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Short communication

Synthesis and primary cytotoxicity evaluation of new 5-nitroindole-2,3-dione derivatives

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Abstract

A new series of 5-nitro-1*H*-indole-2,3-dione-3-thiosemicarbazones (**3a**–**k**) obtained by condensation of 5-nitro-1*H*-indole-2,3-dione (**1**) with N-substituted-thiosemicarbazides (**2a**–**k**) were treated with morpholine or piperidine and formaldehyde to yield 1-morpholino/piperidinomethyl-5-nitroindole-2,3-dione-3-thiosemicarbazones (**4a**–**m**). The structures of all the compounds were determined by analytical and spectral (IR, ^{1}H-NMR, EIMS) methods. Compounds **3b**, **3c**, **3f**, **3k**, **4a**, **4c**, **4f** and **4l** chosen as prototypes were evaluated in the National Cancer Institute's 3-cell line, one dose in vitro primary cytotoxicity assay. All the compounds that passed the criteria for activity in this assay were scheduled automatically for evaluation against the full panel of 60 human tumour cell lines at a minimum of five concentrations at 10-fold dilutions. Sulphorhodamine B (SRB) protein assay was used to estimate cell stability or growth. The most active compound was found to be 1-morpholinomethyl-5-nitroindole-2,3-dione-3-*N*-(chlorophenyl)thiosemicarbazone (**4l**). This compound demonstrated the most marked effects in the National Cancer Institute's 60 human tumour cell line in vitro screen on a non-small cell lung cancer cell line (HOP-62, $\log_{10}$ GI_{50} value < -8.00) and on leukaemia cell lines (HL-60(TB), $\log_{10}$ GI_{50} value -6.30; MOLT-4, $\log_{10}$ GI_{50} value -6.18).

Keywords: 5-Nitro-1*H*-indole-2,3-dione; Thiosemicarbazones; N-Mannich bases; Cytotoxicity

1. Introduction

Several authors found that isatin-β-thiosemicarbazone (1*H*-indole-2,3-dione-3-thiosemicarbazone) and its N-Mannich bases were active against various viruses [1–3]. *N*-Methylisatin-β-thiosemicarbazone (methisazone) (**I**) was one of the first antiviral compounds used in clinical practice. This drug plays an important role as a prophylactic agent against several viral diseases. Compound **I** and isatin-β-thiosemicarbazone (**II**) are active against poxviruses, including variola and vaccinia [4]. Furthermore, inhibition of reverse transcriptase by *N*-methylisatin-β,4′,4′-diethylthiosemicarbazone (**III**) has been reported by Ronen et al. [5–7]. This compound is known to be useful for the treatment of human and animal diseases caused by oncoviruses and foamy viruses [8]. Substituted oxoisoindolines **IV** have been proved to be potent cytotoxic agents in suspended cells derived from solid uterine tumours by Hall et al. [9–11]. Bis(heteroaryl)piperazine derivative bearing the indoline structure, delaviridine **V** have been approved for use in combination with nucleoside reverse transcriptase inhibitors, such as 2′,3′-deoxy-3′-azidothymidine (AZT), for the treatment of human immunodeficiency virus (HIV) [12] (Fig. 1).

In view of these observations, we report here the synthesis, structural determination and primary cytotoxicity evaluation of 5-nitroindole-2,3-dione-3-thiosemicarbazone derivatives.

2. Chemistry

5-Nitro-1*H*-indole-2,3-dione (**1**) was reacted with N-substituted-thiosemicarbazides (**2a**–**k**) in ethanol containing a catalytic amount of sulphuric acid, to give the corresponding 5-nitro-1*H*-indole-2,3-dione-3-thiosemicarbazones (**3a**–**k**). 1-Morpholino/piperidinomethyl-5-

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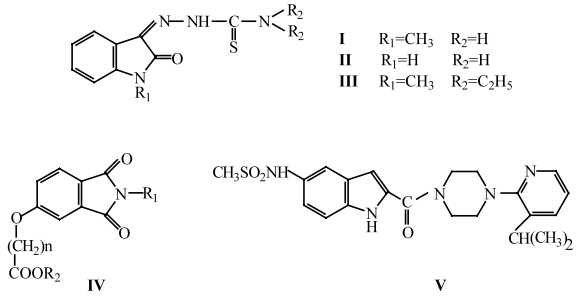

Fig. 1.

nitroindole-2,3-dione-3-thiosemicarbazones (**4a**–**m**) were synthesized from consecutive treatment of **3a**–**k** with formaldehyde solution and morpholine or piperidine [13,14] (Fig. 2) The structures of the compounds were confirmed by analytical and spectral data (IR, ^{1}H-NMR, EIMS) (Tables 1 and 2).

The IR spectra of **3** showed two separate bands resulting from the NH stretchings of the indole and thioamide functions in the 3350–3286 and 3197–3161 cm^{-1} regions. The lactam C=O and the thioamide C=S stretchings were observed in the 1718–1691 and 1205–1149 cm^{-1} regions, respectively. In the IR spectra of **4**, the thioamide NH stretching was observed in the 3350–3195 cm^{-1}, the lactam C=O and the thioamide C=S stretchings were observed in the 1708–1696 and 1175–1139 cm^{-1} regions, respectively [15–17]. The ^{1}H-NMR spectra of **3** displayed the NH protons of the thiosemicarbazone moiety (δ 9.54–11.05 and 12.34–12.60 ppm) and the indole NH proton (δ 11.74–11.80 ppm) as three separate singlets [13,15]. Observation of only two NH signals assigned to the thiosemicarbazone moiety (δ 9.73–11.15 and 12.33–12.47 ppm) and of a singlet due to N–CH_2–N function of morpholino/piperidinomethyl residue (δ 4.57–4.60 ppm) in the ^{1}H-NMR spectra of **4** provided support for N-Mannich base formation [18]. The ^{1}H-NMR spectra of **3** and **4** displayed the indole C_7–H as a doublet at δ 7.09–7.52 ppm. Indole C_6–H, being deshielded due to the electron attracting effect of the nitro function at 5-position, appeared at δ 8.24–8.33 and 8.28–8.35 ppm as a double doublet. Indole C_4–H, experiencing a deshielding effect, due to the inductive effects of the C=N function and the electron attracting effect of the nitro group resonated as a doublet at δ 8.56–8.75 ppm [19–21]. No duplication of indole C_7–H (δ 7.50 ppm) or C_4–H (δ 8.75 ppm) signals were observed while indole C_6–H resonance (δ 8.35 ppm) was observed as a doublet in **4h**. The EI mass specta of **3** showed molecular ions of different intensity which confirmed their molecular weights. The major fragmentation pathway involved the cleavage of the exocyclic NH–CS and endocyclic NH–CO bonds. EIMS of **4** showed the same fragmentation pattern observed in the precursor thiosemicarbazones. Further fragments peculiar to the morpholine or piperidine moiety were also observed in the spectra of **4** [22]. The proposed fragmentation pattern of **4d** is depicted in Fig. 3.

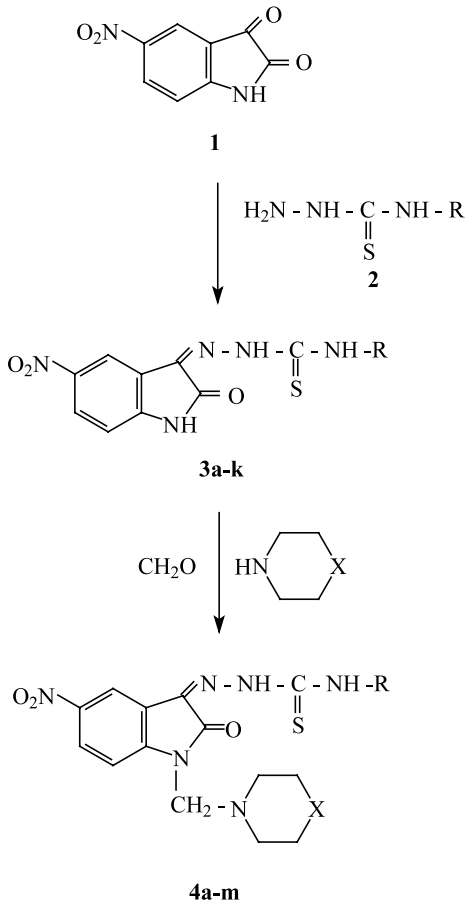

Fig. 2. Synthesis if **3a**–**k** and **4a**–**m**.

3. Cytotoxicity evaluation and discussion

The 5-nitroindole-2,3-dione-3-thiosemicarbazone derivatives **3b**, **3c**, **3f**, **3k**, **4a**, **4c**, **4f** and **4l** chosen as prototypes were evaluated in the 3-cell line panel consisting of NCI-H460 (Lung), MCF7 (Breast), and SF-268 (CNS). Primary anticancer assay was performed in accordance with the protocol of the Drug Evaluation Branch, National Cancer Institute, Bethesda [23–25]. The compounds were added at a single concentration (10^{-4} M) and the culture was incubated for 48 h. End point determinations were made with a protein binding dye, sulphorhodamine B (SRB). Results for each compound were reported as the percent of growth of the treated cells when compared to the untreated control cells. All the compounds which reduced the growth of any one of the cell lines to 32% or less (negative numbers indicate cell kill) were passed on for evaluation in the full panel of 60 human tumour cell lines (Table 3). The cytotoxic and/or growth inhibitory effects of the compounds were tested in vitro against the full panel of 60

Table 1
Physicochemical data of **3a**–**k**

Compound	R	Yield (%)	M.p. (°C)	Formula (M.W.)	Analysis Calc./Found		
					C	H	N
3a	CH_3	98	295	$C_{10}H_9N_5O_3S$ (279.28)	43.00 42.53	3.24 3.28	25.07 24.36
3b	C_2H_5	58	266	$C_{11}H_{11}N_5O_3S$ (293.31)	45.04 44.49	3.78 3.91	23.87 23.71
3c	CH_2–CH=CH_2	97	235–236	$C_{12}H_{11}N_5O_3S \cdot H_2O$ (323.34)	44.57 45.02	4.05 3.72	21.65 21.97
3d	*n*-C_4H_9	89	237–239	$C_{13}H_{15}N_5O_3S$ (321.36)	48.58 48.95	4.70 5.40	21.79 21.38
3e	cycl-C_6H_{11}	86	252	$C_{15}H_{17}N_5O_3S$ (347.40)	51.86 51.51	4.93 4.90	20.15 20.09
3f	C_6H_5	98	251–254	$C_{15}H_{11}N_5O_3S$ (341.36)	52.78 52.70	3.24 3.25	20.51 20.84
3g	$C_6H_4CH_3$(4−)	89	251–253	$C_{16}H_{13}N_5O_3S \cdot H_2O$ (373.41)	51.47 51.42	4.05 4.01	18.76 18.44
3h	C_6H_4Br(4−)	91	235–236	$C_{15}H_{10}BrN_5O_3S \cdot 1/2H_2O$ (429.26)	41.97 41.88	2.58 2.27	16.31 15.87
3i	C_6H_4Cl(4−)	99	260	$C_{15}H_{10}ClN_5O_3S$ (375.80)	47.94 47.54	2.68 2.65	18.63 18.23
3j	C_6H_4F(4−)	79	275	$C_{15}H_{10}FN_5O_3S \cdot 1.5H_2O$ (386.38)	46.63 46.47	3.39 2.77	18.12 17.72
3k	$C_6H_4NO_2$(4−)	78	267–268	$C_{15}H_{10}N_6O_5S \cdot 1/2H_2O$ (395.36)	45.57 45.94	2.80 2.55	21.25 21.06

human tumour cell lines derived from nine neoplastic diseases at 10-fold dilutions of five concentrations ranging from 10^{-4} to 10^{-8} M. The percentage growth was evaluated spectrophotometrically versus controls not treated with test agents. A 48 h continuous drug exposure protocol was followed and a SRB protein assay was used to estimate cell viability or growth. For each compound, the 50% growth inhibition (GI_{50}) and total growth inhibition (TGI) were obtained for all the cell lines. The $\log_{10} GI_{50}$ and $\log_{10} TGI$ were then determined, defined as the mean of the $\log_{10}$'s of the individual GI_{50} and TGI values. Negative values indicated the most sensitive cell lines. Compounds having values -4 and < -4 were declared to be active. The cell lines used in the NCI screen were leukaemia (L) lines CCRF-CEM, HL-60 (TB), K-562, MOLT-4, RPMI-8226, SR; non-small cell lung cancer (NSCLC) lines A549/ATCC, EKVX, HOP-62, HOP-92, NCI-H226, NCI-H23, NCI-H322M, NCI-H460, NCI-H522; colon cancer (CL) lines COLO 205, HCC-2998, HCT-116, HCT-15, HT29, KM12, SW-620; central nervous system cancer (CNSC) lines SF-268, SF-295, SF-539, SNB-19, SNB-75, U251; melanoma (M) lines LOX IMVI, MALME-3M, M14, SK-MEL-2, SK-MEL-28, SK-MEL-5, UACC-257, UACC-62; ovarian cancer (OC) lines IGROV1, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, SK-OV-3; renal cancer (RC) lines 786-O, A498, ACHN, CAKI-1, RXF 393, SN12C, TK-10, UO-31; prostate cancer (PC) lines PC-3, DU-145 and breast cancer (BC) lines MCF7, NCI/ADR-RES, MDA-MB 231/ATCC, HS 578T, MDA-MB-435, MDA-N, BT-549, T-47D (Table 4). Among the compounds tested, 4-chlorophenyl substituted compound **4l** demonstrated the most marked effects in the National Cancer Institute's 60 human tumour cell line in vitro screen on a NSCLC cell line (HOP-62, $\log_{10} GI_{50}$ value < -8.00) and L cell lines (HL-60(TB), $\log_{10} GI_{50}$ value -6.30; MOLT-4, $\log_{10} GI_{50}$ value -6.18). On the same cancer cell line, the $\log_{10} GI_{50}$ values of thioguanine and 5-fluorouracil used as anticancer agents are -6.13, -5.96, -6.57 and -4.30, -4.81, -4.86, respectively. When these data are examined, it is observed that **4l** is much more active than thioguanine and 5-fluorouracil against a NSCLC cell line (HOP-62) and a L cell line (HL-60(TB)). In addition, **3b**, **3c**, **4c** and **4f** are the most active on a L cell line (CCRF-CEM). The $\log_{10} GI_{50}$ values of these compounds are -5.12, -5.29, -5.57 and -6.42, respectively. Compound **3f** is highly active on a NSCLC cell line (EKVX, $\log_{10} GI_{50}$ value -5.92), a OC cell line (IGROVI, $\log_{10} GI_{50}$ value -5.85) and a L cell line (CFRF-CEM, $\log_{10} GI_{50}$ value -5.67) whereas **4a** exhibited high cytotoxicity on a RC cell

Table 2
Physicochemical data of **4a–m**

Compound	R	X	Yield (%)	M.p. (°C)	Formula (M.W.)	Analysis Calc./Found		
						C	H	N
4a	CH_3	O	97	249	$C_{15}H_{18}N_6O_4S \cdot 1/2H_2O$ (387.42)	46.50 46.62	4.94 4.79	21.69 21.28
4b	CH_3	CH_2	88	250	$C_{16}H_{20}N_6O_3S$ (376.44)	51.05 50.80	5.35 5.42	22.32 22.28
4c	$CH_2–CH=CH_2$	O	78	198–200	$C_{17}H_{20}N_6O_4S$ (404.45)	50.48 49.54	4.98 4.79	20.77 21.25
4d	$CH_2–CH=CH_2$	CH_2	93	189	$C_{18}H_{22}N_6O_3S$ (402.47)	53.71 53.43	5.50 5.59	20.88 20.89
4e	C_4H_9	O	79	175–176	$C_{18}H_{24}N_6O_4S$ (420.49)	51.41 51.83	5.75 5.18	19.98 19.96
4f	cycl-C_6H_{11}	O	83	187–189	$C_{20}H_{26}N_6O_4S$ (446.53)	53.79 53.06	5.86 5.91	18.82 18.64
4g	cycl-C_6H_{11}	CH_2	84	177–179	$C_{21}H_{28}N_6O_3S$ (444.55)	56.73 56.59	6.34 6.68	18.90 18.50
4h	C_6H_5	O	89	189–190	$C_{20}H_{20}N_6O_4S$ (440.48)	54.53 55.16	4.57 4.22	19.07 18.72
4i	C_6H_5	CH_2	94	188–190	$C_{21}H_{22}N_6O_3S \cdot 1.5H_2O$ (465.53)	54.18 53.93	5.41 5.13	18.05 17.65
4j	$C_6H_4CH_3(4-)$	O	90	187–191	$C_{21}H_{22}N_6O_4S \cdot 1/2H_2O$ (463.51)	54.41 54.42	5.00 4.83	18.13 17.96
4k	$C_6H_4CH_3(4-)$	CH_2	84	192	$C_{22}H_{24}N_6O_3S$ (452.53)	58.39 58.26	5.34 5.71	18.57 18.28
4l	$C_6H_4Cl(4-)$	O	78	192–193	$C_{20}H_{19}ClN_6O_4S \cdot H_2O$ (492.94)	48.73 48.87	4.29 3.92	17.04 16.65
4m	$C_6H_4Cl(4-)$	CH_2	84	179–180	$C_{21}H_{21}ClN_6O_3S \cdot 1/2H_2O$ (481.96)	52.33 52.11	4.59 4.03	17.43 17.04

line (UO-31, $\log_{10}$ GI_{50} value −5.52) and a M cell line (SK-MEL-2, $\log_{10}$ GI_{50} value −5.19) in the in vitro screen. The $\log_{10}$ GI_{50} values of thioguanine and 5-fluorouracil on a L cell line (CCRF-CEM), a NSCLC cell line (EKVX), a OC cell line (IGROVI), a RC cell line (UO-31) and a M cell line (SK-MEL-2) are −6.81, −5.72, −5.32, −5.83, −6.03, and −4.52, −3.27, −4.77, −5.17, −3.44, respectively. When the results of **3b**, **3c**, **3f**, **3k**, **4a**, **4c**, **4f** and **4l** were compared with thioguanine and 5-fluorouracil, it was speculated that the cytotoxicities of these compounds were comparable to those of anticancer agents. Among 5-nitroindole-1*H*-2,3-dione-3-thiosemicarbazones (**3**) tested, phenyl and 4-nitrophenyl substituted compounds **3f** and **3k** were more active than entries **3b** and **3c** which were alkyl substituted. Furthermore, in *N*-morpholinomethyl derivatives (**4**) the cytotoxicity increased when compared with the starting compounds. Among the synthesized compounds, 4-chlorophenyl substituted compound **4l** showed the most favourable cytotoxicity against a NSCLC cell line (HOP-62). In conclusion, these preliminary results are promising and some of these compounds may be potential candidates for new anticancer agents.

4. Experimental

4.1. Chemistry

M.p.s were estimated with a Büchi 530 melting point apparatus (Flawil, Switzerland) in open capillaries and are uncorrected. Elemental analyses were performed on a Carlo–Erba 1106 elemental analyzer (Milano, Italy). IR spectra were recorded on KBr (BDH, Poole, England) discs, using a Perkin–Elmer Model 1600 FT-IR spectrometer (Norwalk, Connecticut, USA). ^{1}H-NMR spectra were obtained on Bruker AC 200 (200 MHz) (Rheinstätten, Germany) spectrophotometer using DMSO-d_6 (E. Merck, Darmstadt, Germany). EIMS were determined on a VG Zab Spec (70 eV) mass spectrometer (Manchester, England).

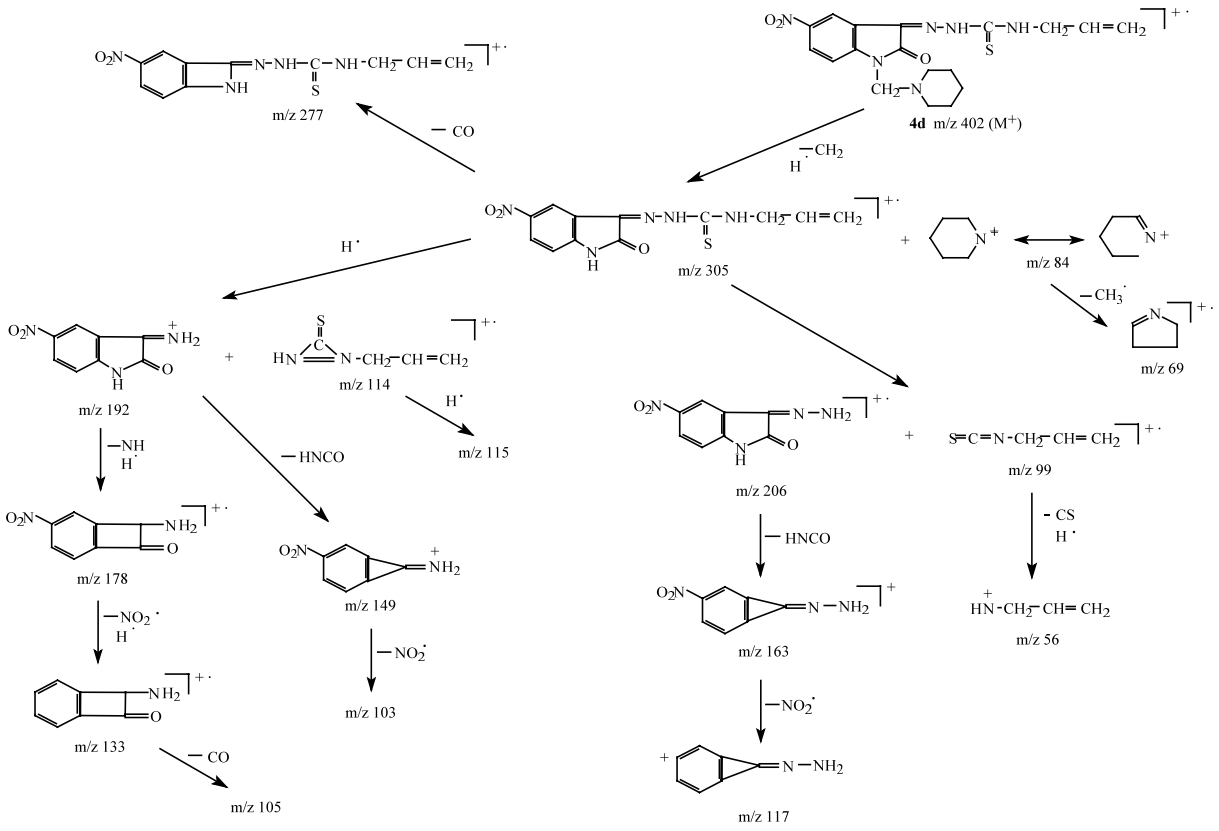

Fig. 3. The proposed fragmentation pattern of **4d**.

Table 3
Primary cytotoxicity assay results of **3b**, **3c**, **3f**, **3k**, **4a**, **4c**, **4f** and **4l** at 10^{-4} M concentration

Compound	(Lung) NCI-H460	(Breast) MCF7	(CNS) SF-268
3b	19	51	45
3c	29	46	47
3f	−42	−8	−59
3k	−20	7	−59
4a	8	−27	−60
4c	11	14	40
4f	−74	−61	−85
4l	−76	−61	−83

*4.1.1. Synthesis of 5-nitro-1H-indole-2,3-dione-3-thiosemicarbazones (**3a**–**k**)*

To a solution of 5-nitro-1*H*-indole-2,3-dione 1 (0.0035 mol) in EtOH (20 mL) was added a solution of N-substituted-thiosemicarbazides **2a**–**k** (0.0035 mol). After addition of a drop of concd. H_2SO_4, the mixture was refluxed on a water bath for 3 h. The product formed after cooling was filtered, washed with EtOH or recrystallised from EtOH.

*4.1.1.1. Spectral data of **3b**.* IR (KBr, cm^{-1}): 3345, 3164 (NH), 1693 (C=O), 1518, 1333 (NO_2), 1157 (C=S). ^{1}H-NMR (DMSO-d_6, δ, ppm): 1.22 (t, *J*: 7.1 Hz, 3H, CH_2–CH_3), 3.67 (quin., *J*: 6.8 Hz, 2H, CH_2CH_3), 7.12 (d, $J_{7,6}$: 8.7 Hz, 1H, indole C_7–H), 8.24, 8.28 (dd, $J_{6,7}$: 8.5 Hz, $J_{6,4}$: 2.3 Hz, 1H, indole C_6–H), 8.56 (d, $J_{4,6}$: 2.2 Hz, 1H, indole C_4–H), 9.54 (s, 1H, CS–NH), 11.74 (br.s, 1H, indole NH), 12.34 (s, 1H, N–NH). EIMS (70 eV): *m*/*z* (%) 293 ([M^+], 19), 265 (18), 206 (5), 192 (20), 168 (24), 149 (10), 146 (6), 141 (29), 136 (5), 135 (10), 133 (13), 131 (10), 129 (25), 119 (8), 117 (6), 115 (23), 111 (28), 105 (15), 103 (12), 97 (48), 95 (30), 94 (29), 91 (13), 83 (52), 69 (68), 67 (23), 60 (40), 56 (29), 57 (100).

*4.1.1.2. Spectral data of **3c**.* IR (KBr, cm^{-1}): 3336, 3180 (NH), 1700 (C=O), 1534, 1340 (NO_2), 1149 (C=S). ^{1}H-NMR (DMSO-d_6, δ, ppm): 4.29 (t, *J*: 5.5 Hz, 2H, CH_2–$CH{=}CH_2$), 5.17, 5.22; 5.18, 5.23 (2dd, *J*: 17.2, 10.4 Hz, *J*: 17.2, 10.2 Hz, 2H, CH_2–$CH{=}CH_2$), 5.89–5.99 (m, 1H, CH_2–$CH{=}CH_2$), 7.13 (d, $J_{7,6}$: 8.6 Hz, 1H, indole C_7–H), 8.27, 8.28 (dd, $J_{6,7}$: 8.6 Hz, $J_{6,4}$: 2.4 Hz, 1H, indole C_6–H), 8.58 (d, $J_{4,6}$: 2.2 Hz, 1H, indole C_4–H), 9.76 (t, *J*: 5.8 Hz, 1H, CS–NH), 11.79 (s, 1H, indole NH), 12.39 (s, 1H, N–NH). EIMS (70 eV): *m*/*z* (%) 305 ([M^+], 2), 271 (3), 257 (3), 206 (5), 192 (8), 189 (5), 178 (7), 159 (7), 149 (4), 148 (14), 146 (20), 136 (1), 135 (2), 133 (7), 131 (5), 119 (3), 117 (3), 115 (8), 111 (6), 105 (6), 103 (4), 99 (17), 92 (79), 91 (100), 89 (6), 78 (8), 69 (4), 65 (14), 64 (28), 60 (5), 57 (16), 56 (13).

Table 4
In vitro tumour cell growth inhibition of **3b**, **3c**, **3f**, **3k**, **4a**, **4c**, **4f** and **4l**

Panel/cell line	**3b**		**3c**		**3f**		**3k**		**4a**		**4c**		**4f**		**4l**	
	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI
Leukaemia																
CCRF-CEM	−5.12	> −4.00	−5.29	−4.38	−5.67	−4.70	−4.73	−4.22	−4.63	−4.14	−5.57	> −4.00	−6.42	−5.56	−5.55	−4.55
HL-60 (TB)	−4.03	> −4.00	−4.26	> −4.00	−5.28	−4.44	−4.57	−4.14	−4.63	−4.16	−4.61	> −4.00	−5.48	−4.76	−6.30	−4.94
K-562	−4.28	> −4.00	−4.49	> −4.00	−5.31	> −4.00	−4.47	> −4.00	> −4.00	> −4.00	−4.72	> −4.00	−5.44	> −4.00	–	−4.61
MOLT-4	−4.65	> −4.00	−4.78	> −4.00	−5.22	> −4.00	−4.44	> −4.00	−4.36	> −4.00	−5.22	> −4.00	−5.77	> −4.00	−6.18	−5.53
RPMI-8226	> −4.00	> −4.00	−4.24	> −4.00	−4.81	> −4.00	−4.46	> −4.00	−4.67	−4.30	−4.48	> −4.00	−4.95	−4.28	–	–
SR	−4.50	> −4.00	−4.99	> −4.00	−5.28	> −4.00	−4.52	> −4.00	−4.27	> −4.00	−5.20	> −4.00	−5.94	−4.67	−5.70	−5.07
Non-small cell lung cancer																
A549 /ATCC	−4.45	> −4.00	−4.47	> −4.00	−5.30	−4.68	−4.68	−4.32	−4.50	> −4.00	−4.98	> −4.00	−5.39	−4.80	−5.09	−4.68
EKVX	−4.30	> −4.00	−4.37	> −4.00	−5.92	−5.04	−4.58	−4.20	−4.81	−4.36	−4.81	> −4.00	−5.50	−4.85	–	–
HOP-62	−4.56	> −4.00	> −4.00	> −4.00	−5.07	−4.66	−4.65	−4.30	−4.85	−4.55	−4.69	> −4.00	−4.99	−4.62	< −8.00	–
HOP-92	−4.47	> −4.00	−4.35	> −4.00	−5.53	−4.66	−4.73	−4.45	−4.67	−4.36	−4.88	−4.08	−5.71	−4.89	−5.07	−4.67
NCI-H226	−4.01	− −4.00	> −4.00	> −4.00	−4.74	−4.18	−4.56	−4.06	−4.80	−4.44	> −4.00	> −4.00	−4.72	−4.18	−5.32	−4.63
NCI-H23	> −4.00	> −4.00	−4.41	> −4.00	−5.03	−4.59	−4.70	−4.39	−4.75	−4.49	−4.35	> −4.00	−5.23	−4.67	−5.68	−5.03
NCI-H322M	−4.43	> −4.00	−4.06	> −4.00	−5.25	−4.63	−4.61	−4.18	−4.80	−4.33	−4.55	> −4.00	−4.99	−4.66	–	–
NCI-H460	−4.69	> −4.00	−4.78	> −4.00	−5.44	−4.84	−4.71	−4.38	−4.63	−4.12	−5.38	> −4.00	−5.60	−4.87	−5.11	−4.69
NCI-H522	–	> −4.00	−4.19	> −4.00	–	–	−4.51	−4.22	–	–	−4.38	> −4.00	−5.00	−4.52	−5.17	−4.63
Colon cancer																
COLO 205	> 4.00	> −4.00	−4.04	> −4.00	−4.93	> −4.00	−4.44	> −4.00	−4.57	−4.04	−4.46	> −4.00	−5.27	−4.63	−4.88	−4.56
HCC-2998	−4.60	−4.14	−4.63	> −4.00	−5.44	−4.91	−4.68	−4.44	−4.83	−4.35	−4.89	−4.04	−5.31	−4.75	–	–
HCT-116	−4.56	> −4.00	−4.61	> −4.00	−5.39	−4.73	−4.69	−4.33	−4.81	−4.54	−5.19	> −4.00	−5.45	−4.79	−5.39	−4.80
HCT-15	−4.31	> −4.00	−5.12	> −4.00	−5.52	−4.77	−4.72	−4.33	−4.89	−4.58	−5.30	> −4.00	−5.62	−4.81	−5.47	−4.94
HT29	−4.30	> −4.00	−4.16	> −4.00	−5.27	−4.72	−4.58	−4.18	−4.39	> −4.00	−4.63	> −4.00	−5.35	−4.75	–	–
KM12	−4.28	> −4.00	−4.33	> −4.00	−5.08	−4.57	−4.73	−4.42	−4.64	−4.01	−4.55	> −4.00	−5.32	−4.74	−4.93	−4.62
SW-620	−4.30	> −4.00	−4.09	> −4.00	−5.05	> −4.00	−4.41	> −4.00	−4.55	> −4.00	−4.21	> −4.00	−5.20	> −4.00	−5.36	−4.51
CNS cancer																
SF-268	−4.35	> −4.00	−4.24	> −4.00	−5.36	−4.78	−4.49	−4.14	−4.69	−4.32	−4.23	> −4.00	−5.36	−4.72	−5.36	−4.78
SF-295	−4.53	> −4.00	−4.54	> −4.00	−4.92	−4.56	−4.70	−4.35	−4.74	−4.27	−5.10	> −4.00	−5.44	−4.78	−5.30	−4.74
SF-539	−4.52	> −4.00	> −4.00	> −4.00	−5.27	−4.56	−4.53	> −4.00	−4.75	−4.48	−4.29	> −4.00	−4.94	−4.60	−4.95	−4.58
SNB-19	−4.34	> −4.00	−4.25	> −4.00	−5.12	−4.67	−4.58	−4.16	−4.66	−4.21	−4.67	> −4.00	−5.10	−4.65	−5.10	−4.68
SNB-75	–	–	–	–	–	–	−4.66	−4.37	−4.66	−4.21	−4.61	−4.05	−4.94	−4.62	−4.89	−4.59
U251	−4.53	> −4.00	−4.19	> −4.00	−5.31	−4.78	−4.63	−4.29	–	–	−4.74	> −4.00	−5.34	−4.76	−5.14	−4.70
Melanoma																
LOX IMVI	−4.21	> −4.00	−4.09	> −4.00	−5.02	−4.61	−4.65	−4.31	−4.76	−4.50	−4.49	> −4.00	−5.30	−4.71	−5.00	−4.62
MALME-3M	−4.24	> −4.00	> −4.00	> −4.00	–	–	−4.48	−4.26	−4.79	−4.36	> −4.00	> −4.00	−4.94	−4.61	−5.21	−4.73
M14	−4.33	> −4.00	−4.21	> −4.00	−5.22	−4.65	−4.71	−4.38	−4.50	> −4.00	−4.76	> −4.00	−5.02	−4.66	−5.00	−4.66
SK-MEL-2	> −4.00	> −4.00	> −4.00	> −4.00	−4.92	−4.50	−4.73	−4.45	−5.19	−4.67	> −4.00	> −4.00	−4.93	−4.59	−4.88	−4.58
SK-MEL-28	−4.32	> −4.00	> −4.00	> −4.00	−4.78	−4.28	−4.58	−4.09	−4.68	−4.26	> −4.00	> −4.00	−4.96	−4.59	−4.96	−4.58
SK-MEL-5	−4.45	> −4.00	−4.52	> −4.00	−5.54	−4.87	−4.80	−4.51	−4.49	> −4.00	−5.01	−4.07	−5.50	−4.95	−5.40	−4.75
UACC-257	−4.27	> −4.00	−4.15	> −4.00	−4.90	−4.56	−4.68	−4.30	−4.60	−4.11	−4.78	> −4.00	−4.98	−4.64	−4.88	−4.58
UACC-62	−4.00	> −4.00	> −4.00	> −4.00	−4.96	−4.54	−4.68	−4.31	−4.82	−4.48	> −4.00	> −4.00	−5.10	−4.69	−5.28	−4.75
Ovarian cancer																
IGROV1	−4.50	> −4.00	−4.63	> −4.00	−5.85	−4.71	−4.75	−4.40	–	–	−5.21	> −4.00	−5.82	−4.81	−5.38	−4.74
OVCAR-3	−4.39	> −4.00	−4.16	> −4.00	−5.00	−4.65	−4.64	−4.32	−4.76	−4.46	−4.61	> −4.00	−5.23	−4.69	−5.40	−4.75
OVCAR-4	−4.59	> −4.00	−4.38	> −4.00	−5.14	−4.53	−4.71	−4.39	−4.83	−4.51	−4.64	> −4.00	−5.34	−4.75	−5.00	−4.67
OVCAR-5	> −4.00	> −4.00	> −4.00	> −4.00	−4.91	−4.61	−4.54	−4.17	−4.64	−4.15	−4.25	> −4.00	−4.95	−4.62	–	–
OVCAR-8	−4.46	> −4.00	−4.30	> −4.00	−5.29	−4.50	−4.50	> −4.00	–	–	−4.90	> −4.00	−5.41	−4.76	−5.25	−4.63
SK-OV-3	−4.40	> −4.00	−4.47	> −4.00	−4.85	−4.55	−4.65	−4.38	−4.77	−4.50	−4.44	> −4.00	−4.89	−4.59	−4.90	−4.58
Renal cancer																
786-O	−4.72	−4.16	−4.68	> −4.00	−5.27	−4.75	−4.77	−4.49	−4.81	−4.54	−4.81	> −4.00	−5.26	−4.73	−5.36	−4.78
A498	−4.64	−4.05	−4.63	−4.04	−5.45	−4.88	−4.74	−4.49	−4.61	−4.18	−5.12	−4.18	−5.32	−4.76	–	–
ACHN	−4.51	> −4.00	−4.85	> −4.00	−5.49	−4.71	−4.61	−4.13	−4.76	−4.45	−5.28	> −4.00	−5.29	−4.74	−5.33	−4.79
CAKI-1	−4.92	> −4.00	−4.77	> −4.00	−5.45	−4.82	−4.67	−4.36	−4.75	−4.19	−5.28	> −4.00	−5.48	−4.84	−5.45	−4.91

Table 4 (*Continued*)

Panel/cell line	**3b**		**3c**		**3f**		**3k**		**4a**		**4c**		**4f**		**4l**	
	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI	Log_{10} GI_{50}	Log_{10} TGI
RXF 393	−4.59	−4.09	−4.45	−4.13	−4.94	−4.58	−4.76	−4.43	−4.79	−4.52	> −4.00	> −4.00	−4.91	−4.54	−5.14	−4.70
SN12C	−4.50	> −4.00	−4.58	> −4.00	−5.23	−4.59	−4.49	> −4.00	−4.79	−4.41	−4.79	> −4.00	−5.43	−4.71	−5.39	−4.80
TK-10	−4.74	> −4.00	−4.51	> −4.00	−5.21	−4.63	−4.61	−4.25	−4.88	−4.53	−5.27	−4.03	−5.17	−4.71	−4.91	−4.61
UO-31	−4.59	> −4.00	–	–	−5.32	−4.66	−4.74	−4.35	−5.52	−4.85	−5.21	> −4.00	−5.20	−4.74	−5.42	−4.80
Prostate cancer																
PC-3	−4.01	> −4.00	> −4.00	> −4.00	−5.26	−4.68	−4.62	−4.31	−4.66	−4.24	−4.62	> −4.00	−5.33	−4.72	−4.99	−4.66
DU-145	−4.48	> −4.00	−4.50	> −4.00	−4.93	−4.17	−4.67	−4.31	−4.86	−4.56	−4.73	> −4.00	−4.96	−4.57	−4.98	−4.65
Breast cancer																
MCF7	−4.39	> −4.00	−4.44	> −4.00	−5.36	−4.61	−4.60	−4.08	−4.69	−4.37	−4.48	> −4.00	−5.39	−4.76	−5.28	−4.73
NCI/ADR-RES	−4.48	> −4.00	−4.75	> −4.00	−5.61	−5.06	−4.69	−4.31	−4.79	−4.42	−5.43	> −4.00	−5.49	−4.73	−5.66	−5.23
MDA-MB 231/ATCC	−4.45	> −4.00	> −4.00	> −4.00	−4.91	−4.61	−4.61	−4.35	−4.92	−4.60	−4.40	> −4.00	−4.80	−4.50	−4.82	−4.51
HS 578T	−4.00	> −4.00	> −4.00	> −4.00	−4.45	> −4.00	−4.28	> −4.00	−4.60	−4.14	> −4.00	> −4.00	−4.82	−4.34	−5.15	−4.55
MDA-MB-435	−4.06	> −4.00	−4.05	> −4.00	−5.00	−4.57	−4.74	−4.41	−4.22	> −4.00	−4.26	> −4.00	−5.17	−4.70	−5.20	−4.72
MDA-N	> −4.00	> −4.00	−4.08	> −4.00	−5.20	−4.66	−4.75	−4.38	−4.17	> −4.00	−4.51	> −4.00	−5.22	−4.70	−5.34	−4.76
BT-549	> −4.00	> −4.00	−4.28	> −4.00	−5.41	-4.70	−4.79	−4.47	−4.72	−4.41	−4.82	> −4.00	−5.31	−4.68	–	–
T-47D	−4.50	> −4.00	−4.59	−4.06	−5.33	−4.41	−4.72	−4.20	–	–	−4.77	−4.02	−5.58	−4.75	−5.36	−4.68
MG-MID	−4.38	−4.01	−4.36	−4.01	−5.20	−4.56	−4.63	−4.26	−4.69	−4.31	−4.69	−4.01	−5.27	−4.66	−5.30	−4.73
Delta	0.77	0.15	0.93	0.37	0.71	0.50	0.17	0.26	0.82	0.54	0.88	0.17	1.15	0.90	2.70	0.80
Range	1.15	0.16	1.29	0.38	1.47	1.06	0.52	0.51	1.51	0.85	1.57	0.18	1.70	1.56	3.18	1.02

*4.1.1.3. Spectral data of **3f**.* IR (KBr, cm^{-1}): 3303, 3169 (NH), 1693 (C=O), 1534, 1351 (NO_2), 1151 (C=S). ^{1}H-NMR (DMSO-d_6, δ, ppm): 7.14 (d, $J_{7,6}$: 8.7 Hz, 1H, indole C_7–H), 7.32 (d, J: 7.1 Hz, 1H, phenyl C_4–H), 7.45 (t, J: 7.5 Hz, 1H, phenyl C_3–H, C_5–H), 7.60 (d, J: 7.9 Hz, 1H, phenyl C_2–H, C_6–H), 8.27, 8.29 (dd, $J_{6,7}$: 8.7 Hz, $J_{6,4}$: 2.3 Hz, 1H, indole C_6–H), 8.70 (d, $J_{4,6}$: 2.4 Hz, 1H, indole C_4–H), 11.04 (s, 1H, CS–NH), 11.80 (br.s, 1H, indole NH), 12.57 (s, 1H, N–NH). EIMS (70 eV): *m/z* (%) 341 ([M^+], 12), 313 (37), 206 (38), 191 (6), 189 (10), 149 (12), 148 (4), 136 (14), 135 (60), 133 (12), 131 (13), 129 (13), 120 (19), 119 (18), 118 (18), 115 (16), 111 (11), 105 (17), 103 (10), 97 (20), 93 (100), 92 (18), 91 (23), 83 (23), 77 (56), 69 (30), 66 (34), 60 (15), 57 (45).

*4.1.1.4. Spectral data of **3i**.* IR (KBr, cm^{-1}): 3312, 3161 (NH), 1697 (C=O), 1527, 1341 (NO_2), 1153 (C=S). ^{1}H-NMR (DMSO-d_6, δ, ppm): 7.09 (d, $J_{7,6}$: 8.9 Hz, 1H, indole C_7–H), 7.50, 7.66 (2d, J: 8.7 Hz, 4H, phenyl–H), 8.28, 8.29 (dd, $J_{6,7}$: 8.7 Hz, $J_{6,4}$: 2.2 Hz, 1H, indole C_6–H), 8.67 (d, $J_{4,6}$: 2.4 Hz, 1H, indole C_4–H), 11.05 (s, 1H, CS–NH), 11.80 (br.s, 1H, indole NH), 12.60 (s, 1H, N–NH). EIMS (70 eV): *m/z* (%) 375 ([M^+], 1), 341 [7 (343, 2)], 303 [8 (305, 3)], 206 (2), 191 (6), 150 [16 (152, 5)], 149 (16), 137 (34), 136 (23), 135 (15), 133 (6), 129 (10), 123 (21), 121 (20), 119 (8), 111 (15), 109 (20), 107 (15), 105 (7), 98 (15), 97 (25), 96 (16), 95 (32), 94 (11), 93 (19), 91 (8), 85 (15), 84 (17), 83 (30), 82 (23), 81 (69), 73 (15), 71 (26), 70 (22), 69 (100), 68 (24), 67 (21), 57 (40).

*4.1.2. Synthesis of 1-morpholino/piperidinomethyl-5-nitroindole-2,3-dione-3-thiosemicarbazones (**4a–m**)*

To a suspension of **3** (0.002 mol) in absolute EtOH (20 mL), 37% formaldehyde solution (0.5 mL) and morpholine or piperidine (0.002 mol) were added dropwise with vigorous stirring. After combining all reagents the reaction mixture was refluxed on a water bath for 4 h. The product formed were filtered, washed with petroleum ether.

*4.1.2.1. Spectral data of **4c**.* IR (KBr, cm^{-1}): 3345, 3227 (NH), 1699 (C=O), 1526, 1343 (NO_2), 1175 (C=S). ^{1}H-NMR (DMSO-d_6, δ, ppm): 2.59 (t, J: 4.2 Hz, 4H, morpholine C_3–H, C_5–H), 3.55 (t, J: 4.2 Hz, 4H, morpholine C_2–H, C_6–H), 4.29 (t, J: 4.6 Hz, 2H, CH_2–CH=CH_2), 4.58 (s, 2H, N–CH_2–N), 5.16, 5.22 (dd, J: 15.8, 9.0 Hz, 2H, CH_2–CH=CH_2), 5.85–6.04 (m, 1H, CH_2–CH=CH_2), 7.50 (d, $J_{7,6}$: 8.8 Hz, 1H, indole C_7–H), 8.31, 8.32 (dd, $J_{6,7}$: 8.8 Hz, $J_{6,4}$: 2.3 Hz, 1H, indole C_6–H), 8.62 (d, $J_{4,6}$: 2.2 Hz, 1H, indole C_4–H), 9.73 (br.s, 1H, CS–NH), 12.35 (br.s, 1H, N–NH). EIMS (70 eV): *m/z* (%) 404 ([M^+], 1), 368 (4), 341 (4), 256 (14), 205 (3), 192 (4), 191 (4), 171 (5), 167 (5), 161 (4), 157 (6), 152 (5), 149 (12), 141 (5), 137 (24), 136 (15), 135 (10), 133 (6), 129 (16), 123 (16), 121 (15), 119 (6), 117 (3), 111 (16), 109 (16), 107 (11), 105 (7), 98 (20), 97 (29), 96 (16), 95 (27), 93 (13), 91 (12), 87 (8), 85 (20), 84 (20), 83 (33), 82 (22), 81 (66), 73 (28), 71 (31), 70 (24), 69 (100), 68 (20), 67 (20), 60 (21), 57 (48), 56 (19).

*4.1.2.2. Spectral data of **4d**.* IR (KBr, cm^{-1}): 3314 (NH), 1701 (C=O), 1522, 1339 (NO_2), 1160 (C=S). ^{1}H-NMR (DMSO-d_6, δ, ppm): 1.36 (br.s, 2H, piperidine C_4–H), 1.47 (br.s, 4H, piperidine C_3–H, C_5–H), 2.55 (br.s, 4H, piperidine C_2–H, C_6–H), 4.30 (t, J: 5.5 Hz, 2H, CH_2–CH=CH_2), 4.57 (s, 2H, N–CH_2–N), 5.17, 5.23 (dd, J: 15.5, 10.1 Hz, 2H, CH_2–CH=CH_2), 5.88–6.04 (m, 1H, CH_2–CH=CH_2), 7.48 (d, $J_{7,6}$: 8.6 Hz, 1H, indole C_7–H),8.32, 8.35 (dd, $J_{6,7}$: 8.8 Hz, $J_{6,4}$: 2.2 Hz, 1H, indole C_6–H), 8.62 (d, $J_{4,6}$: 2.2 Hz, 1H, indole C_4–H), 9.75 (br.s, 1H, CS–NH), 12.33 (s, 1H, N–NH). EIMS (70 eV): *m/z* (%) 402 ([M^+], 2), 305 (14), 277 (13), 206 (22), 192 (100), 178 (13), 163 (17), 162 (20), 149 (11), 133 (23), 117 (17), 115 (79), 114 (20), 105 (17), 103 (15), 101 (11), 99 (17), 85 (23), 84 (33), 83 (20), 81 (40), 78 (81), 73 (17), 71 (16), 70 (15), 69 (25), 63 (90), 61 (24), 60 (21), 57 (51), 56 (98).

*4.1.2.3. Spectral data of **4h**.* IR (KBr, cm^{-1}): 3195 (NH), 1698 (C=O), 1518, 1338 (NO_2), 1164 (C=S). ^{1}H-NMR (DMSO-d_6, δ, ppm): 2.59 (s, 4H, morpholine C_3–H, C_5–H), 3.54 (s, 4H, morpholine C_2–H, C_6–H), 4.60 (s, 2H, N–CH_2–N), 7.32 (d, J: 6.9 Hz, 1H, phenyl C_4–H), 7.44 (d, J: 7.5 Hz, 2H, phenyl C_3–H, C_5–H), 7.50 (s, 1H, indole C_7–H), 7.58 (d, J: 8.2 Hz, 2H, phenyl C_2–H, C_6–H), 8.35 (d, $J_{6,7}$: 8.9 Hz, 1H, indole C_6–H), 8.75 (s, 1H, indole C_4–H), 11.15 (s, 1H, CS–NH), 12.47 (s, 1H, N–NH). EIMS (70 eV): *m/z* (%) 206 (6), 190 (2), 159 (6), 149 (4), 148 (10), 146 (10), 136 (7), 135 (69), 133 (5), 119 (12), 118 (10), 115 (5), 105 (9), 103 (7), 93 (18), 92 (67), 91 (100), 87 (5), 86 (5), 77 (40), 71 (11), 70 (5), 69 (9), 65 (15), 64 (13), 63 (10), 60 (3), 57 (30), 56 (12).

*4.1.2.4. Spectral data of **4j**.* IR (KBr, cm^{-1}): 3261 (NH), 1705 (C=O), 1519, 1335 (NO_2), 1146 (C=S). ^{1}H-NMR (DMSO-d_6, δ, ppm): 2.35 (s, 3H, CH_3), 2.60 (t, J: 4.4 Hz, 4H, morpholine C_3–H, C_5–H), 3.56 (t, J: 4.4 Hz, 4H, morpholine C_2–H, C_4–H), 4.60 (s, 2H, N–CH_2–N), 7.25 (d, J: 8.2 Hz, 2H, phenyl C_3–H, C_5–H), 7.46 (d, J: 8.2 Hz, 2H, phenyl C_2–H, C_6–H), 7.52 (d, $J_{7,6}$: 8.8 Hz, 1H, indole C_7–H), 8.33, 8.35 (dd, $J_{6,7}$: 8.8 Hz, $J_{6,4}$: 2.4 Hz, 1H, indole C_6–H), 8.75 (d, $J_{4,6}$: 2.3 Hz, 1H, indole C_4–H), 11.05 (s, 1H, CS–NH), 12.46 (br.s, 1H, N–NH). EIMS (70 eV): *m/z* (%) 224 (1), 191 (1), 149 (1), 119 (1), 115 (1), 105 (7), 103 (2), 91 (11), 87 (4), 86 (3), 77 (12), 71 (2), 70 (5), 69 (3), 65 (18), 64 (14), 63 (28), 62 (15), 60 (4), 57 (27), 56 (31), 54 (15), 53 (16), 52 (35), 51 (49), 50 (42), 49 (13), 46 (13), 45 (17), 44 (32), 43 (37), 42 (53), 41 (42), 40 (23), 39 (69), 38 (32), 37 (19), 35 (13), 32 (100), 31 (25), 30 (94).

4.1.2.5. Spectral data of ***4k***. IR (KBr, cm^{-1}): 3299 (NH), 1703 (C=O), 1519, 1335 (NO_2), 1148 (C=S). ^{1}H-NMR (DMSO-d_6, δ, ppm): 1.35 (br.s, 2H, piperidine C_4–H), 1.46 (br.s, 4H, piperidine C_3–H, C_5–H), 2.34 (s, 3H, CH_3), 2.55 (br.s, 4H, piperidine C_2–H, C_6–H), 4.58 (s, 2H, N–CH_2–N), 7.25, 7.44 (dd, J: 8.2, 8.2 Hz, 4H, phenyl), 7.50 (d, $J_{7,6}$: 8.9 Hz, 1H, indole C_7–H), 8.33, 8.34 (dd, $J_{6,7}$: 9.1 Hz, $J_{6,4}$: 2.2 Hz, 1H, indole C_6–H), 8.74 (d, $J_{4,6}$: 2.1 Hz, 1H, indole C_4–H), 11.09 (s, 1H, CS–NH), 12.46 (br.s, 1H, N–NH). EIMS (70 eV): *m/z* (%) 206 (1), 191 (1), 159 (4), 149 (16), 148 (11), 146 (9), 136 (1), 135 (1), 133 (6), 132 (6), 131 (4), 119 (4), 117 (5), 106 (9), 105 (7), 92 (81), 91 (100), 89 (8), 85 (4), 84 (3), 78 (11), 71 (6), 70 (4), 69 (5), 65 (13), 64 (18), 60 (1), 57 (14), 56 (6).

4.2. In vitro cytotoxicity screening

The human tumour cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells were inoculated into 96 well microtiter plates in 100 μL at plating densities ranging from 5000 to 40,000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs.

After 24 h, two plates of each cell line were fixed in situ with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs were solubilized in dimethyl sulphoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 μg mL^{-1} gentamicin. Additional four, 10-fold or 1/2 log serial dilutions were made to provide a total of five drug concentrations plus control. Aliquots of 100 μl of these different drug dilutions were added to the appropriate microtiter wells already containing 100 μl of medium, resulting in the required final drug concentrations.

Following drug addition, the plates were incubated for an additional 48 h at 37 °C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 μl of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant was discarded, and the plates were washed five times with tap water and air dried. Sulphorhodamine B (SRB) solution (100 μl) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology was the same except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 μl of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth was calculated at each of the drug concentrations levels. Percentage GI was calculated as:

$$[(\mathrm{Ti}-\mathrm{Tz})/(C-\mathrm{Tz})] \times 100 \text{ for concentrations for which } \mathrm{Ti} \geq \mathrm{Tz}$$

$$[(\mathrm{Ti}-\mathrm{Tz})/\mathrm{Tz}] \times 100 \text{ for concentrations for which } \mathrm{Ti} < \mathrm{Tz}.$$

Three dose response parameters were calculated for each experimental agent. GI_{50} was calculated from $[(\mathrm{Ti}-\mathrm{Tz})/(C-\mathrm{Tz})] \times 100 = 50$, which was the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in TGI was calculated from Ti=Tz. Values were calculated for each of these parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the value for that parameter was expressed as greater or less than the maximum or minimum concentration tested.

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