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RESEARCH ARTICLE

Synthesis and antioxidant activity evaluations of melatonin-based analogue indole-hydrazide/hydrazone derivatives

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Abstract

Melatonin (MLT) is a hormone synthesized from the pineal gland. It is a direct scavenger of free radicals, which is related to its capability to defend cells from oxidative stress. Recently MLT-related compounds are under investigation to establish which exhibit the maximum activity with the lowest side effects. In this study 5-chloroindole hydrazide/hydrazone derivatives were synthesized from 5-chloroindole-3-carboxaldehyde and phenyl hydrazine derivatives. All the compounds characterized and *in vitro* antioxidant activity was investigated against MLT and BHT. Most of the compounds showed strong inhibitory effect on the superoxide radical scavenging assay at 1 mM concentration (79 to 95%). Almost all the tested compounds possessed strong scavenging activity against the DPPH radical scavenging activity with IC_{50} values (2 to 60 μM). Lastly, compound 1j revealed stronger inhibitory activity against MLT in the LP inhibitory assay at 0.1mM concentration (51%) while the rest of the compounds showed moderate inhibition.

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

Introduction

Harmful effects of free radicals to the human body have been studied over the last decade. Overproduction of the free radicals can be responsible for tissue injuries that cause many health problems which include cancer, aging, heart diseases, neurological disorders, Alzheimer's disease, Huntington disease and so on. Free radicals are involved in normal physiological functions in required concentrations but excess formation of free radicals or/and decrease in antioxidant level leads to oxidative stress. The human body has several systems to eliminate the effect of oxidative stress by producing antioxidants, which are either naturally produced, or externally supplied through foods or supplements. Increasing the antioxidant intake can prevent diseases and lower the health problems[1,2].

Melatonin (MLT), the main secretory product of the pineal gland is a well-known antioxidant and free radical scavenger. It is a neurohormone produced from the amino acid tryptophan. Tan et al.[3] described that MLT scavenges a variety of reactive oxygen and nitrogen species including hydroxyl radical, hydrogen peroxide, singlet oxygen, nitric oxide and peroxynitrite anion.

Synthesis of MLT-related compounds such as MLT metabolites and synthetic analogues are under investigation to determine which exhibit the highest activity with the lowest side effects[4,5]. Many antioxidant activity studies of synthetic indole derivatives such as indole-3-propionic acid[6], indole amine-triazoles[7], stobadine[8] are present. Moreover, our group formerly identified the antioxidant activity of MLT anaologue indole derivatives such as 2-phenylindoles[9], and indole-3-propionamides[10]. Recently, we observed the relationship between aldose reductase and superoxide dismutase inhibition capacities of indole-based analogs of MLT derivatives[11].

Research has proved that the indole ring in the MLT molecule is the reactive centre dealing with oxidants due to its high resonance stability and very low activation energy barrier towards free radical reactions[3,12,13]. In our earlier studies, new MLT-based analogues with changes in the 5-methoxy and 2-acylaminoethyl groups of MLT were synthesized and tested for their *in vitro* antioxidant

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potency in the DPPH, superoxide dismutase and lipid peroxidation (LP) assays[14]. With a few exceptions, most of the compounds tested showed significant antioxidant activity at concentrations comparable with or much higher than that of MLT. We also found that N-methylindole derivatives of MLT analogue hydrazide/hydrazone derivatives[15] have protective effect against membrane lipid peroxidation (LP) and determining inhibitory effect hemolysis of human erythrocytes and showed potent antioxidant activity, even higher than MLT.

Since the modifications on the MLT molecule resulted in a set of compounds having different physical property and different substitution at the indole nucleus, it was hoped to have better SAR discussion in view of the effect of substituents with different electronic properties on the antioxidant activity. On the other hand, carbonyl scavenger activity of hydrazine/hydrazone derivatives gave the idea that antioxidant MLT and hydrazine-containing analogues are good candidates against LP in cooperation with antioxidant and carbonyl scavenger activities. It is known that oxidative stress induces LP of cellular membranes resulting in the generation of reactive carbonyl compounds (RCC) that react rapidly with free amino groups resulting with oxidative stress-associated diseases[16]. Some of the suggested metabolism products of synthesized MLT analogue compounds might be hydrazine and aldehyde derivatives. Galvani et al.[17] showed that Phenylhydrazine reacts with RCCs which facilitates the break of the covalent bond RCC–protein, and the binding of RCCs on hydrazine, thus forming hydrazone. The chemical combination between hydrazine and ketones or aldehydes and the formation of hydrazones is a characteristic of the carbonyl scavenger effect of these molecules.

All previous results showed that halogenated aromatic side chain resulted in much more active compounds than 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 radicals. These results prompted us to synthesize more indol-3-aldehyde hydrazone and hydrazide derivatives. With this study based on MLT, new indole imines were developed. These chemically significant modulations of the lead structure were made at mainly two different points (Figure 1): the methoxy group at the 5-position of the indole ring (modification I) and 2-N-acetylaminoethyl side chain including formation of imine (Modification II).

Twenty-one MLT analogue indole hydrazide/hydrazone derivatives were synthesized (Figure 2) and antioxidant activity was investigated *in vitro* by measuring DPPH, superoxide radical scavenging and LP inhibitory activities (Table 1). The results were compared with MLT and BHT. All the analogue compounds except previously synthesized 1a[18] and 1u[19], were characterized on the basis of ^{1}H and ^{13}C NMR, Mass, FT-IR spectra and elemental analysis.

Experimental

Chemistry

Melting points of the compounds were determined with a Buchi SMP-20 apparatus and uncorrected. 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). HPLC studies were determined on Waters LC Module Plus. All spectral analysis was performed at Ankara University, Faculty of Pharmacy, Central Laboratory. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), ascorbic acid (AA), xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), sitocrom c, malondialdehyde (MDA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The chemical reagents used in synthesis were purchased from Sigma (Germany) and Aldrich (US).

The target imines derived from 5-chloro-1*H*-indole-3-carboxaldehyde 1 and appropriate hydrazine or hydrazide derivatives using simple reaction strategies. For the synthesis of compounds 1a-t, a similar method of Kidwai et al.[20] has been used by heating phenyl hydrazine derivatives and compound 1 in the presence of ethanol. The hydrazones 1u and 1v were also prepared from the reaction of equimolar amounts of hydrazide (isonicotinic hydrazide for 1u, anisic acid hydrazide for 1v) with compound 1 in ethanol. Finally, N,N'-bis-(5-chloro-1*H*-indole-3-ylmethylene)hydrazine (1y) was synthesized using equimolar amounts of hydrazine hydrate with compound 1 in the presence of ethanol. All the new compounds were characterized on the basis of spectral data except 1a[18] and 1u[19]. Compound 1u was previously described in view of its electrochemical behaviour by our group. ^{13}C spectra of compounds 1g and 1h showed some

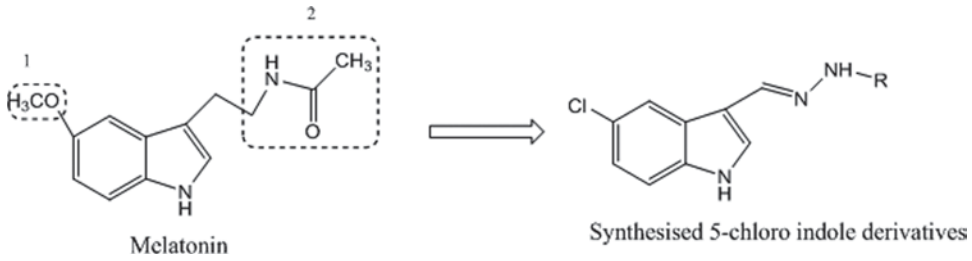

Figure 1. Modifications made on MLT molecule.

difficulty of interpreting fluorine magnetic shielding. These spectra are not given.

General procedure for the synthesis of 5-Chloro-1H-indole-3-carboxaldehyde phenylhydrazones (1a-t)

5-Chloro-1*H*-indole-3-carboxaldehyde (0.1mmol) was reacted with phenyl hydrazine or its derivatives (0.13mmol) in 10 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 and washed with cold EtOH to give 1a-t with 10–92% yield.

5-Chloro-1H-indole-3-carboxaldehyde (2,4-dimethylphenyl) hydrazone 1b

Yield 77.8%, m.p 148–149°C; ^{1}H NMR (400 MHz): 2.19 (6H, d), 6.86 (1H, s), 6.96 (1H, d), 7.18-7.21 (2H, dd), 7.44 (1H, d), 7.71 (1H, d), 8.23 (1H, d), 8.31 (1H, s), 8.98 (1H, s, hydrazine-NH) 11.53 (1H, s, indole-NH); ^{13}C NMR: 17.38, 20.06, 111.14, 112.54, 113.18, 120.21, 120.57, 122.03, 124.31, 125.16, 126.15, 127.04, 128.53, 130.78, 135.37, 141.63 (azomethine-C): ESI mass m/z 298 (M+1, %100), 300 (M+2); Analysis for $C_{17}H_{16}ClN_3$; Calcd: C; 68.57, H; 5.42, N; 14.11. Found: C; 68.87, H; 5.08, N; 13.51. FT-IR (KBr) cm^{-1} 1609 C=N (azomethine) stretch band, 3316 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (2,3-dimethylphenyl) hydrazone 1c

Yield 80.5%, m.p 145–146°C; ^{1}H NMR (400 MHz): 2.12 (3H, s), 2.23 (3H, s), 6.60 (1H, d), 7.04 (1H, t), 7.18-7.21 (1H, dd), 7.26 (1H, dd), 7.45 (1H, d), 7.72 (1H, s), 8.23 (1H, d), 8.35 (1H, d), 9.07 (1H, s, hydrazine-NH) 11.55 (1H, s,

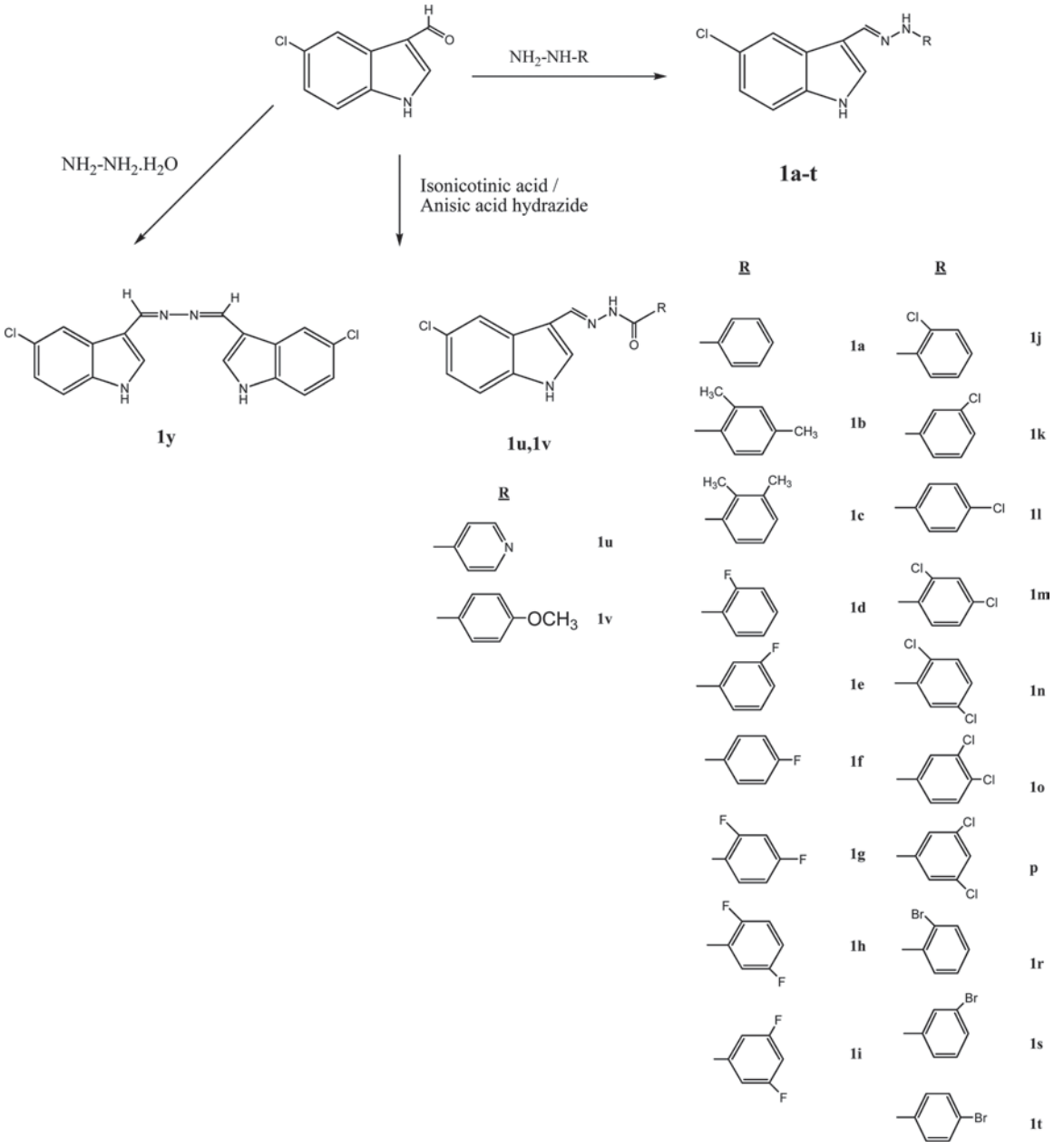

Figure 2. Synthetic pathway of melatonin analogues.

Table 1. Antioxidant activity results of synthesized compounds.

	Superoxide radical inhibition (%)		DPPH scavenging activity	LP (% inhibition)
Compounds[a]	1 mM	0.1 mM	IC_{50} μM	0.1 mM
1a	64 ± 3.0	P	42 ± 2	20.68 ± 0.55
1b	P	P	60 ± 2	31.03 ± 0.71
1c	P	P	60 ± 1	34.48 ± 0.36
1d	39 ± 7.5	P	19 ± 2	37.93 ± 0.25
1e	7.0 ± 1.5	P	21 ± 1	37.93 ± 0.33
1f	NA	NA	55 ± 4	20.68 ± 0.44
1g	P	P	22 ± 3	41.38 ± 0.29
1h	93 ± 2.4	P	20 ± 2	44.83 ± 0.36
1i	45 ± 2.0	NA	22 ± 1	41.37 ± 0.28
1j	79 ± 2.5	NA	2 ± 0.4	51.72 ± 0.22
1k	88 ± 4.8	P	24 ± 2	37.93 ± 0.12
1l	46 ± 1.7	P	23 ± 1	41.37 ± 0.19
1m	93 ± 3.0	P	15 ± 2	37.93 ± 0.84
1n	91 ± 2.0	P	28 ± 1	27.58 ± 0.29
1o	89 ± 5.2	NA	20 ± 4	27.58 ± 0.52
1p	95 ± 1.0	NA	20 ± 2	31.03 ± 0.55
1r	86 ± 4.5	P	30 ± 5	31.03 ± 0.61
1s	81 ± 5.7	P	19 ± 3	34.48 ± 0.54
1t	88 ± 3.5	P	24 ± 1	34.48 ± 0.32
1u	P	P	650 ± 19	44.82 ± 0.54
1v	P	25 ± 1.2	—	27.58 ± 0.60
1y	P	NA	—	41.38 ± 0.56
Melatonin	80 ± 1.5	20 ± 1.4	780 ± 25	46 ± 1.4
BHT	—	—	15 ± 2	—

P= prooxidant, NA = no activity.
[a]Compounds were diluted with DMSO (solvent showed no antioxidant activity).

indole-NH); ^{13}C NMR: 12.61, 20.16, 109.42, 112.51, 113.19, 118.56, 119.93, 120.56, 122.06, 124.34, 125.15, 125.85, 128.67, 135.37, 135.71, 136.05, 143.79 (azomethine-C): ESI mass m/z 298 (M+1, %100), 300 (M+2); Analysis for $C_{17}H_{16}Cl\ N_3$; Calcd: C; 68.57, H; 5.42, N; 14.11. Found: C; 69.00, H; 5.73, N; 13.84. FT-IR (KBr) cm^{-1} 1582 C=N (azomethine) stretch band, 3410 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (2-fluorophenyl) hydrazone 1d

Yield 48%, m.p 198–199°C; ^{1}H NMR (400 MHz): 6.69-6.74 (1H, m), 7.10-7.18 (2H, m), 7.19 (1H, d), 7.22 (1H, d), 7.41-7.47 (1H, m), 7.75 (1H, d), 8.22 (1H, s), 8.34 (1H, s), 9.82 (1H, s, hydrazine-NH) 11.59 (1H, s, indole-NH); ^{13}C NMR: 112,94, 113,51, 114,04, 115,50, 115,68, 118,09, 118,16, 121,31, 122,94, 125,27, 125,75, 125,86, 130,08, 134,84, 134,94, 136,16, 137,94 (azomethine-C): ESI mass m/z 288 (M+1, %100), 290 (M+2); Analysis for $C_{15}H_{11}FClN_3$; Calcd: C; 62.62, H; 3.85, N; 14.60. Found: C; 62.70, H; 3.96, N; 14.11. FT-IR (KBr) cm^{-1} 1622 C=N (azomethine) stretch band, 3751 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (3-fluorophenyl) hydrazone 1e

Yield 35.1%, m.p 176–177°C; ^{1}H NMR (400 MHz): 6.48 (1H, t), 6.79 (2H, d), 7.23 (2H, t), 7.46 (1H, d), 7.77 (1H, s, -CH), 8.16 (2H, d), 10.18 (1H, s, hydrazine-NH) 11.59 (1H, s, indole-NH); ^{13}C NMR: 98.17, 98.43, 104.25, 104.46, 108.17, 112.83, 114.05, 121.24, 122.93, 125.26, 125.84, 130.09, 131.32, 131.42, 136.14, 136.22, 148.57, 148.68 (azomethine-C), 162.94, 165.32: ESI mass m/z 288 (M+1, %100), 290 (M+2); Analysis for $C_{15}H_{11}FClN_3$; Calcd: C; 62.62, H; 3.85, N; 14.60. Found: C; 62.71, H; 3.85, N; 13.37. FT-IR (KBr) cm^{-1} 1616 C=N (azomethine) stretch band, 3675 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (4-fluorophenyl) hydrazone 1f

Yield 35.4%, m.p 153–154°C; ^{1}H NMR (400 MHz): 6.95-6.98 (2H,m), 7.04-7.09 (2H, t), 7.15-7.18 (1H, dd), 7.42 (1H, s), 7.70 (1H, d), 8.06 (1H, s, -CH), 8.17 (1H, d), 9.85 (1H, s, hydrazine-NH) 11.51 (1H, s, indole-NH); ^{13}C NMR: 112.75, 112.81, 113.06, 113.99, 116.21 116.43, 121.25, 122.87, 125.14, 125.85, 129.58, 135.29, 136.12, 143.47 (azomethine-C), 154.82, 157.13: ESI mass m/z 288 (M+1, %100), 290 (M+2); Analysis for $C_{15}H_{11}FClN_3$; Calcd: C; 62.62, H; 3.85, N; 14.60. Found: C; 62.46, H; 3.70, N; 14.44. FT-IR (KBr) cm^{-1} 1507 C=N (azomethine) stretch band, 3462 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (2,4-difluorophenyl) hydrazone 1g

Yield 87.2%, m.p 128–129°C; ^{1}H NMR (400 MHz): 7.03-7.08 (1H, m), 7.14-7.20 (2H, m), 7.33-7.43 (2H, m), 7.72 (1H, d), 8.15 (1H, d), (1H, s), 8.30 (1H, s), 9.72 (1H, s, hydrazine-NH) 11.57 (1H, s, indole-NH); ESI mass m/z

306 (M+1, %100), 308 (M+3); Analysis for $C_{15}H_{10}F_2ClN_3$; Calcd: C; 58.93, H; 3.30, N; 13.75. Found: C; 58.87, H; 3.07, N; 13.35. FT-IR (KBr) cm^{-1} 1517 C=N (azomethine) stretch band, 3452 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (2,5-difluorophenyl) hydrazone 1h

Yield 10.4%, m.p 152–153°C; ^{1}H NMR (400 MHz): 6.40-6.45 (1H, m), 6.57-6.60 (2H, dd), 7.20-7.23 (1H, dd), 7.47 (1H, d), 7.81(1H, s, -CH), 8.14 (1H, d), 9.78 (1H, s, hydrazine-NH) 10.40 (1H, s, indole-NH), 11.64 (1H, s, indole-NH); ESI mass m/z 306 (M+1, %100), 308 (M+3); Analysis for $C_{15}H_{10}F_2ClN_3$; Calcd: C; 58.93, H; 3.30, N; 13.75. Found: C; 58.78, H; 3.17, N; 13.48%. FT-IR (KBr) cm^{-1} 1517 C=N (azomethine) stretch band, 3407 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (3,5-difluorophenyl) hydrazone 1i

Yield 90%, m.p 159–160°C; ^{1}H NMR (400 MHz): 6.47-6.48 (1H, m), 7.07-7.23 (3H, m), 7.46 (1H, d), 7.79 (1H, s), 8.17 (1H, d), 8.36 (1H, s), 10.12 (1H, s, hydrazine-NH) 10.66 (1H, s, indolamine); ^{13}C NMR: 92.49, 92.76, 93.02, 94.48, 94.76, 112.51, 114.13, 116.40, 121.17, 123.01, 125.39, 125.79, 130.68, 136.15, 137.33, 148.38, 148.74, 148.98, 149.14, 149.27 (azomethine -C), 150.69, 153.38, 162.92, 163.09, 165.33, 165.49; ESI mass m/z 306 (M+1, %100), 308 (M+3); Analysis for $C_{15}H_{10}F_2ClN_3$; Calcd: C; 58.93, H; 3.30, N; 13.75. Found: C; 59.12, H; 3.98, N; 13.47. FT-IR (KBr) cm^{-1} 1635 C=N (azomethine) stretch band, 3428 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (2-chlorophenyl) hydrazone 1j

Yield 62.3%, m.p 144–145°C; ^{1}H NMR (400 MHz): 6.73-6.77 (1H, m), 7.20-7.23 (1H, dd), 7.31 (2H, t), 7.48 (2H, t), 7.77 (1H, s, -CH), 8.20 (1H, d), 8.48 (1H, s), 9.48 (1H, s, hydrazine-NH) 11.64 (1H, s, indole-NH); ^{13}C NMR: 112.78, 113.59, 114.10, 116.40, 119.23, 121.23, 122.99, 125.34, 125.85, 128.80, 130.01, 130.38, 136.17, 138.96, 142.63 (azomethine -C); ESI mass m/z 304 (M^+, %100), 306 (M+2).; Analysis for $C_{15}H_{11}Cl_2N_3$; Calcd: C; 59.23, H; 3.65, N; 13.81. Found: C; 59.29, H; 3.42, N; 13.67. FT-IR (KBr) cm^{-1} 1598 C=N (azomethine) stretch band, 3323 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (3-chlorophenyl) hydrazone 1k

Yield 57.2%, m.p 123–124°C; ^{1}H NMR (400 MHz): 6.69-6.72 (2H, tt), 6.90-6.92 (1H, dd), 7.04 (1H, s), 7.19-7.26 (2H, m), 7.77 (1H, s), 8.11 (1H, s), 8.18 (1H, d), 10.16 (1H, s, hydrazine-NH) 11.59 (1H, s, indole-NH); ^{13}C NMR: 110.69, 111.27, 112.83, 114.09, 117.68, 121.27, 122.96, 125.30, 125.86, 130.16, 131.46, 134.54, 136.15, 136.43, 148.08 (azomethine -C); ESI mass m/z 304 (M^+, %100), 306(M+2); Analysis for $C_{15}H_{11}Cl_2N_3$; Calcd: C; 59.23, H; 3.65, N; 13.81. Found: C; 59.29, H; 3.48 N; 13.52. FT-IR (KBr) cm^{-1} 1594 C=N (azomethine) stretch band, 3444 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (4-chlorophenyl) hydrazone 1l

Yield 76.8%, m.p 185–186°C; ^{1}H NMR (400 MHz): 7.00 (2H, m), 7.18-7.21 (3H, m), 7.27 (1H, d), 7.45 (1H, d), 7.75 (1H, s), 8.10 (1H, s, -CH), 8.18 (1H, d), 10.06 (1H, s, hydrazine-NH) 11.58 (1H, s, indole-NH); ^{13}C NMR: 112.11, 112.52, 113.24, 120.42, 120.67, 122.14, 124.42, 125.03, 128.82, 129.14, 135.20, 135.34, 144.78 (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; 3.65, N; 13.81. Found: C; 59.03, H; 3.41 N; 13.62. FT-IR (KBr) cm^{-1} 1595 C=N (azomethine) stretch band, 3448 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (2,4-dichlorophenyl) hydrazone 1m

Yield 57%, m.p 197–1986°C; ^{1}H NMR (400 MHz): 7.20-7.23 (1H, dd), 7.37-7.48 (4H, m), 7.79 (1H, s, -CH), 8.15 (1H, d), 8.49 (1H, s), 9.63 (1H, s, hydrazine-NH) 11.67 (1H, s, indole-NH); ^{13}C NMR: 112.60, 114.19, 114.51, 116.84, 121.15, 121.69, 123.09, 125.46, 125.81, 128.88, 129.27, 130.81, 136.22, 139.84, 141.80 (azomethine -C); ESI mass m/z 338 (M^+, %100), 340 (M+2, %100), 342 (M+4); Analysis for $C_{15}H_{10}Cl_3N_3$; Calcd: C; 53.20, H; 2.98, N; 12.42. Found: C; 54.34, H; 2.82 N; 12.12. FT-IR (KBr) cm^{-1} 1596 C=N (azomethine) stretch band, 3442 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (2,5-dichlorophenyl) hydrazone 1n

Yield 68%, m.p 171–172°C; ^{1}H NMR (400 MHz): 6.76-6.79 (1H, dd), 7.21-7.24 (1H, dd), 7.35 (1H, d) 7.43-7.49 (2H, m), 7.82 (1H, s), 8.18 (1H, d), 8.52 (1H, s), 9.77 (1H, s, hydrazine-NH) 11.68 (1H, s, indole-NH); ^{13}C NMR: 112.54, 112.93, 114.22, 114.95, 118.41, 121.21, 123.07, 125.52, 125.86, 130.95, 131.43, 133.43, 136.22, 140.15, 143.74 (azomethine -C); ESI mass m/z 338 (M^+, %100), 340 (M+2, %100), 342 (M+4); Analysis for $C_{15}H_{10}Cl_3N_3$; Calcd: C; 53.20, H; 2.98, N; 12.42. Found: C; 52.50, H; 2.78 N; 12.48. FT-IR (KBr) cm^{-1} 1596 C=N (azomethine) stretch band, 3428 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (3,4-dichlorophenyl) hydrazone 1o

Yield 65%, m.p 142–143°C; ^{1}H NMR (400 MHz): 66.89–6.92 (1H, dd), 7.15-7.20 (2H, m), 7.42 (2H, t), 7.77 (1H, d), 8.10 (2H, s, CH), 8.14 (1H, d), 10.24 (1H, s, hydrazine-NH), 11.59 (1H, s, indole-NH); ^{13}C NMR: 112.29, 112.63, 112.77, 114.11, 118.82, 121.20, 123.00, 125.36, 125.80, 130.47, 131.64, 132.24, 136.15, 137.12, 146.63 (azomethine -C); ESI mass m/z 338 (M^+, %100), 340 (M+2, %100), 342 (M+4); Analysis for $C_{15}H_{10}Cl_3N_3$; Calcd: C; 53.20, H; 2.98, N; 12.42. Found: C; 52.90, H; 2.92 N; 12.24. FT-IR (KBr) cm^{-1} 1593 C=N (azomethine) stretch band, 3426 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (3,5-dichlorophenyl) hydrazone 1p

Yield 61%, m.p 168–169°C; ^{1}H NMR (400 MHz): 6.79 (1H, t), 6.95 (2H, d), 7.20-7.23 (1H, dd), 7.47 (1H, d), 7.82 (1H, s), 8.15 (2H, t), 10.37 (1H, s, hydrazine-NH), 11.64 (1H, s, indole-NH); ^{13}C NMR: 109.36, 111.71, 113.38, 116.03, 120.40, 122.24, 124.64, 125.03, 129.96, 134.54, 135.37, 136.95, 147.90 (azomethine-C); ESI mass m/z 338 (M^+, %100), 340 (M+2, %100), 342 (M+4); Analysis for $C_{15}H_{10}Cl_3N_3$; Calcd: C; 53.20, H; 2.98, N; 12.42. Found: C; 52.90, H; 2.92 N; 12.24. FT-IR (KBr) cm^{-1} 1594 C=N (azomethine) stretch band, 3453 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (2-bromophenyl) hydrazone 1r

Yield 77.3%, m.p 183–184°C; ^{1}H NMR (400 MHz): 6.64-6.68 (1H, m), 7.18-7.21 (1H, dd), 7.30-7.34 (1H, m), 7.44-7.47 (3H, m), 7.74 (1H, s, -CH), 8.19 (1H, d), 8.48 (1H, s), 9.21 (1H, s, hydrazine-NH), 11.5 (1H, s, indole-NH); ^{13}C NMR: 106.41, 112.77, 114.08, 114.12, 119.99, 121.24, 123.02, 125.40, 125.87, 129.34, 130.39, 133.24, 136.18, 139.14, 143.62 (azomethine-C); ESI mass m/z 348 (M^+, %100), 350 (M+2, %100), 352 (M+4); Analysis for $C_{15}H_{11}ClBrN_3$; Calcd: C; 51.68, H; 3.18, N; 12.05. Found: C; 51.32, H; 3.22 N; 11.85. FT-IR (KBr) cm^{-1} 1588 C=N (azomethine) stretch band, 3447 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (3-bromophenyl) hydrazone 1s

Yield 92%, m.p 133–134°C; ^{1}H NMR (400 MHz): 6.82-6.85 (1H, dd), 6.93-6.96 (1H, m), 7.15-7.22 (3H, m), 7.46 (1H, d), 7.77 (1H, d), 8.10 (1H, s, -CH), 8.18 (1H, d), 10.14 (1H, s, hydrazine-NH), 11.59 (1H, s, indole-NH); ^{13}C NMR: 111.02, 112.81, 114.07, 114.18, 120.53, 121.26, 122.94, 123.18, 125.28, 125.83, 130.14, 131.75, 136.13, 136.43, 148.18 (azomethine-C); ESI mass m/z 348 (M^+, %100), 350 (M+2, %100), 352 (M+4); Analysis for $C_{15}H_{11}ClBrN_3$; Calcd: C; 51.68, H; 3.18, N; 12.05. Found: C; 51.34, H; 3.03 N; 11.84. FT-IR (KBr) cm^{-1} 1591 C=N (azomethine) stretch band, 3446 N-H stretch band.

5-Chloro-1H-indole-3-carboxaldehyde (4-bromophenyl) hydrazone 1t

Yield 80.3%, m.p 167–168°C; ^{1}H NMR (400 MHz): 6.93 (2H, d), 7.16-7.19 (1H, dd), 7.34-7.43 (3H, m), 7.72 (1H, d), 8.08 (1H, s, -CH), 8.15 (1H, d), 10.05 (1H, s, hydrazine-NH), 11.55 (1H, s, indole-NH); ^{13}C NMR: 108.96, 112.90, 113.87, 114.04, 121.22, 122.94, 125.24, 129.97, 132.43, 136.09, 136.13,145.92 (azomethine -C); ESI mass m/z 348 (M^+, %100), 350 (M+2, %100), 352 (M+4); Analysis for $C_{15}H_{11}ClBrN_3$; Calcd: C; 51.68, H; 3.18, N; 12.05. Found: C; 51.35, H; 3.04 N; 11.99. FT-IR (KBr) cm^{-1} 1594 C=N (azomethine) stretch band, 3422 N-H stretch band.

General procedure for the synthesis of compounds (1u-y)

A solution 5-chloro-1*H*-indole-3-carboxaldehyde (0.05 mmol) and izonicotinic acid hydrazide (for 1u) or anisic acid hydrazide (for 1v; 0.05 mmol), hydrazine hydrate (for 1y; 0.1 mmol) in 50 mL of EtOH was heated for 2.5 h (4h for 1y) on the hot water bath. On cooling, the precipitate was collected and washed with cold EtOH to give 1u-y with 20–45% yield.

N-(4-methoxybenzoyl)-N′-(5-chloro-1H-indolyl-3-methylene) hydrazone 1v

Yield 42%, m.p 249–250°C; ^{1}H NMR (400 MHz): 3.04 (3H, s), 7.07 (2H, d), 7.21-7.23 (1H, dd), 7.47 (1H, s), 7.92 (3H, t), 8.33 (1H, d), 8.59 (1H, s, -CH), 11.49 (1H, s, hydrazine-NH), 11.77 (1H, s, indole-NH); ^{13}C NMR: 56.10, 112.26, 114.10, 114.37, 121.87, 123.28, 125.69, 126.04, 126.61, 130.02, 132.34, 136.21, 144.53 (azomethine -C), 162.45 (C=O), 162.71; ESI mass m/z 328 (M+1, %100), 330 (M+3); Analysis for $C_{17}H_{14}ClN_3O_2$; Calcd: C; 62.30, H; 4.31, N; 12.82. Found: C; 62.82, H; 4.60 N; 12.09. FT-IR (KBr) cm^{-1} 1647 C=N (azomethine) stretch band, 3330 N-H stretch band.

N,N′-bis-(5-chloro-1H-indole-3-ylmethylene)hydrazine 1y

Yield 45.4%, m.p 285–286°C; ^{1}H NMR (400 MHz): 7.23-7.26 (2H, dd), 7.51 (2H, d), 8.02 (2H, s, -CH), 8.35 (2H, d), 8.93 (2H, s), 11.90 (2H, brs, indole-NH); ^{13}C NMR: 112.34, 114.27, 121.85, 123.29, 125.89, 126.41, 134.20, 136.33, 155.95 (azomethine -C); ESI mass m/z 355 (M^+, %100), 357 (M+2, %100), 359 (M+4); Analysis for $C_{18}H_{12}Cl_2N_4$; Calcd: C; 60.86, H; 3.41, N; 15.77. Found: C; 60.75, H; 3.28 N; 13.48. FT-IR (KBr) cm^{-1} 1646 C=N (azomethine) stretch band, 3420 N-H stretch band.

Measurement of E/Z isomer ratio

Mass spectra showed that compounds 1d, 1j and 1r which have o-halogenated phenyl ring in the molecule exist as *E* and *Z* isomers. The isomers could not separated by chromatographic methods. However the *E/Z* isomer ratio was measured by HPLC. Literature data[21,22] indicated that the major product might be (*E*)-isomer. Therefore (*E*)-isomer was assumed as major product (Compound 1d 99.5%, 1j 99.9% and 1r 91.71); the minor (*Z*)-isomer was detected in small amounts (Compound 1d 0.5%, 1j 0.09% and 1r 8.29).

A high-performance liquid chromatographic (HPLC) method was established for the enantiomeric determination[21,23] of compounds 1d, 1j and 1r. Experimentation was achieved under normal-phase chiral stationary phase liquid chromatography using the Chiralpak AS chiral column (250 mm × 4.6 mm). The optimized chromatographic conditions were n-hexane-isopropyl alcohol (87:13:1) as the mobile phase with a flow rate of 1mL/min, and detection at the wavelength of 254 nm. The column temperature was set at room temperature.

In vitro antioxidant activity studies

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 and BHT.

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)[24]. The stock solutions of the compounds were prepared at 10^{-2} M concentration in DMSO. A series of stock solution in DMSO were diluted to varying concentrations in 96-well microplates. Then, methanolic DPPH solution (100 μM) was added to each well. The plate was shaken and placed into the dark. After 30 min, the optical density of the solution was read at the wavelength 517 nm. The methanolic solution of DPPH served as a control. Percentage inhibition was calculated using the following formula:

$$\text{Radical scavenging activity \%} = \left(\left[\frac{A_{Control} - A_{Sample}}{A_{Control}}\right]\right) \times 100$$

Where: $A_{control}$: absorption of blank sample; A_{sample}: absorption of tested solution

A dose response curve was plotted to determine the IC_{50} values. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests and analyses were run in triplicate and averaged. The standard used in this assay was MLT and BHT.

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 inhibition of cytochrome c reduction[25,26]. 100 μl of 4 mM xanthine, 400 μl sitocrom c, 50mL of 50mM phosphate buffer (pH 7.8, 1mM 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 sitocrom c reduction decrease of the test group versus the control group after 1 min. Superoxide radical scavenging capacity was calculated as follows:

$$\text{Superoxide radical scavenging activity (\%)} = \frac{\left(A_{Control} - A_{Sample}\right)}{\left(A_{Control} - A_{blank}\right)} \times 100$$

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 Each experiment was triplicated. MLT was used as positive controls.

Assay of lipid peroxidation

The effect of synthesised MLT analogues on rat liver homogenate induced with $FeCl_2$-ascorbic acid and LP was determined by the method of modified Mihara et al.[27] 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 anaesthesia. 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 mmol malondialdehyde (MDA)/g tissue. A typical optimized assay mixture contained 0.5 mL of liver homogenate, 0.1mL 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 1mL of 0.6% TBA were added and shaken vigorously. The mixture was boiled for 30 min. After cooling, 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:

$$\text{Lipid peroxidation inhibitory activity (\%)} = \frac{\left(A_{Control} - A_{Sample}\right)}{\left(A_{Control} - A_{blank}\right)} \times 100$$

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. MLT was used as positive controls.

Results

The synthesized compounds were 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 and BHT. The results are shown in Table 1.

Ten of the synthesized compounds showed strong inhibitory effect on the superoxide radical scavenging assay at 1 mM concentration (79 to 95%), with the exception of compounds 1d, 1e, 1i, 1l and the ones that have pro-oxidant activity (1b, 1c, 1g, 1u, 1v and 1y). Rest of the tested compounds displayed higher antioxidant activity then that of MLT. Compound 1p demonstrated the best inhibitory activity with 95%. Compounds 1m, 1h, 1n, 1o, 1k, 1t, 1r, 1s and 1j followed compound 1p with the inhibitory concentrations of 93, 93, 91, 89, 88, 88, 86 and 79%, respectively. Most of the compounds showed pro-oxidant activity at 0.1 mM concentration.

Almost all the tested compounds possessed strong scavenging activity against the DPPH radical scavenging activity with IC_{50} values (2 to 60 μM). Compound that have anisic acid hydrazide 1v in the 3rd position of indole ring showed no free radical scavenging effect while Compound that have nicotinic acid hydrazide 1u in the 3rd position showed the lowest scavenging activity. Compound 1j established the best inhibitory activity with 2 μM IC_{50} value. Rest of the compounds showed very strong antioxidant activity compare to MLT. Compound

1j showed stronger antioxidant activity compare while compound 1m exhibited similar activity to BHT. Rest of the compounds which showed significantly good activity against MLT showed similar DPPH scavenging activity to BHT a well-known antioxidant with 15 μM IC_{50} value.

Lastly, compound 1j revealed stronger inhibitory activity against MLT in the LP inhibitory assay at 0.1mM concentration (51%). Compounds 1h, 1u, 1g, 1l, 1i, 1m, 1e, 1d, 1s and 1t followed compound 1j with the moderate inhibitory concentration of 44, 44, 41, 41, 41, 37, 37, 37, 34 and 23%, respectively.

Discussion

This study was performed to investigate the potential *in vitro* antioxidant effects of MLT-based analogues of indole hydrazide/hydrazone derivatives. A series 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 in DPPH scavenging and LP assays. The only exception was in DPPH assay, compounds that have anisic acid hydrazide 1v and 5-chloro-1*H*-indole 1y in the 3rd position of indole ring. These compounds were also found prooxidant in superoxide radical scavenging activity assay. This finding was found in agreement with our earlier studies[14,15].

Replacement the 5-methoxy group of MLT by Cl 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 methoxygroup in 5-position of the indole ring of MLT is still under investigation. Although by replacement of the methoxy group, the antioxidant capacity of the molecule may be enhanced[3], we did not observed significant difference between compounds bearing Br or H in the 5th position of the indole ring in our earlier studies[14,15]. In this study, addition of Cl in the 5th position did not change the earlier suggestions. Similar to our findings, Poeggeler et al.[29,30] showed that the MLT derivatives bearing Cl in the 5th position performed parallel activity to MLT.

According to the DPPH scavenging and LP assays results, although the most active compound was 1j (o-Cl substitution on phenyl), in generally the active compounds were established as di-flouro substituted (1g-1i) in LP assay, di-flouro and di-chloro substituted (1g-1p) in DPPH assay. The results of two assays were found similar but the superoxide dismutase assay showed some differences. The best activity was observed with compound 1p (3,5 dichloro substitution on phenyl) but there was no significant activity obtained with compound 1a-1g in superoxide dismutase assay.

MLT has redox properties because of the presence of an electron-rich aromatic ring system, which allows the indoleamine to easily function as an electron donor[12,13,31]. It is possible that making the indole ring more stable electronically helped to act as a better electro donor. MLT scavenges the radicals via nitrogen centred radical, the indolyl (or melatonyl) cation radical[32]. 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 in the synthesized compounds.

Conclusions

The present work aimed to synthesize, characterize and investigate the potential antioxidant effects of indole-based MLT analogue hydrazide/hydrazone derivatives.

This study proposes a new approach for the *in vitro* antioxidant activity properties and structure activity relationship of 3- and 5-substituted indole ring as MLT analogue.

It is possible that the synthesized compounds may undergo some hydrolysis under the in vivo conditions of the LP assay. However, in our earlier studies, *in vitro* electrochemical data showed that oxidation starts on the nitrogen atom in the indole ring which leads finally to the hydroxylation of the benzene ring[12,13,19,31]. Regardless of their medicinal importance, few biological systems metabolize hydrazides and hydrazones and not much is known about those that attack the double bond of the hydrazone groups. Peptidylglycine hydroxylase (EC 1.14.17.3)[33] and glutamine transaminase (EC 2.6.1.15)[34] are the recognized enzymes that react with hydrazones. Since hydrazones are not their physiological substrates, how biological systems employ hydrazones remains unclear. Hydrazones can be chemically hydrolyzed to relevant hydrazines and carbonyl compounds under acidic conditions. However, the biological system metabolizes hydrazones via the more complex NAD^+-dependent oxidation reaction[35]. This is a remarkable difference between the chemical and biological systems for degrading hydrazones.

In conclusion, majority of synthesized indole derivatives related to MLT showed significant antioxidant activity in three *in vitro* assays. In general, lack of methoxy group and introduction of Cl in the 5-position did not effect the antioxidant capacity of the new indole derivatives infect the in vitro assays showed that many of the compounds were much more active than MLT itself. 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. For the antioxidant activity of melatonin analogue indole derivatives, not only the indole ring is

important, but so is the side chain containing the amide group.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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