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Design, synthesis and in vitro cytotoxicity evaluation of 5-(2-carboxyethenyl)isatin derivatives as anticancer agents

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ABSTRACT

Forty four di- or trisubstituted novel isatin derivatives were designed and synthesized in 5–6 steps in 25–45% overall yields. Their structures were confirmed by ^{1}H NMR and ^{13}C NMR as well as LC–MS. The anticancer activity of these new isatin derivatives against three human tumor cell lines, K562, HepG2 and HT-29, were evaluated by MTT assay in vitro. SAR studies suggested that the combination of 1-benzyl and 5-[*trans*-2-(methoxycarbonyl)ethen-1-yl] substitution greatly enhance their cytotoxic activity, whereas an intact carbonyl functionality on C-3 as present in the parent ring is required to such a potency. This study leads to the identification of two highly active molecules, compounds **2h** (IC_{50} = 3 nM) and **2k** (IC_{50} = 6 nM), against human leukemia K562 cells.

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Isatin is an indole derivative widely present endogenously in both human and other mammalian tissues and fluids likely as a result of the tryptophan metabolic pathway. The versatility of isatin's molecular architecture makes it an ideal platform for structural modification and derivatization as evidenced by the fact that many isatin derivatives exhibit a broad range of biological activities such as anticancer,[1,2] antidepressant,[3] anticonvulsant,[4] antifungal,[5] anti-HIV[6] and anti-inflammatory,[7] etc. In the last several decades, increasing numbers of researchers from both industry and academia have embarked on the development of new isatin-based anticancer agents.[8–16] Eshba's group found that 5-bromoisatin 3-(2-nitrophenyl)hydrazone and a series of 5-[5-bromo-(2-oxo-3-indolinylidene)]amino thiazolidine-2,4-diones substituted by various Mannich bases exhibit antileukemic activity against P388 lymphocytic leukemia in mice, respectively.[12] Popp et al. has reported that some 3-hydroxy-3-substituted oxindoles obtained from condensation of substituted isatins with cyclic ketones possess anticonvulsant activity.[13] SU11248 (Sutent), a 5-fluoro-3-substituted isatin derivative, was approved by the FDA in 2006 for the treatment of advanced renal carcinoma and gastrointestinal stromal tumors.[14,15] C5- and C6-substituted isatin analogues were shown to be selective MAO B inhibitors with 5-(4-phenylbutyl)isatin exhibiting the highest activity and being 18,500-fold more potent than isatin.[16] Recently, the groups of Mach[17,18] and Reichert[19] synthesized several *N* alkyl and C-5 sulfonamido isatin analogues as small molecule caspase-3 and caspase-7 inhibitors.

As part of our research program on the SAR study of isatin derivatives' anticancer property, herein we wish to report the synthesis and antitumor activity of a series of N-benzylated isatins possessing an acrylate moiety at C5 against three human cancer cell lines, including human leukemia K562, human liver cancer HepG2, and human colon HT-29.

As shown in Scheme 1, 5-substituted isatins **1a**–**1g** were prepared in two steps in 55–80% yield by following literature procedures.[20] Microwave-assisted Heck coupling reaction was employed to converted 5-bromo isatin (**1d**) 5-*trans*-(2-methoxycarbonylethenyl)isatin (**1h**) in 70%. Further derivatization led to the synthesis of compounds **2a**–**2p** in good to excellent yield by N-alkylation with alkyl halides in the presence of K_2CO_3.

Based on the identification of several major structure motifs that main potentially improve isatin's antitumor activity as illustrated in Figure 1, the investigation was started with phenyl portion's substitution pattern which was followed by N-derivatization and then C-3 variation.

The in vitro antitumor activities of the 5-substituted isatins **1a**–**1h** against three human tumor cells, K562, HepG2 and HT-29 were evaluated by MTT assay with Camptothecin (CPT) as the positive control.[21,22] As can be seen from the data in Table 1, isatin derivatives **1a**–**1f** possessing simple substituent groups on the C5 position with different sizes and varing electronic properties do not have significant impact on their activities. Interestingly, when

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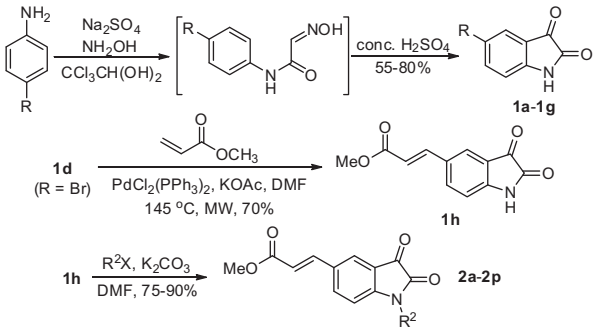


Scheme 1. Synthesis of isatin derivatives (**1a**–**1h**, **2a**–**2p**).

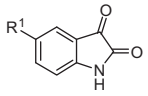
a methyl acrylate group was introduced at C5, the resulting compound **1h** has a potency that is at least 130–230 times stronger than isatin itself. However, compound **1g**, which contains an *n*-butyl group at C-5, has only a very minimal improvement in potency. This observation suggests that the good inhibitory activity of **1h** does not simply correlate with the size of the acrylate moiety and the limited spatial orientation due to its conjugation with the aromatic ring may play a role.

With the identification of compound **1h** as a potential lead, our SAR study was then focused on the N-derivatized analogues. Recently, some *N*-benzyl isatin derivatives have been reported to exhibit a broad range of biological activities, including activity related to the treatment of Neuropathic Pain,[23] reverse transcriptase inhibitor,[24] caspase inhibitory activity[17,25] and in vitro cytotoxicity.[2,8,26] Therefore, it seemed highly likely that the combination of C-5 olefination with an acrylate moiety and N-substitution may further enhance their potency.

As shown in Table 2, simple alkyl groups such as the *N*-methyl and *N*-ethyl (compounds **2a** and **2b**) do not result in a significant change in the cell growth inhibition compared to **1h**. This observation also suggests that the N–H group is not a necessary structure unit for the activity of **1h**. Recognizing that the *N*-methyl and *N*-ethyl groups maybe too small to cause a significant effect in potency, a benzyl group was introduced to the N-1 position. Pleasingly, compound **2c** was found to exhibit a potency with a 5–9 fold improvement, which suggests that larger groups at the N-1 position could increase the potency. Further test on compounds **2d**–**2f** (with an electron withdrawing group) and **2g**, **2p** (with an electron donating group) showed that these derivatives do not differ significantly with the benzyl **2c** in their potency.

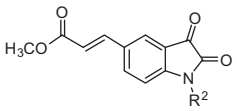
However, compounds **2h**[27] and **2k**[28] containing a 4-methoxy and 4-bromo benzyl group, respectively, exhibit excellent activity against human cancer cell lines. For K562, their potency reached

Table 1
Antitumor activities of compounds **1a**–**1h** in vitro



Compd	R^1	IC_{50} (μM)		
		K562	HepG2	HT-29
CPT	—	0.040 ± 0.02	0.050 ± 0.02	0.060 ± 0.01
1a	H	>10	>10	>10
1b	F	>10	>10	>10
1c	Cl	>10	>10	>10
1d	Br	>10	>10	>10
1e	CH_3	>10	>10	>10
1f	OCH_3	>10	>10	>10
1g	$CH_2CH_2CH_2CH_3$	>10	>10	>10
1h	$CH{=}CHCOOCH_3$	0.22 ± 0.06	0.37 ± 0.07	0.27 ± 0.01

Table 2
Antitumor activities of compounds **2a**–**2p** in vitro



Compd	R^2	IC_{50} (μM)		
		K562	HepG2	HT-29
CPT	—	0.040 ± 0.02	0.050 ± 0.02	0.060 ± 0.01
1h	H	0.22 ± 0.06	0.37 ± 0.07	0.27 ± 0.01
2a	CH_3	0.34 ± 0.09	0.53 ± 0.19	0.30 ± 0.05
2b	CH_2CH_3	0.17 ± 0.11	0.40 ± 0.01	0.21 ± 0.05
2c	$CH_2C_6H_5$	0.040 ± 0.02	0.040 ± 0.01	0.070 ± 0.02
2d	$CH_2C_6H_4F$[a]	0.030 ± 0.03	0.040 ± 0.02	0.060 ± 0.02
2e	$CH_2C_6H_4CN$[a]	0.030 ± 0.001	0.050 ± 0.01	0.050 ± 0.001
2f	$CH_2C_6H_4CF_3$[a]	0.040 ± 0.02	0.070 ± 0.04	0.040 ± 0.001
2g	$CH_2C_6H_4CH_3$[a]	0.040 ± 0.01	0.040 ± 0.01	0.030 ± 0.01
2h	$CH_2C_6H_4OCH_3$[a]	0.0030 ± 0.001	0.030 ± 0.02	0.030 ± 0.01
2i	$CH_2C_6H_4Cl$[a]	0.030 ± 0.01	0.040 ± 0.01	0.060 ± 0.03
2j	$CH_2C_6H_3Cl$[b]Cl[a]	0.060 ± 0.03	0.16 ± 0.09	0.21 ± 0.08
2k	$CH_2C_6H_4Br$[a]	0.0060 ± 0.001	0.040 ± 0.05	0.030 ± 0.01
2l	$CH_2C_6H_4Br$[b]	0.030 ± 0.01	0.17 ± 0.08	0.24 ± 0.02
2m	$CH_2C_6H_4Br$[c]	0.070 ± 0.05	0.26 ± 0.07	0.24 ± 0.11
2n	$CH_2C_{10}H_7$	0.040 ± 0.001	0.090 ± 0.08	0.10 ± 0.06
2o	$CH_2CH_2OC_6H_5$	0.060 ± 0.02	0.21 ± 0.22	0.20 ± 0.02
2p	$CH_2C_6H_3CH_3$[b]CH_3[b]	0.040 ± 0.03	0.15 ± 0.10	0.070 ± 0.03

[a] Substitution at the *para* position.
[b] Substitution at the *meta* position.
[c] Substitution at the *ortho* position.

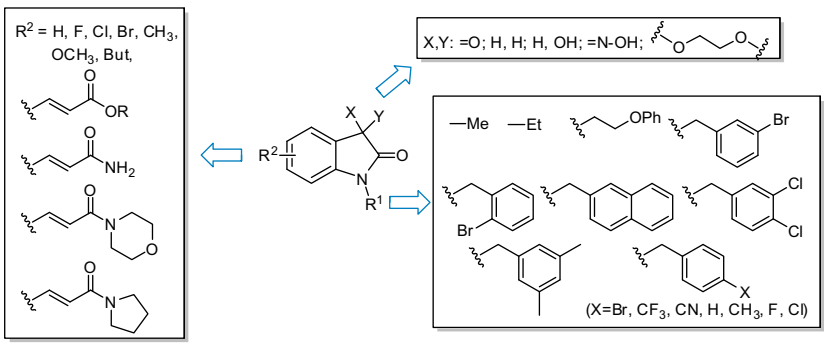
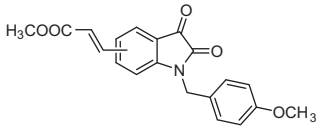


Figure 1. Structure modifications of isatin derivatives for SAR study.

Table 3
In vitro antitumor activities of compounds **2q–2s**



Compd	Position	IC_{50} (μM)		
		K562	HepG2	HT-29
CPT	—	0.040 ± 0.02	0.050 ± 0.02	0.060 ± 0.01
2h	5	0.0030 ± 0.001	0.030 ± 0.02	0.030 ± 0.01
2q	4	0.35 ± 0.05	5.49 ± 2.23	3.12 ± 0.96
2r	6	4.86 ± 0.56	>10	>10
2s	7	4.13 ± 1.27	>10	>10

3 and 6 nM, respectively. It was also noticed that the potency of the meta and *ortho* bromo-substituted benzyl compounds **2l** (0.030 μM) and **2m** (0.070 μM) dropped by 5–11 folds when compared to their para counterpart **2k** (0.0060 μM). Moreover, when the N-1 substitution was changed to the more bulky 2-naphthylmethyl (**2n**) or the phenoxyethyl (**2o**) groups, the potency also dinimished. The above results illustrated the importance of the size and orientation for N-substitution in the acrylate-containing isatin analogues for their anticancer activity.

To clarify the importance for the position of the acrylate moiety in **2h**, regioisomers **2q–2s** were synthesized by using a similar procedure for the synthesis of **2h** (Table 3). The 6- and 7-substituted **2r** and **2s** were found to be much less potent than **2h** (1000 folds) and the 4-substituted counterpart **2q** (10 folds). Along with the inferior potency of **2q** as compared to **2h**, these data suggests that the presence of a methyl acrylate group at the *para*-position found in **2h** is vital to maintain a high level of potency, probably as the result of electronic and/or geometric preference.

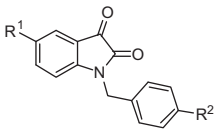
After the N-substitution and C-substitution patterns were established, target compounds **3a–3o** were synthesized by microwave-assisted Heck coupling reaction in a similar way for the synthesis of **2h** and their potency evaluated (Table 4). The results indicated that the potency generally decreases with the increase in the size of the alkyl group (**3a**, **3c**, **3g** and **3e** vs **2h**; **3b**, **3d**, **3h** and **3f** vs **2k** in going from Me to Et, Bu, 2-hydroxyethyl and *t*-Bu). The presence of a free acrylic acid (**3i**) brought about a drastic diminishment in potency, pointing to a detrimental effect of the highly polar and readily ionizable free carboxyl group. All the amide analogues examined (**3j–3o**) were found to possess low activities relative to the esters either duo to the high polarity of these groups or an increase in their sized in the case of **3l**/**3m** and **3n**/**3o**.

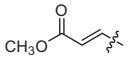
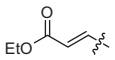
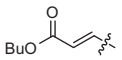
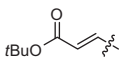
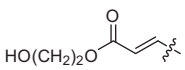
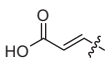
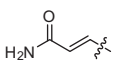
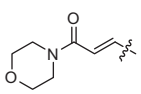
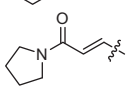
To examine the importance of the C-3 carbonyl functionality, eight new isatin derivatives (**3p–3w**) were synthesized from **2h** and **2k** through an appropriate carbonyl group derivatization reaction (Scheme 2). All these derivatives proved to be inferior candidates as inhibitors of K562, HepG2, HT-29 (Table 5), suggesting that the carbonyl functionality at C-3 is essential in order to maintain the observed high antitumor activity.

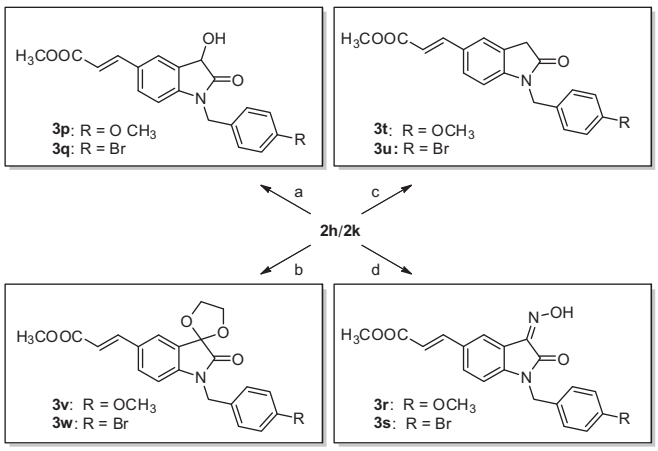
Preliminary results from a flow cytometric analysis conducted in our laboratory revealed that compounds **2h** and **2k** could significantly induce the levels of apoptosis in K562 cells in vitro at low micro molar concentrations (Fig. 2).[29]

In conclusion, a series of 1,5-disubstituted and 1,3,5-trisubstituted isatin derivatives were synthesized and tested for their in vitro antitumor activity against three strains of cancer cell lines K562, HepG2, HT-29. The SAR study of these compounds led to the identification of two new isatins, **2h** and **2k**, as highly potent anticancer compounds with IC_{50} = 3 nM, IC_{50} = 6 nM, respectively, against human leukemia K562 cells. Further chemo-biological study of these two compounds with regards to their antitumor pathway as well as their enzymatic targets and in vivo investigation are ongoing in this laboratory.

Table 4
In vitro antitumor activities of compounds **3a–3q**

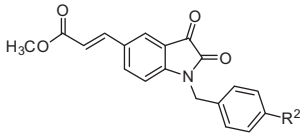


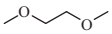
Compd	R^1	R^2	IC_{50} (μM)		
			K562	HepG2	HT-29
CPT	—	—	0.040 ± 0.02	0.050 ± 0.02	0.060 ± 0.01
2h		OCH_3	0.0030 ± 0.001	0.030 ± 0.02	0.030 ± 0.01
2k		Br	0.0060 ± 0.001	0.040 ± 0.05	0.030 ± 0.01
3a		OCH_3	0.010 ± 0.002	0.090 ± 0.01	0.060 ± 0.01
3b		Br	0.030 ± 0.01	0.29 ± 0.31	0.050 ± 0.04
3c		OCH_3	0.040 ± 0.02	0.63 ± 0.17	0.42 ± 0.04
3d		Br	0.15 ± 0.01	0.65 ± 0.21	0.60 ± 0.06
3e		OCH_3	0.050 ± 0.04	0.62 ± 0.02	0.48 ± 0.02
3f		Br	0.29 ± 0.04	2.05 ± 0.21	0.73 ± 0.01
3g		OCH_3	0.040 ± 0.01	0.86 ± 0.13	0.55 ± 0.16
3h		Br	0.050 ± 0.03	3.66 ± 1.51	0.68 ± 0.16
3i		OCH_3	>10	>10	>10
3j		OCH_3	0.20 ± 0.02	0.89 ± 0.18	0.56 ± 0.16
3k		Br	0.30 ± 0.01	>10	0.55 ± 0.15
3l		OCH_3	0.72 ± 0.24	7.38 ± 2.26	4.24 ± 0.74
3m		Br	1.38 ± 0.40	7.79 ± 1.66	4.59 ± 0.55
3n		OCH_3	0.46 ± 0.11	7.81 ± 0.55	5.23 ± 0.34
3o		Br	1.71 ± 0.53	6.30 ± 0.90	4.42 ± 0.58



Scheme 2. C-3 modification of isatin derivatives (**3p–3w**). Reagents and conditions: (a) $NaBH_4$, MeOH, rt,50–55%; (b) ethylene glycol, pTSA, reflux, 85–90%; (c) hydrazine monohydrate, EtOH, reflux, 60–65%; (d) $NH_2OH{\cdot}{\cdot}HCl$, EtOH, reflux, 80–90%.

Table 5
Antitumor activity of the C-3 derivatized compounds **3p-3w**



Compds	R^2	C-3	IC_{50} (μM)		
			K562	HepG2	HT-29
CPT	—	—	0.040 ± 0.02	0.050 ± 0.02	0.060 ± 0.01
2h	OCH_3	=O	0.0030 ± 0.010	0.030 ± 0.02	0.030 ± 0.01
2k	Br		0.0060 ± 0.001	0.040 ± 0.05	0.030 ± 0.01
3p	OCH_3	–OH·H	0.030 ± 0.01	0.38 ± 0.07	0.32 ± 0.03
3q	Br		0.26 ± 0.03	0.67 ± 0.12	0.66 ± 0.17
3r	OCH_3	=NOH	2.11 ± 1.13	3.56 ± 1.54	4.94 ± 2.54
3s	Br		2.92 ± 0.34	9.96	3.64 ± 1.19
3t	OCH_3	–H·H	0.99 ± 0.56	9.17	3.72 ± 0.03
3u	Br		3.16 ± 0.21	>10	>10
3v	OCH_3		4.89 ± 0.19	>10	>10
3w	Br		2.63 ± 0.03	7.47 ± 2.15	5.11 ± 1.38

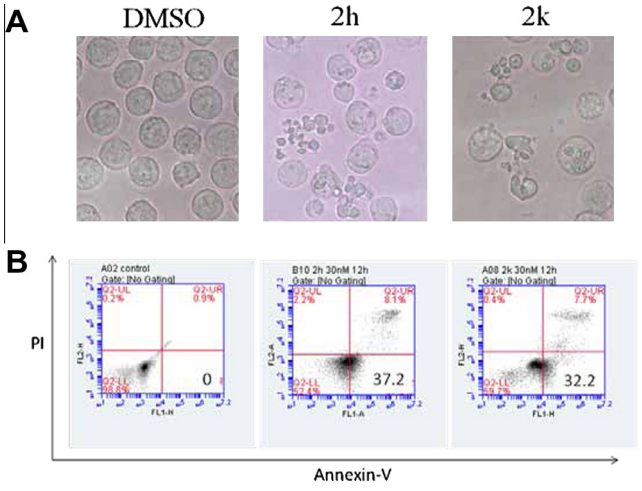


Figure 2. Compounds **2h** and **2k** induced apoptosis in K562 cells. (A) Phase contrast images of K562 cells after culture with 30 nM 2 h or **2k** for 12 h. (B) Apoptosis was evaluated by Annexin-V and PI staining of K562 cells cultured in the absence or presence of 30 nM **2h** or **2k** for 12 h.

Acknowledgments

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Supplementary data

Supplementary data (synthetic procedures and analytical data of all compounds and the ^{1}H NMR spectra, ^{13}C NMR spectra and LC–MS data of **2h** and **2k**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.12.001.

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22. Cells (100 μL) were cultured in 96-well plates at a density of 5×10^4 cells/mL for 2 h (K562) or overnight (HepG2 and HT-29). Compounds (DMSO solution of 0.5 μL) were added to each well to culture for another 48 h. MTT assay was performed using thermo microplate reader. The DMSO-treated controls were calculated as a cell viability value of 100%. The IC_{50} values were obtained by nonlinear regression using GraphPad Prism 4.0. IC_{50} measurements for each compound were done three times.
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27. *Analytical data of **2h**:* ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.78 (s, 3H), 3.80 (s, 3H), 4.88 (s, 2H), 6.33–6.37 (d, 1H, J = 16.0 Hz), 6.83 (d, 1H, J = 8.4 Hz), 6.87 (d, 2H, J = 8.4 Hz), 7.26 (d, 2H, J = 8.8 Hz), 7.58 (d, 1H, J = 16.0 Hz), 7.62 (m, 1H), 7.77 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 43.78, 51.85, 55.31, 111.43, 114.52, 114.52, 118.08, 118.16, 124.10, 126.10, 128.95, 128.95, 130.49, 137.93, 142.43, 151.56, 158.11, 159.60, 166.92, 182.77. ESI-MS: 352.1 [M+H]+, 374.1 [M+Na]+.
28. *Analytical data of **2k**:* ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.80 (s, 3H), 4.90 (s, 2H), 6.34–6.38 (d, 1H, J = 16.0 Hz), 6.77–6.79 (d, 1H, J = 8.0 Hz), 7.20–7.22 (d, 2H, J = 8.4 Hz), 7.49 (d, 2H, J = 8.4 Hz), 7.59 (d, 1H, J = 16.0 Hz), 7.63 (m, 1H), 7.79 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 43.69, 51.88, 111.22, 118.10, 118.43, 122.42, 124.26, 129.14, 129.14, 130.80, 132.35, 132.35, 133.20, 138.00, 142.26, 151.11, 158.09, 166.85, 182.34. ESI-MS: 400.1 [M+H]+.
29. For flow cytometric analysis of apoptosis, phosphatidylserine exposure on the outer leaflet of the plasma membrane was detected using the TACS Annexin V Kit (Trevigen, Gaithersburg, MD, USA) according to the manufacturer's instructions. In brief, 1×10^6 cells were resuspended in 100 μL of binding buffer containing of annexin V-FITC and propidium iodide (PI) and incubated for 15 min. After 400 μL of binding buffer was added, the cells were analysed using a Becton Dickinson FACScan flow cytometer (Mountain View, CA, USA).