent supporting electrolytes, which are indicated in Table 2. These supporting electrolytes with a constant amount of methanol were chosen for the subsequent experiments. The effects of methanol amount on peak current and potential were also studied. As expected, the peak current decreased with changing ionic strength and viscosity of the medium. Good correlations were obtained in different concentration ranges using both techniques, which were reported in Table 2. Above the linearity high limit concentration, a loss of linearity was probably due to the adsorption of the compounds on the electrode surface. The characteristics of the calibration plots are summarized in Table 2. The detection limit and quantification limit of the procedures (Table 2) were calculated according to the 3 s/m and 10 s/m criterions, respectively, where s is the standard deviation of the peak currents (five runs) and m is the slope of related calibration graph[45–47]. The low values of SE of slope and intercept and greater than 0.999 correlation coefficient nearly in all media established the precision of the proposed method. The developed methods were validated according to the standard procedures and the results obtained are shown in Table 2. Accuracy, precision and reproducibility of the proposed method were assessed by performing replicate analysis of the standard solutions in supporting electrolytes within the calibration curves. The within day and between day precision, accuracy and reproducibility were determined as the RSD% (relative standard deviation) and the results are shown in Table 2 [31,47,48].

Conclusions

The present work aimed to synthesize, characterize investigate the potential antioxidant effects, and electrochemical evaluation of indole-based MLT analogue hydrazide/hydrazone derivatives. In general all the synthesized indole derivatives were found to have potent antioxidant activity, even higher than MLT itself, according to the results of *in vitro* antioxidant experiments revealing differences in their relative potencies probably related to electronic distribution. Having halogenated aromatic side chain increase the antioxidant activity. Antioxidant effect of indole-analogs on H_2O_2-induced oxidation of DCFH in erythrocytes showed that all analogues except **1d** and **1r** were effective.

We considered several possible interactions during the fluorescence measurements and performed several negative controls. We examined the fluorescence of untreated loaded cells. The healthy cells exhibited lower levels of fluorescence that was relatively stable during the experiment (with a slight gradual increase possibly due to auto oxidation) to make sure that the cells are not progressing to death or another oxidative event is not taking place. The fluorescence intensity of the compounds in the same test environment without DCFH was analyzed to assure that the added compounds are not giving any fluorescence at the same excitation and emission wavelengths (which can cause false positive results). None of the compounds were found to have any. Finally in order to make sure that added compounds do not have a quenching effect we examined the absorbance spectrum of the compounds and determined that the absorbance peaks were not overlapping with either the excitation and emission peak of the oxidized dye.

Although we have thought about the possible esterase inhibition because of the following reasons such an interaction for the present compounds is not possible. First of all two known esterases were identified in RBC cytosol; acetylcholinesterase and arylesterase. The esterases are reported to exhibit specificities for certain substrates and inhibitors but a drug is often hydrolysed by more than one esterase at different sites. Among the most common acetylcholinesterase inhibitors are phosphorus-based compounds, which are designed to bind to the active site of the enzyme. The structural requirements are a phosphorus atom bearing two lipophilic groups, a leaving group (such as a halide or thiocyanate), and a terminal oxygen[50,51]. As a conclusion none of the compounds that we tested are structurally thought to be an inhibitor of the esterases as well as even one of the isozyme or the type of the esterase in RBC cytosol is inhibited by any of our compound we think that there should still be enough activity to hydrolyse DCFH-DA.

Structural investigation of the rest of the active compounds showed that having o- and m-halogenated aromatic side chain increase the antioxidant activity (such as compounds **1f**, **1k**, and **1m**). These are the most promising compounds that should be kept in mind for designing new MLT-based indole derivatives for our ongoing study.

Lack of a methoxy group in the five positions did not affect the antioxidant capacity of the new indole derivatives. In fact, the *in vitro* assays showed that lack of a methoxy group, introduction of a methyl group at the nitrogen in the indole ring and a halogenated aromatic side chain resulted in much more active compounds than MLT itself. 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 radicals.

The use of electrochemical techniques to study mechanisms directly with the electrodes and the determination of compounds of pharmaceutical interest is becoming a mature field[23].

Electrochemical mechanisms are important to all redox chemistry including biological systems usually concerning electron transport chains. Such reactions are most often studied with electrode techniques such as CV[35]. In this study electrochemical behavior of selective MLT analogues through mechanistic studies was investigated. It was observed that oxidation occurred firstly on the nitrogen atom of indole ring leading finally to hydroxylation of the benzene ring[36–39,42]. This investigation gives some suggestions about the possible *in vivo* metabolism of newly synthesized compounds.

This study presents supportive data and will contribute for the design of indole-related drugs with improved antioxidant activity.

Declaration of interest

This work was supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK) Research and Development Grant 109S099. Cell culture laboratory where the antioxidant activity studies performed was established with the TÜBİTAK Research and Development Grant 108S202.

The authors report no conflicts of interest.

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