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Novel 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-one derivatives as potential anti-cancer agents

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ABSTRACT

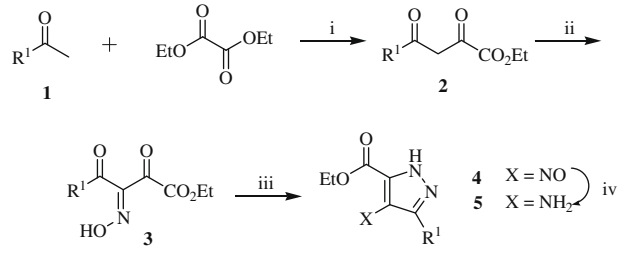
A novel series of 3,5,6-trisubstituted pyrazolo[4,3-*d*]pyrimidin-7-one derivatives, especially 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-ones were synthesized and evaluated for their in vitro anticancer activities against various human cancer cell lines. The inhibitory activities for several kinases have also been tested. The prepared compounds library exhibited significant anticancer activity towards HT-29 colon and DU-145 prostate cancer cell lines. The structure–activity relationships of the 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-one scaffold at R^1, R^2 and R^3 have been elucidated. Among the synthesized compounds, **12b** was the most active compound with GI_{50} value of 0.44 μM and 1.07 μM against HT-29 and DU-145 cell lines, respectively, and **13a** was the most selective compound towards colon cancer cell line.

Cancer, a diverse group of diseases characterized by uncontrolled growth of abnormal cells, is a major worldwide problem. It is a fatal disease standing next to the cardiovascular disease. Although the cancer research has led to a number of new and effective solutions, the medicines used as treatments have clear limitations and unfortunately cancer is projected as the primary cause of death in the future.[1,2] Currently there is a huge scientific and commercial interest in the discovery of new anticancer drugs. Therefore the search for potent, safe and selective anticancer compounds is a crucial aspect of modern cancer research.

Pyrazolopyrimidines have extremely rich biological activities because of their structural similarities with purines.[3] The research by other groups have confirmed that pyrazolo[4,3-*d*]pyrimidin-7-one derivatives are potent and selective inhibitors of type 5 cyclic guanosine-3′,5′-monophosphahate phosphodiesterase (cGMP) PDE-5[4] and, as such, have utility in the treatment of erectile dysfunction.[5] The other pharmacological applications such as memory improvement,[6] CNS stimulation,[7] anti-inflammation,[8] and treatment of heart diseases[9] have been reported. Substituted pyrazolopyridmidinones are also useful as cardiotonic,[10] herbicidal,[11] anticancer[12] and antiviral[13] agents. Although much attention has been paid to the complexes and the biological activities thereof, our interests have been focused onto the relationships between structure of pyrazolo[4,3-*d*]pyrimidinone and their anticancer activities. Herein, we report the synthesis and structure–activity relationship (SAR) of a series of 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-one derivatives as anti-cancer agents.

The general procedure to obtain 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-one derivatives is shown in Schemes 1 and 2. Based on the consideration of simple processes and ample diversity, we derived a concise strategy for synthesizing 3,5,6-trisubstituted pyrazolo[4,3-*d*]pyrimidin-7-one derivatives.

The synthesis of the key intermediate ethyl 4-amino-3-substituted-pyrazol-5-carboxylate **5** is depicted in Scheme 1. The diketo ester **2** was prepared from the commercially available corresponding ketone **1** and diethyl oxalate using sodium ethoxide as a base in 90–95% yield. Addition of sodium nitrite to the ester **2** in glacial acetic acid gave the oxime **3** in 50–60% yield.[14] Treatment of diketo



Scheme 1. Synthesis of ethyl 4-amino-3-substituted-pyrazol-5-carboxylate intermediate. Reagents and conditions: (i) NaOEt, EtOH, rt, overnight; (ii) Aq $NaNO_2$, AcOH, 1 h; (iii) hydrazine hydrate, EtOH, 3 h; (iv) In, HCl, aq THF, 4 h.

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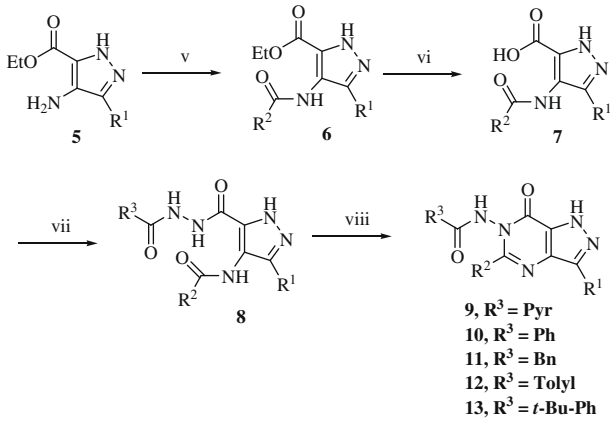


Scheme 2. Synthesis of 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-one derivatives. Reagents and conditions: (v) R_2COCl, pyridine, DMAP, CH2Cl2, rt, 2 h; (vi) 0.5 N NaOH, EtOH, 70 °C, 2.5 h; (vii) $R_3CONHNH_2$, DIC, EtOAc, rt, 3 h; (viii) PPA, Benzene, 80 °C, overnight.

oxime **3** with hydrazine hydrate in EtOH yielded nitroso pyrazole **4** in 95–100%,[15] which was then reduced via indium mediated reduction in the presence of HCl at room temperature in aqueous THF,[16] and purified by neutral alumina column chromatography to give 55–60% yield of desired intermediate ethyl 4-amino-3-substituted-pyrazol-5-carboxylate **5**. By introducing various R^1 groups at position 3 of pyrazole ring, several ethyl 4-amino-3-substituted-pyrazol-5-carboxylate **5** were synthesized and further reactions were carried out.

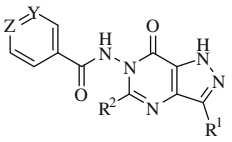
The 4-amine group of pyrazole was acylated by using substituted carbonyl chlorides (Scheme 2). It was carried out according to the previously reported procedure to obtain **6** in 90–95% yield.[17] Hydrolysis of the ester released the free acid **7** in 95–100% yield.[18] The coupling reaction between acid **7** and various arylhydrazine were carried out by activating the carboxyl group with diisopropylcarbodiimide (DIC) in ethyl acetate. After completion of the reaction, pure solid **8** was collected with a good yield of 60–70%.[19] The polyphosphoric acid (PPA) induced cyclo-condensation of **8** in toluene resulted in the final cyclized 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-one derivatives **9–13** in 50–70% yield.[20] The structures of the synthesized compounds were determined by analyses with ^{1}H NMR and ^{13}C NMR spectra.[21]

All the isolated 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-one derivatives were evaluated for anticancer activity against various human cancer cell lines using doxorubicin as internal standard by SRB assay.[22] The cell lines used for this study were the human lung cancer (A-549), human prostate cancer (DU-145), human colon adenocarcinoma (HT-29), human malignant melanoma (SK-MEL-2) and human ovarian carcinoma (SK-OV-3).

As preliminarily evaluation for anticancer activity, the inhibition percentage of selected compounds at 100 μM concentration was evaluated against five human cancer cell lines. The results of the assay are summarized in Table 1.

The structure–activity relationships of 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-one scaffold have been studied based on the in vitro cytotoxicity (Table 1). The data were divided into two groups. The first group of compounds from **9a** to **9k** is comprised of one-nitrogen atom in Y or Z (i.e., a pyridine on R^3) and the second group from **10a** to **10j** has carbon atoms on both Y and Z making R^3 a phenyl group. All the compounds in the first group showed low inhibition percentage within 50% on all cancer cell lines. The best compound in the first group was **9e** (R^1 = 4-ClPh) with the inhibition percentages of 45.97% (HT-29), 54.41% (DU-145), 46.02% (A-549), 39.57% (SK-MEL-2) and 42.48% (SK-OV-3). Compounds **9a**, **9b** and **9h** having alkyl and heterocyclic functionality on R^1 showed poor inhibition indicating the importance of *p*-chlorophenyl group on R^1 position. The second group consisting phenyl

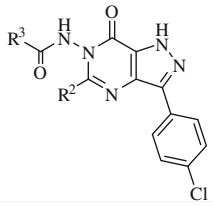
Table 1
Cytotoxicity of selected 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-one derivatives against various human cancer cell lines



Compounds	Substituents				Cytotoxicity (% inhibition, 100 μM)				
	R^1	R^2	Y	Z	HT-29	DU-145	A-549	SK-MEL-2	SK-OV-3
9a	*n*-Pr	3-NO_2Ph	CH	N	−0.83	7.51	9.95	14.21	15.11
9b	*n*-Pr	3-OMePh	CH	N	0.07	9.13	11.18	11.41	4.04
9c	*i*-Bu	3-NO_2Ph	CH	N	ND	55.29	ND	38.73	35.77
9d	4-ClPh	3-NO_2Ph	N	CH	15.12	30.98	19.66	16.28	30.19
9e	4-ClPh	3-OMePh	CH	N	45.97	54.41	46.02	39.57	42.48
9f	4-FPh	3-NO_2Ph	N	CH	24.73	24.86	28.84	24.50	19.28
9g	(4-Piperidine)Ph	3-OMePh	CH	N	28.09	36.99	32.4	23.38	18.22
9h	2-Furan	3-NO_2Ph	N	CH	11.73	1.66	1.51	19.18	-0.61
9i	2-Furan	3-OMePh	N	CH	12.8	30.08	14.87	34.14	24.99
9j	2-Thiophen	3-NO_2Ph	N	CH	3.9	32.77	10.22	3.26	ND
9k	2-Phenethyl	3-NO_2Ph	N	CH	20.12	22.46	18.98	28.49	30.15
10a	*n*-Pr	3-NO_2Ph	CH	CH	18.66	27.76	33.23	22.85	28.82
10b	*n*-Pr	3-OMePh	CH	CH	6.27	23.56	20.73	16.70	9.96
10c	3-ClPh	3-NO_2Ph	CH	CH	89.77	93.82	ND	78.23	ND
10d	3-ClPh	3-OMePh	CH	CH	90.97	93.54	ND	81.87	ND
10e	4-ClPh	3-NO_2Ph	CH	CH	87.81	94.25	88.64	88.95	86.81
10f	4-ClPh	3-OMePh	CH	CH	87.28	94.10	81.79	84.94	81.65
10g	4-FPh	3-NO_2Ph	CH	CH	92.03	90.81	80.68	88.47	77.67
10h	2-Furan	3-NO_2Ph	CH	CH	21.18	16.05	18.74	23.01	17.88
10i	2-Furan	3-OMePh	CH	CH	49.83	50.54	53.78	43.16	35.19
10j	2-Phenethyl	3-NO_2Ph	CH	CH	89.68	92.51	73.88	91.96	ND
Doxorubicin	—	—	—	—	60.89	62.79	ND	ND	ND

ND, not determined.

Table 2
GI_{50} values of 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-one derivatives



Compounds	Substituents		HT-29		DU-145	
	R^2	R^3	%Inhibition, (100 μM)	GI_{50}[a] (μM)	% Inhibition, (100 μM)	GI_{50}[a] (μM)
10e	3-NO_2Ph	Ph	87.81	18.45 ± 1.5	94.25	17.09 ± 2.3
10f	3-OMePh	Ph	87.28	20.36 ± 1.7	94.10	17.90 ± 0.9
10k	Ph	Ph	92.03	20.56 ± 1.6	90.21	20.10 ± 1.3
10l	3-OMeBn	Ph	89.26	22.08 ± 4.0	93.19	44.92 ± 2.719
10m	2-Phenethyl	Ph	86.49	23.44 ± 7.58	85.59	86.72 ± 10.08
10n	*n*-Pr	Ph	91.09	9.26 ± 3.27	89.37	29.24 ± 12.15
10o	*n*-Bu	Ph	89.48	0.71 ± 0.29	93.05	29.13 ± 5.97
10p	*n*-Pent	Ph	90.14	2.26 ± 0.94	88.68	22.10 ± 5.71
10q	2-Cyclopentylethyl	Ph	87.29	1.80 ± 0.59	89.61	10.17 ± 1.98
11a	*n*-Pr	Bn	91.38	15.23 ± 1.44	89.68	15.96 ± 2.54
11b	cyclohexylmethyl	Bn	85.53	6.39 ± 0.80	79.78	15.09 ± 3.71
12a	*n*-Pent	3-Tolyl	90.54	5.37 ± 2.40	88.76	12.86 ± 1.51
12b	Cyclohexylmethyl	3-Tolyl	88.67	0.44 ± 0.31	85.86	1.07 ± 0.67
13a	Cyclohexylmethyl	4-*t*-BuPh	72.35	1.06 ± 0.51	65.17	>100
13b	2-Cyclopentylethyl	4-*t*-BuPh	76.77	20.57 ± 1.89	64.16	21.48 ± 0.74
Doxorubicin	—	—	60.89	0.85 ± 0.05	62.79	0.35 ± 0.02

ND, not determined.
[a] Data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM) by MTT assay.

functionality on R^3 have better cytotoxic activities of over 90% inhibition when compared with those of the first group compounds. In the case of colon cancer cell line HT-29, the maximum inhibition of 92.03% was observed for **10g** followed by **10d** with 90.97% inhibition. For cell lines DU-145 and A-549, the maximum inhibition were observed for **10e (**94.25%, 88.64%) and **10f (**94.10%, 81.79%), respectively. In case of SK-MEL-2 cancer cell line, 91.96% inhibitory potential was observed for **10j**. Finally in the case of ovarian cancer cell line SK-OV-3, the maximum inhibition was 86.81% for **10e**. The second series compounds such as **10c–10g** and **10j** which have a substituted phenyl at R^1 and a phenyl at R^3 showed good inhibitory potential. The detailed study of SAR helped to point out that the improvements in growth inhibition of cancer cell lines can be achieved as follows: (i) The alkyl and heterocyclic groups at R^1 weaken the inhibition towards all cell lines; therefore it must be excluded in order to improve the growth inhibition. (ii) The growth inhibition can be maximized by changing pyridine ring with phenyl group in the region R^3. (iii) In most of the cell lines, the phenyl group with chloride at R^1 increases the percentage growth inhibition.

Based on the structure–activity relationship studies, R^1 group was fixed as *p*-chlorophenyl group which was the most effective group for enhancing the cytotoxicity, and further modifications were made. Selected compounds which showed over 80% inhibition at 100 μM concentration were evaluated for their GI_{50} values against two cell lines, HT-29 and DU-145. GI_{50} values, which indicate the concentration required to inhibit cancer cell proliferation by 50% after exposure of cells to test compounds, have been determined by MTT assay[22] using doxorubicin as internal standard. The results are tabulated in Table 2.

As shown in Table 2, the modification of R^2 and R^3 substituent led to significant change in anticancer activity. Compound **12b** (R^2 = cyclohexylmethyl, R^3 = tolyl) was the most active compound on HT-29 cell line with GI_{50} value of 0.44 μM. It was found that compounds **10e**, **10f** and **10k** with variable R^2 have no major differences in GI_{50} values. The electron withdrawing or electron donating group attached to phenyl ring and phenyl ring itself were not effective as R^2 in improving the activity. The GI_{50} values for these compounds remained in the range of 17–20 μM. Furthermore, the compounds **10l** and **10m** having flexible phenyl ring and their analogs on R^2 drastically lost anticancer activity on DU-145 cell line.

In an effort to increase the anticancer activity, we introduced aliphatic and alicylcic substituents at R^2 region. Interestingly, compound **10n** (R^2 = *n*-Pr, GI_{50} = 9.26 μM) showed better activity compared to **10k** (R^2 = Ph, GI_{50} = 20.56 μM) against HT-29 cell line. To our surprise, elongation of one carbon chain (compound **10o**, R^2 = *n*-Bu, GI_{50} = 0.71 μM) resulted in 13-fold increase in potency compared to **10n** on HT-29 cell line. However, further increase of the chain length decreased the activity by threefold (compound **10p,** R^2 = *n*-Pen, GI_{50} = 2.26 μM). The increase in activity was observed when the aliphatic groups on R^2 were replaced by alicyclic groups. The most active compound of the set was **12b,** which showed GI_{50} values of 0.44 μM and 1.07 μM against HT-29 and DU-145 cell lines, respectively. For HT-29 cell line, most of the prepared compounds having aliphatic and alicyclic chain on R^2 showed potent anticancer activities, while they showed low cytotoxic activities against DU-145 cell line except the compound **12b**.

Having identified potent anticancer compounds, we next profiled them against a panel of kinases as shown in Table 3. GSK 3β and Aurora-A kinase in the panel were effectively targeted by all

Table 3
Kinase activity of 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-one derivatives

Kinases	Compounds (% of remaining enzyme activity at 10 μM)				
	10o	**10p**	**11b**	**12a**	**12b**
Aurora-A(h)	16	9	26	7	18
EGFR(h)[a]	86	76	113	82	85
GSK3β(h)[b]	11	8	11	6	11
PDGFRα(h)[c]	105	99	77	92	95

[a] Human epithelial growth factor receptor.
[b] Human glycogen synthase kinase 3-beta.
[c] Human platelet-derived growth factor receptor alpha.

the five compounds with 6–11% (89–94% of inhibition) and 7–26% (74–93% of inhibition) of remaining activity at 10 μM concentration, respectively. The compounds, however, showed negligible activity on EGFR and PDGFRα kinases indicating moderate selectivity towards these kinases. Thus, it could be found that 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-one derivatives selectively inhibit serine/threonine kinases rather than tyrosine kinases.

In summary, an extensive library of 6-*N*-arylcarboxamidopyrazolo[4,3-*d*]pyrimidin-7-one derivatives was assembled and examined for trends with respect to anticancer potency, selectivity and SARs. The in vitro anticancer activity tests indicated that compound **12b** was the most cytotoxic agent against both colon and prostate cancer cell lines. While **13a** was highly selective towards colon (HT-29) cancer cell line, a few other compounds such as **10o, 10p** and **10q** exhibited significant anticancer activity against HT-29. The alicyclic and aliphatic groups on R^2 were found to be vital for potency. Further studies on the structure modification and anticancer activity evaluation are in progress.

Acknowledgements

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21. *Spectral data of selected 6-N-arylcarboxamidopyrazolo[4,3-d]pyrimidin-7-one derivatives*: *Compound* **10o**: White Solid (362 mg, 75.5%); mp 226.0; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.62 (s, 1H, NH), 8.33 (d, J = 8.4 Hz, 2H), 8.04–7.99 (m, 2H), 7.67–7.58 (m, 5H), 2.86–2.64 (m, 2H), 1.81–1.70 (m, 2H), 1.45–1.35 (m, 2H), 0.89 (t, J = 7.3 Hz, 3H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 173.2, 166.5, 157.5, 133.2, 131.7, 129.9, 129.4, 129.3, 129.3, 128.7, 128.2, 128.0, 127.0, 123.6, 33.1, 28.4, 22.0, 14.1; FABMS: m/z 422.138 [M$^+$+H]; *Compound* **12b**: White Solid (328.8 mg, 52.4%); mp 253.7; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 14.16 (s, 1H, NH), 9.61 (s, 1H, NH), 7.85 (d, J = 8.8 Hz, 2H), 7.73 (d, J = 8.2 Hz, 2H), 7.59–7.40 (m, 4H), 2.43 (s, 3H), 2.22 (d, J = 6.6 Hz, 2H), 1.73–1.56 (m, 6H), 1.17–1.10 (m, 3H), 0.98–0.91 (m, 2H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 172.4, 163.9, 139.4, 133.9, 133.2, 129.9, 129.3, 128.8, 127.4, 124.2, 123.5, 117.0, 102.1, 43.7, 34.9, 33.0, 26.2, 25.9, 21.3; FABMS: m/z 476.185 [M$^+$+H]; *Compound* **13a**: White Solid (211.1 mg, 55.8%); mp 296.5; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.61 (s, 1H, NH), 7.97 (d, J = 8.1 Hz, 2H), 7.72 (d, J = 8.2 Hz, 2H), 7.65 (d, J = 8.2 Hz, 2H), 7.57 (d, J = 8.0 Hz, 2H), 2.21 (d, J = 6.7 Hz, 2H), 1.69–1.53 (m, 6H), 1.32 (s, 9H), 1.10–1.07 (m, 3H), 0.92 (t, J = 10.6 Hz, 2H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 172.4, 163.9, 155.6, 133.9, 129.5, 129.3, 128.8, 128.6, 128.2, 126.9, 126.7, 125.7, 120.9, 117.0, 43.7, 35.3, 34.9, 33.1, 31.2, 26.2, 25.9; FABMS: m/z 518.232 [M$^+$+H].
22. *In vitro cytotoxicity evaluation*: Cytotoxic activities of the anticancer drugs against human cancer cell lines were investigated using the SRB assay or MTT assay. Human lung cancer (A-549), Human colon adenocarcinoma (HT-29), Human prostate cancer (DU-145), human ovarian cancer (SK-OV-3) and human melanoma cancer cell lines (SK-MEL-2) were supplied from the Korean Cell Line Bank, Seoul National University. All cell lines were grown in RPMI 1640 (Gibco BRL) supplemented with 10% (V/V) heat inactivated Fetal Bovine Serum (FBS) and maintained at 37 °C in a humidified atmosphere with 5% CO_2. SRB assay; SRB (Sulforhodamine B) were purchased from Sigma. The cells ($3–7 \times 10^3$ cells/well) were seeded into 96-well plate. Various concentrations of samples were added to each well in duplicate, then incubated at 37 °C with 5% CO_2 for two days such that time cells are in the exponential phase of growth at the time of drug addition. After incubation, the 100 μL of formalin solution were gently added to the wells. Microplates were left for 30 min at room temperature, washed five times with tap water. The 100 μL of 0.4% SRB solution was added to each well and left at room temperature for 30 min. SRB was removed and the plates washed five times with 1% acetic acid before air drying. Bound SRB was solubilized with 200 μL 10 mM unbuffered Tris-base solution (Sigma) and plates were left on a plate shaker for at least 10 min. The optical density was measured using a microplate reader (Versamax, Molecular Devices) with a 520 nm wavelength and the anticancer effective concentration was expressed as an GI_{50}. $F(x)$= $(T2 - T0)/T0 * 100$, $T2 < T0$ $(T2 - T0)/(C - T0) * 100$, $T2 > T0$ or $T2 = T0$.; MTT assay; The cells (5×10^4 cells/mL) were seeded into 96-well plate. Various concentrations of samples were added to each well in duplicate, then incubated at 37 °C with 5% CO_2 for two days such that time cells are in the exponential phase of growth at the time of drug addition. Add 15 μL of the Dye solution (Promrga, Cell Titer96) to each well. Incubate the plate at 37 °C for up to 4 h in a humidified, 5% CO_2 atmosphere. After incubation, add 100 μL of the solubilization solution/stop mix (Promrga, Cell Titer96) to each well. Allow the plate to stand overnight in a sealed container with a humidified atmosphere at room temperature to completely solubilize the formazan crystals. The optical density was measured using a microplate raeder (Versamax, Molecular Devices) with a 570 nm wavelegth and the anticancer effective concentration was expressed as a GI_{50}.