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Original article

Synthesis of substituted 3-amino-N-phenyl-1H-indazole-1-carboxamides endowed with antiproliferative activity

Benedetta Maggio [a], Maria Valeria Raimondi [a], Demetrio Raffa [a], Fabiana Plescia [a], Stella Cascioferro [a], Salvatore Plescia [a], Manlio Tolomeo [b], Antonietta Di Cristina [b], Rosaria Maria Pipitone [b], Stefania Grimaudo [b], Giuseppe Daidone [a,*]

[a] Dipartimento di Chimica e Tecnologie Farmaceutiche, Università degli Studi di Palermo, Via Archirafi, 32, 90123-Palermo, Italy
[b] Centro Interdipartimentale di Ricerca in Oncologia Clinica e Dipartimento Biomedico di Medicina Interna e Specialistica, Università di Palermo, Palermo, Italy

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ABSTRACT

Several new N-phenyl-1H-indazole-1-carboxamides **1c**–**h** and **4l**,**m** were prepared by reacting phenyl isocyanate derivatives **3a**,**b** with 3-amino-1H-indazole derivatives **2c**,**e**,**g** or 1H-indazole **2l** respectively. Chemical transformations of compounds **1a**,**b** and **1g**,**h** gave 3-acetamido-N-phenyl-1H-indazole-1-carboxamide derivatives **5a**,**b**, and 3,5-diamino-N-phenyl-1H-indazole-1-carboxamide derivatives **4i**, **j** respectively. Finally, 3,5-diacetamido-N-phenyl-1H-indazole-1-carboxamide derivatives **6a**,**b** were prepared by acetylation of **4i**, **j**. Some of synthesized compounds were evaluated for their *in vitro* antiproliferative activity against the full NCI tumor cell lines panel derived from nine clinically isolated cancer types (leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate and breast). Compound **1c**, the most active of the series, was able to inhibit cell growth showing GI_{50} values in the 0.041–33.6 μM range, mean GI_{50} 1.90 μM, being very effective against colon and melanoma cell lines. Cell cycle analysis in K562 cells showed that **1c** causes a marked increase of cells in G0–G1 phase. Moreover, it increases the ratio between hypophosphorylated pRb and total pRb.

1. Introduction

In the literature are reported several examples of indazole derivatives endowed with antineoplastic activity [1–7].

Recently our research group has reported the synthesis of some 3-amino-N-phenyl-1H-indazole-1-carboxamide derivatives of type **1** (Fig. 1). Among these, compounds **1a** and **1b** were able to inhibit at low micromolar concentrations the cell growth of many neoplastic cell lines of the NCI 60 human cell lines panel, being the benzyl derivative **1a** quite more active than the butoxy analogue **1b** [8]. Compounds **1a**,**b** caused a marked arrest of cells in G_0–G_1 phase and a decrease of the phosphorylated form of retinoblastoma protein (pRb) which is involved in conferring uncontrolled growth to many neoplastic cells [9–11].

The above compounds are not substituted in the indazole nucleus and this gives the hope to obtain more active compounds if a suitable substituent is bore by the above nucleus. In fact, it is well documented in the literature that substitution may greatly influence the activity. For this reason, we synthesized new 3-amino-N-phenyl-1H-indazole-1-carboxamide derivatives of type **1a** and **1b**, substituted in the indazole nucleus, in order to estimate as different substituents can influence the antiproliferative activity. Moreover, the influence of the 3-amino group on the activity was evaluated by comparing the activity of **1b** in respect of **4m** which is devoid of this group. Finally, we verified whether the substitution of the amino group with the acetamido one in compounds **1b** and **4j** may afford some advantage for the antiproliferative activity.

2. Results

2.1. Chemistry

The 1H-indazole-1-carboxamide derivatives **1c**–**h**, **4i**–**m**, **5a**,**b** and **6a**,**b** were obtained as reported in the Scheme 1. In particular, derivatives **1c**–**h** and **4l**,**m** were synthesized reacting 3-amino-indazoles **2c**,**e**,**g** or indazole **2l** respectively with the isocyanates **3a**,**b** under the same experimental conditions utilized for the obtaining of **1a**,**b** [8]. Products of type 7 derived from the reaction of the indazole 3-amino group with one molecule of isocyanate derivative **3** were not isolated from the reaction mixtures. As regards the 3,5-diaminoderivatives **4i**, **j** , they were obtained reducing with hydrogen and 10% Pd–C the parent 5-nitroderivatives **1g**,**h**. Finally,

* Corresponding author. Tel.: +39 91 23891916.
E-mail address: gdaidone@unipa.it (G. Daidone).

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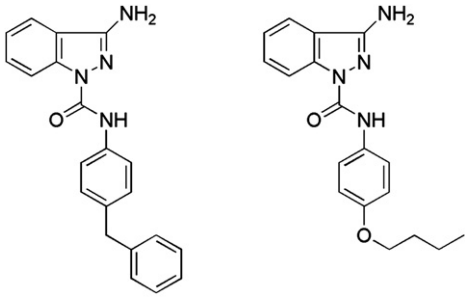


Fig. 1. Structure of 3-amino-N-phenyl-1H-indazole-1-carboxamide derivatives.

the acetyl derivatives **5a**,**b** and **6a**,**b** were obtained from the reaction of acetic anhydride with **1a**,**b** and **4i**, **j** respectively.

All the above compounds were identified on the basis of satisfactory elemental and spectroscopic data. In particular, the structure assignation for compounds **1c**–**h** and **4i**, **j** was based on the presence of the 3-NH_2 signal as a singlet integrated for two protons in the ^{1}H NMR spectra at 6.03–6.07 δ which, as a consequence, ruled out a structure of type **7**. All the above results accorded with our previously reported data for the 3-amino-N-phenyl-1H-indazole-1-carboxamide derivatives which are not substituted in the indazole nucleus, e.g. **1a**,**b** [8]. In the case of **4i**, **j** the spectra showed also the signal for the further 5-amino group at about 5.06 δ. Finally, the ^{1}H NMR spectra of all compounds showed the signal for the 1-carboxamide NH proton in the 9.17–10.33 δ range. ^{13}C NMR spectra of four representative compounds (**4m**, **1f**, **4j**, and **6a**) confirmed the proposed structures.

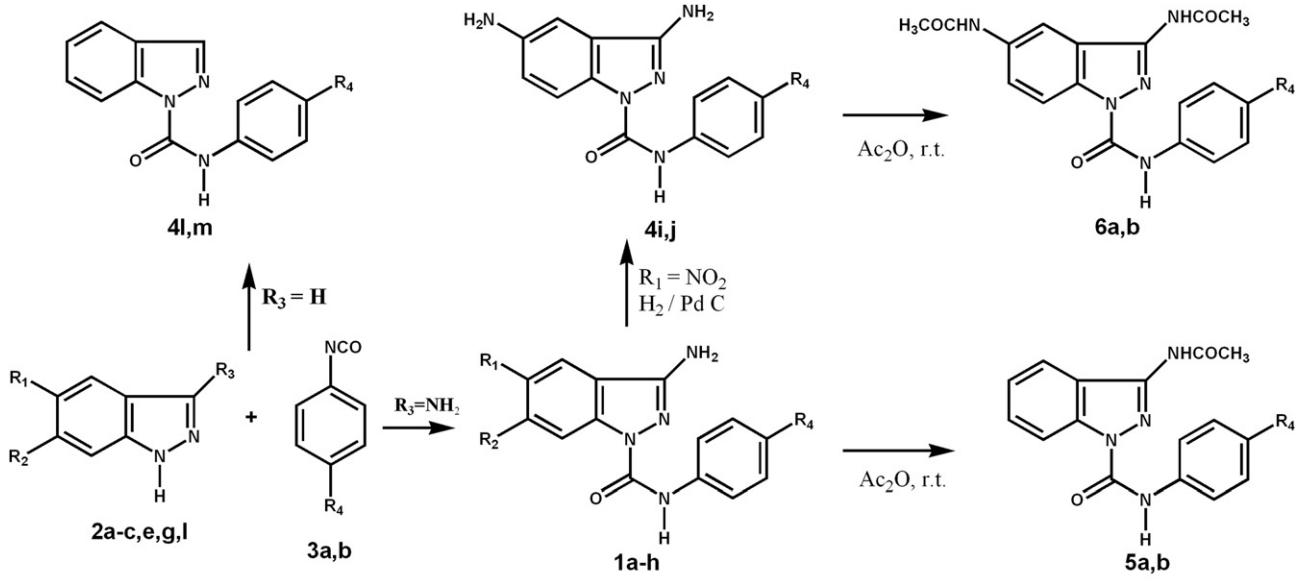
2.2. Biology

2.2.1. Tumor cell line screening

Among the synthesized indazole derivatives, that is **1c**–**h**, **4i**–**m**, **5a**,**b** and **6a**,**b** , eight of them (**1c**,**d**, **4i**, **j**,**m**, **5b**, **6a**,**b**) were selected by the National Cancer Institute (NCI), Bethesda, USA, and tested initially at a single dose (10 μM) in the full NCI cells panel derived from nine clinically isolated cancer types (leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate and breast) [12]. Compounds **1c** and **4i** resulted to be quite more active than the remaining compounds **1d**, **4j**,**m**, **5b** and **6a**,**b**. Moreover, they satisfied the NCI pre-determined threshold inhibition criteria and therefore were also evaluated in the 5-dose screening (0.01, 0.1, 1, 10, 100 μM). The antiproliferative activity of **1c**, **4i** and the previously synthesized derivative **1a**, considered for comparison purposes, is showed in Table 1. The antiproliferative activity of a test compound is given by three parameters for each cell line; GI_{50} (compound's molar concentration inhibiting 50% net cell growth), TGI (compound's molar concentration for total inhibition of net cell growth), and LC_{50} (compound's molar concentration inducing 50% net cell death).

2.2.2. Cell cycle analysis

In consideration of the above results for **1c**, and in order to obtain more insight on its mechanism of action, we thought it



	R_2	R_3	R_4
a	H	NH_2	$CH_2C_6H_5$
b	H	NH_2	OC_4H_9
c	Cl	NH_2	$CH_2C_6H_5$
d	Cl	NH_2	OC_4H_9
e	H	NH_2	$CH_2C_6H_5$
f	H	NH_2	OC_4H_9
g	NO_2	NH_2	$CH_2C_6H_5$
h	NO_2	NH_2	OC_4H_9
i	NH_2	NH_2	$CH_2C_6H_5$
j	NH_2	NH_2	OC_4H_9
l	H	H	$CH_2C_6H_5$
m	H	H	OC_4H_9

Scheme 1. Synthetic pathway of compounds **1c**–**h**, **4i**–**m**, **5a**,**b** and **6a**,**b**.

Table 1
In vitro antiproliferative activity (μM) for **1a**, **1c** and **4i** against the full NCI cell lines panel derived from nine clinically isolated human cancer types.

Cancer types		**1a**			**1c**			**4i**		
	Panel/cell line	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
Leukemia	CCRF-CEM	Nt	Nt	Nt	1.23	34.4	>100	2.07	18.8	>100
	HL-60 (TB)	0.466	15.2	54.9	2.62	42.1	>100	3.46	17.2	72.2
	K-562	1.20	33.8	>100	0.0357	14.7	>100	2.24	11.4	>100
	MOLT-4	0.636	20.4	>100	2.41	55.3	>100	4.48	24.3	>100
	RPMI-8226	1.86	25.8	84.0	2.58	46.5	>100	5.14	35.0	>100
Non-small cell lung	A549/ATCC	12.9	48.6	>100	2.52	>100	>100	6.58	57.1	>100
	EKVX	11.2	92.0	>100	3.19	97.6	>100	14.0	36.7	96.1
	HOP-62	13.7	46.5	>100	1.21	16.4	59.7	11.2	27.0	65.3
	HOP-92	14.2	42.6	>100	10.7	33.7	>100	10.4	24.4	57.4
	NCI-H226	13.5	58.2	>100	15.4	66.7	>100	16.1	38.3	91.4
	NCI-H23	16.8	83.5	>100	5.34	68.4	>100	16.9	39.5	92.4
	NCI-H322M	27.8	>100	>100	18.2	>100	>100	13.0	27.5	58.3
	NCI-H460	0.408	13.0	84.6	0.0645	14.7	60.6	4.64	23.8	>100
	NCI-H522	2.90	>100	>100	15.6	>100	>100	14.7	39.6	>100
Colon	COLO 205	13.4	37.7	>100	0.588	23.8	>100	13.1	26.5	53.9
	HCC-2998	4.27	35.9	>100	0.238	1.53	>100	4.17	14.8	40.9
	HCT-116	7.03	>100	>100	0.467	25.1	77.2	6.79	20.8	50.0
	HCT-15	1.08	>100	>100	0.0458	>100	>100	2.75	14.2	50.5
	HT29	2.39	17.6	56.3	1.15	24.0	79.1	4.51	17.2	46.0
	KM12	2.94	>100	>100	0.0726	>100	>100	3.34	15.1	41.3
	SW-620	3.62	41.5	>100	0.0565	76.5	>100	4.48	18.7	58.5
CNS	SF-268	4.66	32.3	>100	13.0	47.7	>100	13.0	30.7	72.5
	SF-295	0.530	>100	>100	0.0515	24.2	>100	3.96	15.1	45.4
	SF539	0.495	5.36	>100	1.51	11.5	38.8	8.70	22.1	51.8
	SNB-19	22.8	>100	>100	14.6	43.3	>100	13.9	27.3	53.6
	SNB75	17.0	47.4	>100	14.0	32.3	74.5	17.4	31.5	57.1
	U251	13.9	30.2	65.9	12.5	27.0	58.4	6.53	20.9	52.0
Melanoma	LOX IMVI	0.477	16.1	61.5	0.0503	18.4	53.2	3.38	16.0	46.7
	MALME-3M	19.2	64.1	>100	6.69	54.8	>100	18.0	89.8	>100
	M14	0.772	>100	>100	0.0888	14.0	>100	4.59	17.8	51.9
	MDA-MB-435	Nt	Nt	Nt	0.0813	>100	>100	7.36	23.8	65.5
	SK-MEL-2	36.4	>100	>100	33.6	>100	>100	26.1	48.5	90.1
	SK-MEL-28	11.5	>100	>100	18.5	>100	>100	13.7	27.8	56.2
	SK-MEL-5	2.22	8.64	34.3	0.283	21.1	80.2	6.52	19.8	46.4
	UACC-257	10.0	50.9	>100	4.92	>100	>100	14.5	29.7	60.8
	UACC-62	0.353	>100	>100	0.0412	>100	>100	2.90	12.6	39.3
Ovarian	IGROV1	6.98	48.8	>100	13.7	>100	>100	16.4	54.1	>100
	OVCAR-3	6.23	63.1	>100	6.66	20.8	48.2	12.1	28.3	66.7
	OVCAR-4	10.7	40.4	>100	5.73	34.3	>100	8.29	24.1	63.2
	OVCAR-5	31.3	>100	>100	18.8	>100	>100	22.6	60.5	>100
	OVCAR-8	21.9	63.9	>100	14.9	>100	>100	20.2	>100	>100
	NCI/ADR-RES	Nt	Nt	Nt	11.0	39.1	>100	17.4	75.1	>100
	SK-OV-3	36.9	>100	>100	15.8	50.5	>100	15.3	37.7	92.8
Renal	786-0	3.76	26.2	92.7	3.92	28.2	94.4	12.4	25.9	54.3
	A498	16.7	31.5	59.6	16.1	60.7	>100	14.4	28.4	56.1
	ACHN	1.69	33.4	>100	0.0468	17.4	>100	3.37	13.7	39.6
	CAKI-1	21.1	58.0	>100	0.217	>100	>100	4.30	26.8	>100
	RXF 393	22.2	58.2	>100	13.5	31.5	73.4	21.4	43.9	90.1
	SN12C	12.9	28.0	60.6	14.6	42.9	>100	13.4	30.9	71.1
	TK-10	27.2	>100	>100	15.6	38.7	96.3	19.4	46.0	>100
	UO-31	0.347	>100	>100	3.12	84.5	>100	4.72	32.6	>100
Prostate	PC-3	13.5	51.1	>100	2.90	44.3	>100	4.33	24.0	87.0
	DU-145	1.02	28.3	>100	10.4	28.9	80.0	10.9	24.0	52.6
Breast	MCF7	0.555	8.82	89.1	0.395	18.8	84.0	6.92	26.5	85.4
	MDA-MB-231/ATCC	0.847	43.9	>100	5.33	37.6	>100	7.74	22.7	54.3
	HS 578T	17.2	71.8	>100	13.3	41.7	>100	17.3	43.0	>100
	BT-549	13.5	39.6	>100	5.88	44.0	>100	14.6	33.5	76.7
	T-47D	1.55	>100	>100	0.0741	27.6	>100	5.80	22.8	66.0

Nt = not tested, GI_{50} = compound's molar concentration inhibiting 50% net cell growth, TGI = compound's molar concentration for total inhibition of net cell growth, LC_{50} = compound's molar concentration inducing 50% net cell death.

would be of interest to study the effect of this compound on the cell cycle distribution. The effect of **1c** on cell cycle distribution was analyzed in K562 cells. Cells were cultured for 24 h in the presence of **1c** used at 20, 40 and 60 μM and then evaluated after this time. Flow cytometric analysis of cell cycle was carried out as described in the Experimental Section and is reported in Fig. 2. The analysis of cell cycle performed at 60 μM showed a marked block in G0–G1 phase.

2.2.3. Effects of retinoblastoma protein (pRb)

The retinoblastoma gene encodes a nuclear phosphoprotein (pRb) expressed in the majority of vertebrates normal cells and acts as a tumor suppressor factor, playing a key role in the control of cell division [9]. The pRb hypophosphorylated form is mainly found in resting cells, whereas the highly phosphorylated forms are present in proliferating cells. Growth factors act to promote cells through the G1 and S phase of cell cycle and, during this time, pRb

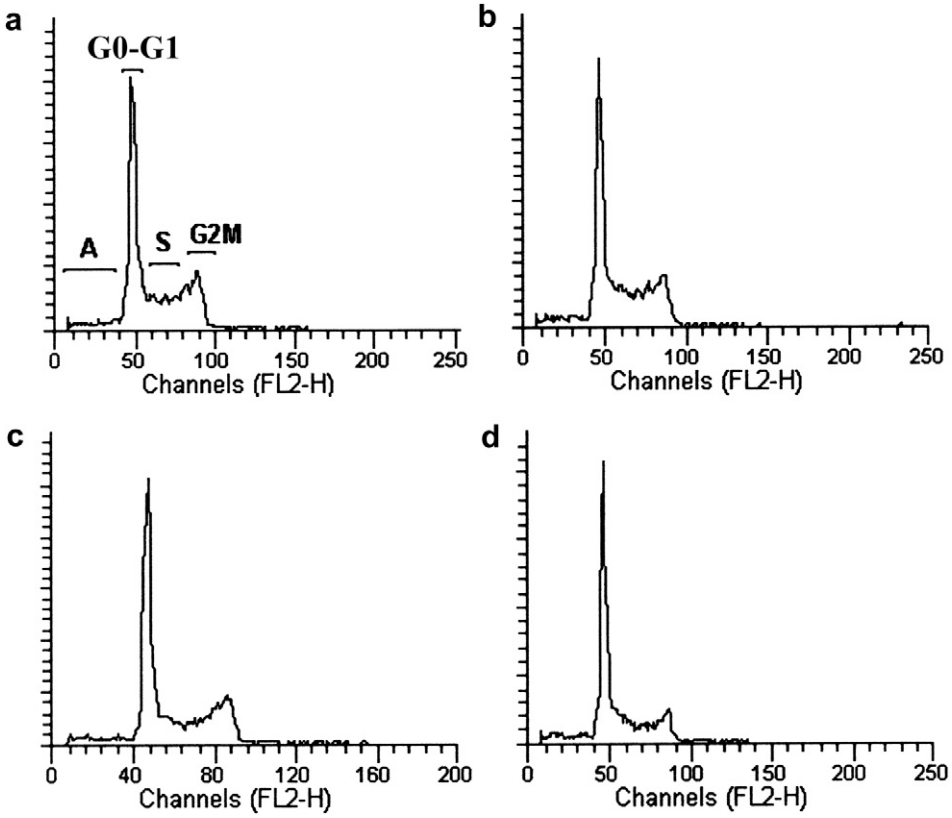


Fig. 2. Effects of compound **1c** on DNA content following the treatment of K562 cells for 24 h. The cells were cultured a) without compounds, or with each compound used at the following concentrations: b) 20 μM; c) 40 μM; d) 60 μM. Cell-cycle distribution was analyzed by the standard propidium iodide procedure. Sub-G0–G1 (A), G0–G1, S, and G2–M cells are indicated in panel a).

undergoes sequential phosphorylation. The pRb hypophosphorylated form binds to critical regulatory proteins (including E2F) and represses the transcription of genes involved in cell cycle progression from the G0–G1 to the S phase [10]. Fig. 3 shows the effects of **1c** on total pRb and hypophosphorylated pRb levels in K562 cells. Flow cytometric analysis of pRb and hypophosphorylated pRb were carried out as described in the Experimental Section. Compound **1c** induced a marked increase of hypophosphorylated pRb form and only a slow increase of total pRb. This is congruent with the G0–G1 block observed in Fig. 2.

3. Discussion

The pharmacological results of all the tested compounds showed that the 5-chloro derivative **1c** produced the best antiproliferative profile. The remarkable difference of activity between **1c** and the analogous derivative **1d** is due only to the different nature of substituent R4: benzyl for **1c** and butoxy for **1d**. As the benzyl group is more lipophilic in character than the butoxy one, it seem that lipophilicity plays a role in the antiproliferative activity of **1c**,**d**. This trend was previously observed for the compounds **1a** and **1b** [8]. Compound **1c** showed antiproliferative activity against almost every type of tumor cell lines studied. It was particularly efficacious against all types of colon cancer cell lines and some other cell lines such as leukemia (K562), non-small cell lung cancer (NCI-H460), CNS cancer (SF-295), melanoma (LOX IMVI, M14, MDA-MB-435, SK-MEL-5, UACC-G2), renal cancer (ACHN, CAKI-1), breast cancer (MCF7, T-47D). Good activity was also showed, with the exception of UO-31 and SK-MEL-2, against the remaining cell lines, being the GI_{50} values in the 1.21–18.8 μM range (see Table 1). Growth inhibition data of **1c** against the most sensitive cell lines are reported in Table 2.

The ratio obtained by dividing the compound's GI_{50} full panel mean graph midpoint (MG-MID, the average sensitivity of all cell lines against a test compound) by its individual sub-panel mean GI_{50} value (the average sensitivity of each sub-panel against the same compound) is considered as a measure of compound selectivity. Ratios between 3 and 6 refer to moderate selectivity, ratio >6 indicate high selectivity towards the corresponding cancer type, while compounds meeting neither of these criteria are rated non-selective [13,14]. Compound **1c** resulted to be highly selective against colon cancer with selectivity index of 9.5 and a sub-panel mean GI_{50} value of 0.20 μM.

As regards the 5-amino derivative **4i**, it proved to be non-selective and, though less active than **1c**, showed good activity against all the cancer cell lines, with the exception of NCI-H460, SK-MEL-2 and RXF 393. The best results were observed against leukemia cell, with GI_{50} values in the 2.07–5.14 μM range (see Table 1). The most significant values of GI_{50} of **4i** against sensitive cell lines are reported in Table 2.

The decreased activity of **4i** than **1c** is due to the substitution in **1c** of the chloro atom with the amino group. As the chloro atom is lipophilic in character ($\Pi = 0.71$) whereas the amino group is essentially hydrophilic ($\Pi = -1.23$), it is possible that the difference of activity between **1c** and **4i** be correlated to higher lipophilicity of **1c** than that of **4i**. This agrees with the pharmacological results observed for **1a**/**1b** [8] and **1c**/**1d**.

The presence of the 3-amino group in the indazole derivatives is on the whole of some advantage for the activity. In fact, comparing the full panel mean growth percent values at 10 μM of **1b** (80.93%) and **4m** (95.19%) we can observe that **4m**, devoid of the amino

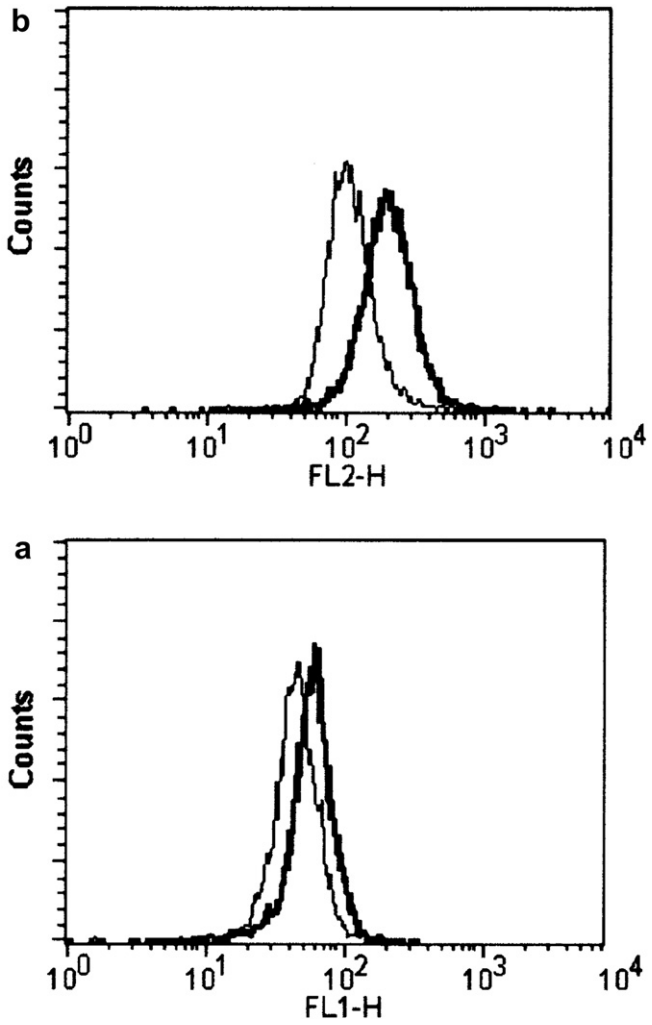


Fig. 3. Effects of **1c** on total Rb and hypophosphorylated Rb expression evaluated by flow cytometry after staining K562 cells with a fluorescein isothiocyanate (FITC)-conjugated anti-Rb mAb, or a phycoerythrin (PE)-conjugated mAb raised against hypophosphorylated Rb. a) Expression of total Rb in cells treated with (dark-gray line) or without (light-gray line) 60 μM 1c. b) Expression of hypophosphorylated Rb in cells treated with (dark-gray line) or without (light-gray line) 60 μM 1c.

group, is less active than **1b**. Moreover, acetylation of the 3-amino group of **1b** to give **5b** (mean GI%=85.29) does not take any advantage for activity. Analogous results have been obtained with the contemporary 3 and 5-acetylation of **4j** to give **6b** (mean GI % = 94.98).

Compounds **1c** and **4j** were tested for their toxicity towards normal human fibroblast cells showing GI_{50} values of 36 and 32 μM respectively. The above values, when compared than the GI_{50} values of **1c** and **4j** obtained for the most sensitive tumor cell lines (see Table 2), indicated a selective effect of these compounds towards the tumor cell lines. Compound **1c** was particularly selective, showing very valuable therapeutic indexes (1020–6.28 range).

To understand the mechanism/s of action of **1c**, cell cycle analysis was performed. The effect of **1c** on cell cycle was studied on K562 to avoid the appearance of a sub-G0–G1 apoptotic peak that could mask the real effect of the compound on the G0–G1–S–G2–M phases of cell cycle. In fact, K562 cells are resistant to early drug-induced apoptosis. Compound **1c** induced an increase of cells in G0–G1 peak and a decrease of cells in S a G2–M phases when used at a concentration of or higher than 40 μM. This could be due to a block of G1–S transition caused by the ability of **1c** to increase the hypophosphorylate pRb/total pRb ratio. In fact, The pRb hypophosphorylated form binds to critical regulatory proteins (including E2F) and represses the transcription of genes involved in cell cycle progression from the G0–G1 to the S phase [10].

Table 2
In vitro antiproliferative activity (μM) against the cell lines in which the compounds **1c** and **4i** resulted mostly active.

	Panel/cell line	**1c**			**4i**		
		GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
Leukemia	CCRF-CEM	1.23	34.4	>100	2.07	18.8	>100
	K-562	0.0357	14.7	>100	2.24	11.4	>100
Non-small cell	NCI-H460	0.0645	14.7	60.6	4.64	23.8	>100
Colon cancer	COLO 205	0.588	23.8	>100	13.1	26.5	53.9
	HCC-2998	0.238	1.53	>100	4.17	14.8	40.9
	HCT-116	0.467	25.1	77.5	6.79	20.8	50.0
	HCT-15	0.0458	>100	>100	2.75	14.2	50.5
	HT29	1.15	24.0	79.1	4.51	17.2	46.0
	KM12	0.0726	>100	>100	3.34	15.1	41.3
	SW-602	0.0565	76.5	>100	4.48	18.7	58.5
CNS cancer	SF-295	0.515	24.2	>100	3.96	15.1	45.4
Melanoma	LOX IMVI	0.0503	18.4	53.2	3.38	16.0	46.7
	M14	0.0888	14.5	>100	4.59	17.8	51.9
	MDA-MB-435	0.0813	>100	>100	7.36	23.8	65.5
	SK-MEL-5	0.2834	21.1	80.2	6.52	19.8	46.4
	UACC-62	0.0412	>100	>100	2.90	12.6	39.3
Ovarian cancer	OVCAR-4	5.73	34.3	>100	8.29	24.1	63.2
Renal cancer	ACHN	0.0468	17.4	>100	3.37	13.7	39.6
	CAKI-1	0.217	>100	>100	4.30	26.8	>100
Prostate cancer	PC-3	2.90	44.3	>100	4.33	24.0	87.0
Breast cancer	MCF7	0.395	18.8	84.0	6.92	26.5	85.4
	T-47D	0.0741	27.6	>100	5.80	22.8	66.0

GI_{50} = compound's molar concentration inhibiting 50% net cell growth, TGI = compound's molar concentration for total inhibition of net cell growth, LC_{50} = compound's molar concentration inducing 50% net cell death.

4. Conclusions

The biological data obtained for **1c** have demonstrated that the presence of the chloro atom in the indazole nucleus increases the antiproliferative activity of the parent compound **1a**, accomplishing in this way the aim of our research. The ability of **1c** to block cancer cells in the G0–G1 phase by inhibiting the phosphorylation of pRb can be considered of interest because mutations in the retinoblastoma gene (RB-1) have been described in a wide variety of neoplasms and constitutive phosphorylation of pRb has been implicated in conferring uncontrolled growth to many cancer cells [11,15].

5. Experimental

5.1. Chemistry

5.1.1. General

Reaction progress was monitored by TLC on silica gel plates (Merck 60, F_{254}, 0.2 mm). Organic solutions were dried over Na_2SO_4. Evaporation refers to the removal of solvent on a rotary evaporator under reduced pressure. All melting points were determined on a Büchi 530 capillary melting point apparatus and are uncorrected. IR spectra were recorded with a Perkin Elmer Spectrum RXI FT-IR System spectrophotometer as solid in KBr disc. ^1H NMR and ^{13}C NMR spectra were obtained in DMSO-d_6 at 300.13 and 75.47 MHz respectively, using a Bruker AC series 300 MHz spectrometer (tetramethylsilane as an internal standard): chemical shifts are expressed in δ values (ppm). Merck silica gel (Kiesegel 60/230–400 mesh) was used for flash chromatography columns. Analyses indicated by the symbols of the elements were within ±0.3% of the theoretical values. They were obtained by an

Elemental Vario EL. III apparatus. Yields refer to purified products and are not optimized. The name of the compounds was obtained using the ACD/I-Lab Web service (ACD/IUPAC Name Free 8.05).

5.1.2. General procedure for the preparation of compounds ***1c–h,l,m***

A solution of equimolar amounts of indazole derivative **2c**,**e** [16], **g** [17] or indazole **2l** [18] and phenyl isocyanate derivative **3a**,**b** [18] (7 mmol) in THF (20 mL) was stirred at room temperature for 24 h. Then, the solvent was removed under reduced pressure and the solid residue was crystallized from a suitable solvent or purified as described below.

*5.1.2.1. 3-Amino-N-(4-benzylphenyl)-5-chloro-1H-indazole-1-carboxamide (**1c**).* The solid collected was crystallized from ethanol the first time and then from methanol. Yield: 55%; m.p.: 142–143 °C; IR (KBr) ν [cm^{-1}]: 3459–3352 (NH, NH_2), 1700 (CO); ^1H NMR (DMSO-d_6) δ [ppm]: 3.91 (s, 2H, CH_2), 6.39 (s, 2H, slowly exchangeable, NH_2,), 7.18–8.18 (a set of signals, 12H, aromatic protons), 9.47 (s, 1H, exchangeable, NH). Anal. ($C_{21}H_{17}ClN_4O$) C,H,N.

*5.1.2.2. 3-Amino-N-(4-butoxyphenyl)-5-chloro-1H-indazole-1-carboxamide (**1d**).* The product was purified by crystallization from ethanol and preparative TLC (silica gel plate, layer thickness 2 mm, chloroform as eluent). Yield: 55%; m.p.: 116–117 °C (ethanol); IR (KBr) ν [cm^{-1}]: 3385–3303 (NH, NH_2), 1698 (CO); ^1H NMR (DMSO-d_6) δ [ppm]: 0.93 (t, 3H, CH_3), 1.44 (m, 2H, CH_2), 1.68 (m, 2H, CH_2), 3.94 (t, 2H, CH_2), 6.37 (s, 2H, exchangeable, NH_2,), 6.89–8.18 (a set of signals, 7H, aromatic protons), 9.43 (s, 1H, slowly exchangeable, NH). Anal. ($C_{18}H_{19}ClN_4O_2$) C,H,N.

*5.1.2.3. 3-Amino-N-(4-benzylphenyl)-6-chloro-1H-indazole-1-carboxamide (**1e**).* The product was purified by crystallization from ethanol and preparative TLC (silica gel plate, layer thickness 2 mm, chloroform as eluent). Yield: 73%; m.p.: 140–142 °C (ethanol); IR (KBr) ν [cm^{-1}]: 3454–3352 (NH, NH_2), 1693 (CO); ^1H NMR (DMSO-d_6) δ [ppm]: 3.88 (s, 2H, CH_2), 6.43 (s, 2H, exchangeable, NH_2,), 7.16–8.19 (a set of signals, 12H, aromatic protons), 9.47 (s, 1H, exchangeable, NH). Anal. ($C_{21}H_{17}ClN_4O$) C,H,N.

*5.1.2.4. 3-Amino-N-(4-butoxyphenyl)-6-chloro-1H-indazole-1-carboxamide (**1f**).* The product was purified by crystallization from ethanol and preparative TLC (silica gel plate, layer thickness 2 mm, chloroform as eluent). Yield: 56%; m.p.: 124–126 °C (ethanol); IR (KBr) ν [cm^{-1}]: 3386–3305 (NH, NH_2), 1701 (CO); ^1H NMR (DMSO-d_6) δ [ppm]: 0.93 (t, 3H, CH_3), 1.43 (m, 2H, CH_2), 1.67 (m, 2H, CH_2), 3.91 (t, 2H, CH_2), 6.43 (s, 2H, slowly exchangeable, NH_2,), 6.84–8.21 (a set of signals, 7H, aromatic protons), 9.46 (s, 1H, exchangeable, NH). ^{13}C NMR (DMSO-d_6) δ [ppm]: 13.67 (CH_3), 18.70 (CH_2), 30.74 (CH_2), 67.19 (CH_2), 113.47 (CH), 114.32 (2× CH), 117.60 (C), 121.78 (2× CH), 122.29 (CH), 122.49 (CH), 130.80 (C), 134.10 (C), 139.81 (C), 149.15 (C), 151.10 (C), 154.89 (CO). Anal. ($C_{18}H_{19}ClN_4O_2$) C,H,N.

*5.1.2.5. 3-Amino-N-(4-benzylphenyl)-5-nitro-1H-indazole-1-carboxamide (**1g**).* Yield: 66%; m.p.: 231–233 °C (ethyl acetate); IR (KBr) ν [cm^{-1}]: 3469–3342 (NH, NH_2), 1716 (CO); ^1H NMR (DMSO-d_6) δ [ppm]: 3.92 (s, 2H, CH_2), 6.74 (s, 2H, exchangeable, NH_2), 7.20–9.05 (a set of signals, 12H, aromatic protons), 9.70 (s, 1H, exchangeable, NH). Anal. ($C_{21}H_{17}N_5O_3$) C,H,N.

*5.1.2.6. 3-Amino-N-(4-butoxyphenyl)-5-nitro-1H-indazole-1-carboxamide (**1h**).* Yield: 72%; m.p.: 181–183 °C (ethyl acetate); IR (KBr) ν [cm^{-1}]: 3454–3365 (NH, NH_2), 1716 (CO); ^1H NMR (DMSO-d_6) δ [ppm]: 0.93 (t, 3H, CH_3), 1.43 (m, 2H, CH_2), 1.68 (m, 2H, CH_2), 3.93 (t, 2H, CH_2), 6.70 (s, 2H, exchangeable NH_2), 6.89–9.04 (a set of signals, 7H, aromatic protons), 9.60 (s, 1H, exchangeable NH). Anal. ($C_{18}H_{19}N_5O_4$) C,H,N.

*5.1.2.7. N-(4-Benzylphenyl)-1H-indazole-1-carboxamide (**4l**).* Yield: 80%; m.p.: 92–94 °C (ethanol); IR (KBr) ν [cm^{-1}]: 3382 (NH), 1727 (CO); ^1H NMR (DMSO-d_6) δ [ppm]: 3.93 (s, 2H, CH_2), 7.19–8.51 (a set of signals, 14H, aromatic protons), 10.33 (s, 1H, exchangeable NH). Anal. ($C_{21}H_{17}N_3O$) C,H,N.

*5.1.2.8. N-(4-butoxyphenyl)-1H-indazole-1-carboxamide (**4m**).* Yield: 60%; m.p.: 54–56 °C (ethanol); IR (KBr) ν [cm^{-1}]: 3373–3359 (NH), 1722 (CO); ^1H NMR (DMSO-d_6) δ [ppm]: 0.94 (t, 3H, CH_3), 1.43 (m, 2H, CH_2), 1.69 (m, 2H, CH_2), 3.95 (t, 2H, CH_2), 6.93–8.49 (a set of signals, 9H, aromatic protons), 10.23 (s, 1H, slowly exchangeable, NH). ^{13}C NMR (DMSO-d_6) δ [ppm]: 13.64 (CH_3), 18.70 (CH_2), 30.73 (CH_2), 67.18 (CH_2), 114.08 (CH), 114.28 (2× CH), 121.55 (CH), 122.43 (CH), 123.35 (2× CH), 125.49 (C), 128.90 (CH), 130.52 (C), 138.01 (CH), 138.75 (C), 149.16 (C), 155.22 (CO). Anal. ($C_{18}H_{19}N_3O_2$) C,H,N.

5.1.3. General procedure for the preparation of compounds ***4i,j***

To a solution of 1.3 mmol of compound **4g**,**h** in 250 mL of warm solvent (ethyl acetate for **4g** and methanol for **4h**), 50 mg of 10% Pd–C as catalyst was added. The mixture was left under hydrogenation in a Parr apparatus at 50 psi for 20 h. The suspension was filtered and the filtrate was evaporated affording the crude compound **4i**, **j** which was purified by flash chromatography and crystallization as described below.

*5.1.3.1. 3,5-Diamino-N-(4-benzylphenyl)-1H-indazole-1-carboxamide (**4i**).* Flash chromatography procedure on silica gel (0.040–0.063 mm): external diameter of the column 6 cm, ethyl acetate/petroleum ether b.p. 40–60 °C (8:2 v/v) as eluent (2.3 L). Fractions 26–46 (each 25 mL) were collected and evaporated affording a product which was crystallized from diethyl ether to give **4i**. Yield: 93%; m.p.: 129–131 °C; IR (KBr) ν [cm^{-1}]: 3456–3232 (2× NH_2, NH), 1684 (CO); ^1H NMR (DMSO-d_6) δ [ppm]: 3.89 (s, 2H, CH_2), 5.07 (s, 2H, exchangeable, NH_2), 6.06 (s, 2H, exchangeable, NH_2), 6.84–7.85 (a set of signals, 12H, aromatic protons), 9.17 (s, 1H, exchangeable, NH). Anal. ($C_{21}H_{19}N_5O$) C,H,N.

*5.1.3.2. 3,5-Diamino-N-(4-butoxyphenyl)-1H-indazole-1-carboxamide (**4j**).* Flash chromatography procedure on silica gel (0.040–0.063 mm): external diameter of the column 4 cm, ethyl acetate as eluent (1 L). Fractions 15–20 (each 25 mL) were collected and evaporated affording a product which was crystallized from ethanol. to give **4j**. Yield: 86%; m.p.: 133–134 °C; IR (KBr) ν [cm^{-1}]: 3428–3211 (2× NH_2, NH), 1706 (CO); ^1H NMR (DMSO-d_6) δ [ppm]: 0.92 (t, 3H, CH_3), 1.42 (m, 2H, CH_2), 1.65 (m, 2H, CH_2), 3.92 (t, 2H, CH_2), 5.06 (s, 2H, exchangeable, NH_2), 6.03 (s, 2H, exchangeable, NH_2), 6.86–7.88 (a set of signals, 7H, aromatic protons), 9.12 (s, 1H, exchangeable, NH). ^{13}C NMR (DMSO-d_6) δ [ppm]: 13.63 (CH_3), 16.69 (CH_2), 30.73 (CH_2), 67.16 (CH_2), 102.00 (CH), 114.32 (3× CH), 118.62 (CH), 119.81 (C), 121.16 (2× CH), 131.38 (C), 132.50 (C), 144.07 (C), 149.16 (C), 150.88 (C), 154.46 (CO). Anal. ($C_{18}H_{21}N_5O_2$) C,H,N.

5.1.4. General procedure for the preparation of compounds ***5a,b***

A mixture of compound **1a**,**b** [8] (1 mmol), and acetic anhydride (16 mL) was stirred at room temperature overnight. The solid collected was washed with diethyl ether, then chromatographed and/or crystallized from a suitable solvent.

*5.1.4.1. 3-(Acetylamino)-N-(4-benzylphenyl)-1H-indazole-1-carboxamide (**5a**).* Yield: 89%; m.p.: 174–175 °C (ethyl acetate); IR (KBr) ν [cm^{-1}]: 3383–3264 (NH), 1727 (carboxamide CO), 1676 (CO); ^1H

NMR (DMSO-d_6) δ [ppm]: 2.23 (s, 3H, CH_3), 3.92 (s, 2H, CH_2), 7.25–8.33 (a set of signals, 13H, aromatic protons), 9.88 (s, 1H, exchangeable carboxamide NH), 10.77 (s, 1H, exchangeable, NH). Anal. ($C_{23}H_{20}N_4O_2$) C,H,N.

*5.1.4.2. 3-(Acetylamino)-N-(4-butoxyphenyl)-1H-indazole-1-carboxamide (**5b**).* The product was purified by preparative TLC (silica gel plate, layer thickness 2 mm, ethyl acetate/petroleum ether b.p. 40–60 °C (1:1 v/v) as eluent) and crystallization from ethyl acetate. Yield: 82%; m.p.: 142–144 °C; IR (KBr) ν [cm^{-1}]: 3378–3250 (NH), 1728 (carboxamide CO), 1672 (acetamido CO); ^{1}H NMR (DMSO-d_6) δ [ppm]: 0.96 (t, 3H, CH_3), 1.46 (m, 2H, CH_2), 1.70 (m, 2H, CH_2), 2.23 (s, 3H, CH_3), 3.96 (t, 2H, CH_2), 6.96–8.32 (a set of signals, 8H, aromatic protons), 9.80 (s, 1H, exchangeable, carboxamido NH), 10.73 (s, 1H, exchangeable, NH). Anal. ($C_{20}H_{22}N_4O_3$) C,H,N.

*5.1.5. General procedure for the preparation of compounds **6a**,**b***

A mixture of compound **4i**, **j** (1 mmol) and acetic anhydride (4 mL) was stirred at room temperature overnight, and then cold water was added until precipitation. The solid was filtered off and then crystallized from dioxane to give **6a**,**b**.

*5.1.5.1. 3,5-Bis(acetylamino)-N-(4-benzylphenyl)-1H-indazole-1-carboxamide (**6a**).* Yield: 82%; m.p.: 236–238 °C; IR (KBr) ν [cm^{-1}]: 3373–3259 (3× NH), 1727, 1679 (CO); ^{1}H NMR (DMSO-d_6) δ [ppm]: 2.07 (s, 3H, CH_3), 2.19 (s, 3H, CH_3), 3.92 (s, 2H, CH_2), 7.24–8.19 (a set of signals, 12H, aromatic protons), 9.87 (s, 1H, exchangeable, carboxamido NH), 10.16 (s, 1H, exchangeable, NH), 10.71 (s, 1H, exchangeable, NH). ^{13}C NMR (DMSO-d_6) δ [ppm]: 22.89 (CH_3), 23.83 (CH_3), 40.42 (CH_2), 111.37 (CH), 114.12 (CH), 120.24 (C), 120.55 (2× CH), 122.57 (CH), 125.84 (CH), 128.32 (2× CH), 128.54 (2× CH), 128.84 (2× CH), 134.57 (C), 135.63 (C), 136.01 (C), 136.76 (C), 141.32 (C), 143.40 (C), 148.69 (CO), 168.20 (CO), 169.06 (CO). Anal. ($C_{25}H_{23}N_5O_3$) C,H,N.

*5.1.5.2. 3,5-Bis(acetylamino)-N-(4-butoxyphenyl)-1H-indazole-1-carboxamide (**6b**).* Yield: 74%; m.p.: 200–203 °C; IR (KBr) ν [cm^{-1}]: 3277–3177 (3× NH), 1712, 1654 (CO); ^{1}H NMR (DMSO-d_6) δ [ppm]: 0.94 (t, 3H, CH_3), 1.45 (m, 2H, CH_2), 1.69 (m, 2H, CH_2), 2.08 (s, 3H, CH_3), 2.20 (s, 3H, CH_3), 3.95 (s, 3H, CH_3), 6.93–8.19 (a set of signal, 7H, aromatic protons), 9.83 (s, 1H, exchangeable, carboxamido NH), 10.12 (s, 1H, exchangeable, NH), 10.67 (s, 1H, exchangeable, NH). Anal. ($C_{22}H_{25}N_5O_4$) C,H,N.

5.2. Biology

The *in vitro* antiproliferative activity values were obtained by the Screen Services of the National Cancer Institute (USA) [19].

5.2.1. Flow cytometric analysis of cell cycle distribution

The effects of compound **1c** on cell cycle distribution were studied on K562 cells (myeloblastic leukemia) by flow cytometric analysis after staining with propidium iodide. Cells were exposed 24 h. After treatment cells were washed once in ice-cold phosphate buffered saline medium (PBS–Sigma) and resuspended at 10^6/mL in a hypotonic fluorochrome solution of propidium iodide (Sigma, St Louis, Mo) (50 μg/mL) and nonidet P-40 (Sigma) [0.03% (v/v)] in 0.1% sodium citrate. After 30 min of incubation, the fluorescence of each sample was analyzed as single-parameter frequency histograms by using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The distribution of cells in the cell cycle was analyzed with the ModFit LT3 program (Verity Software House, Inc., Topsham, Me, USA).

5.2.2. Flow cytometric evaluation of pRb

Cells (1.5×10^6) were washed twice with PBS (Sigma) and resuspended in 100 μL Cytofix/Cytoperm solution (Becton Dickinson). After 20 min cells were washed twice with BD Perm/Wash™ buffer solution (Becton Dickinson) and incubated with 20 μL FITC conjugated anti-pRb or Pe conjugated anti-hypophosphorylated pRb monoclonal antibodies (Becton Dickinson) at 4 °C. After 30 min cells were washed twice and analyzed by flow cytometry.

5.2.3. Toxicity evaluation on normal cells

Human normal fibroblasts (2×10^5) were seeded in 1 mL of complete medium constituted by RPMI 1640, fetal calf serum (10%), penicillin (1%) and streptomycin (1%) in 16 mm wells (tissue culture cluster 24 wells, Costar) and incubated at 37 °C in a CO_2 atmosphere. Compounds were added after 24 h. The number of living cells was evaluated after 72 h by trypan blue exclusion method and expressed as percent of control proliferation.

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[18] The compounds are commercially available.
[19] The methodology can be found at http://dtp.nci.nih.gov/.