European Journal of Medicinal Chemistry 44 (2009) 2923–2929

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and antitumor activity of optically active thiourea and their 2-aminobenzothiazole derivatives: A novel class of anticancer agents

S.N. Manjula [a], N. Malleshappa Noolvi [b], K. Vipan Parihar [a], S.A. Manohara Reddy [a], Vijay Ramani [a], Andanappa K. Gadad [c], Gurdial Singh [d], N. Gopalan Kutty [a], C. Mallikarjuna Rao [a,*]

[a] Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Madhav Nagar, Manipal 576104, Karnataka, India
[b] Department of Pharmaceutical Chemistry, KLE's College of Pharmacy, Hubli 580031, Karnataka, India
[c] School of Pharmacy, Faculty of Medical Sciences, The University of the West Indies, (St. Augustine), Champs Fleurs, Mount Hope, Trinidad and Tobago, WI, USA
[d] Department of Chemistry, University of the West Indies, Trinidad and Tobago, WI, USA

ARTICLE INFO

Article history:
Received 15 May 2008
Received in revised form
7 November 2008
Accepted 8 December 2008
Available online 16 December 2008

Keywords:
2-Aminobenzothiazoles
Thiourea
Comet assay
MTT assay
DNA damage
MCF-7

ABSTRACT

A novel series of optically active 2-aminobenzothiazole derivatives were synthesized by reaction of optically active amine (**I**) with thiophosgene to obtain optically active isothiocyanates (**IIa–h**) which on condensation with 4-fluoro-3-chloro aniline (**III**) yielded various optically active thioureas (**IVa–h**). Further oxidative cyclisation in the presence of bromine and chloroform yielded title compounds (**Va–h**). The structures of these compounds were established by IR, ^{1}H NMR, ^{13}C NMR, Mass and HRMS. The compounds (**IVa–h** and **Va–h**) were evaluated for in vitro cytotoxicity against mouse Ehrlich Ascites Carcinoma (EAC) and two human cancer cell lines (MCF-7 and HeLa). In preliminary MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cytotoxicity studies the optically active thiourea derivatives (**IVe**, **IVf** and **IVh**) were found most effective. In EAC cells the IC_{50} values for **IVe**, **IVf**, **IVh** and **Vg** were found in the range of 10–24 μM, whereas in MCF-7 and HeLa cells the IC_{50} values were observed in the range of 15–30 μM and 33–48 μM, respectively. In alkaline comet assay the compounds (**IVe** and **IVf**) showed dose-dependent DNA damaging activity.

1. Introduction

Genotoxicity and cytotoxicity of anticancer drugs to the normal cells are major problems in cancer therapy and engender the risk of inducing secondary malignancy [1]. A dose of anticancer drug sufficient to kill tumor cells is often toxic to the normal tissue and leads to many side effects, which in turn, limits its treatment efficacy. In recent years, there has been a concerted search for the discovery and development of novel selective antitumor agents, devoid of many of the unpleasant side effects of conventional antitumor agents.

In the efforts to develop drugs with such capabilities, scientists have focused upon many different aspects of cancer biology during their research. Among the antitumor drugs discovered in the recent years, various benzothiazoles [2–4] as well as urea and thiourea derivatives [5–7] possess potent anticancer properties. Currently, a benzothiazole derivative, 2-(4-amino-3-methylphenyl)-5-fluorobenzthiazole, acting through a novel mechanism, has entered the clinical trial phase I in UK. It shows high sensitivity towards the tumor cells by acting through aryl hydrocarbon receptor (AhR) signaling pathway. The activation of AhR pathway in sensitive tumor cells leads to induction of the microsomal enzyme CYP1A1. This converts the drug into highly reactive metabolites that form adducts with DNA causing cell death [8]. Further, the combinations of urea and thiourea derivatives with benzothiazoles have produced DNA topoisomerase [9,10] or HIV reverse transcriptase inhibitors [11,12]. Based on these reports, we herein report the syntheses and in vitro evaluation of antitumor activity of a series of novel optically active thioureas and their derivatives 2-aminobenzothiazole.

The present study involves the syntheses of optically active thioureas and 2-aminobenzothiazole followed by preliminary cytotoxicity screening against mouse Ehrlich Ascites Carcinoma (EAC) and two human cancer cell lines (MCF-7 and HeLa) using MTT assay at 24 h of exposure. The promising molecules were subjected to cytotoxicity assay at different time points along with the DNA damaging potential of two compounds in sensitive cells (MCF-7).

2. Results and discussion

2.1. Chemistry

We have synthesized newer optically active 2-aminobenzothiazoles with chiral carbon attached in the side chain shown

* Corresponding author. Tel.: +91 820 2571201x22482.
E-mail address: cmallikin@yahoo.co.in (C. Mallikarjuna Rao).

0223-5234/$ – see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2008.12.002

in Scheme 1. The compounds were synthesized using various optically active isothiocynates (**IIa–h**), which were prepared from different optically active aromatic and aliphatic primary amines [13]. Prepared isothiocynates yielded thioureas (**IVa–h**) on condensation with 4-fluoro-3-chloro-aniline (**III**). Oxidative cyclisation by bromine resulted in the synthesis of proposed compounds (**Va–h**). The derivatives were characterized by spectral studies using IR, ^{1}H NMR, ^{13}C NMR, DIPMS and HRMS. Optical rotation was measured by using polarimeter.

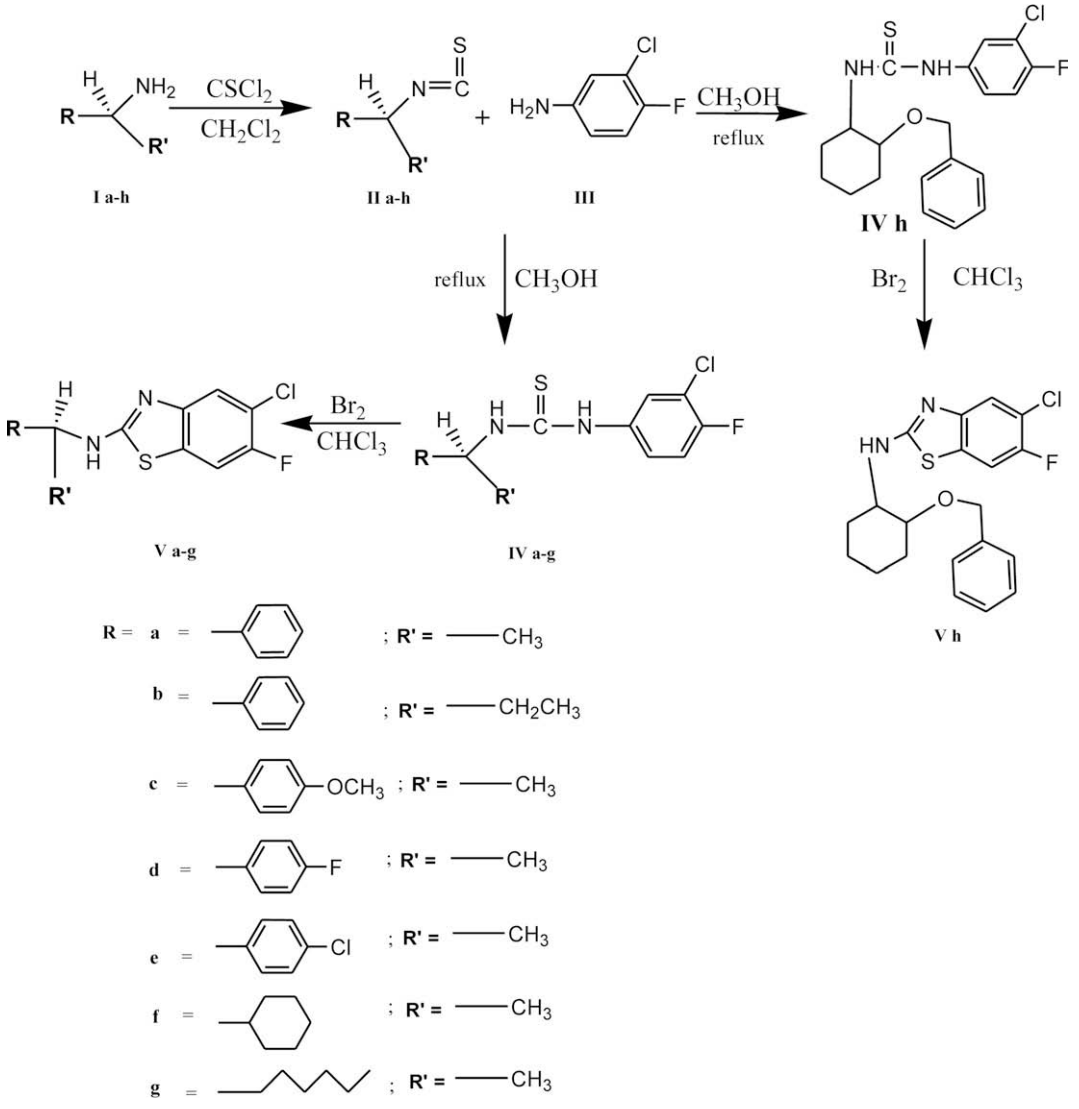
The structures of optically active thiocynates were confirmed through the following spectral data. IR absorption peak at 2000–2200 cm^{-1} corresponding to –SCN, ^{1}H NMR showing a doublet at δ 2.00–1.40 ppm corresponding to –CH_3 on chiral carbon, δ 4.90–4.65 ppm corresponding proton on same chiral carbon, δ 3.90–3.84 ppm for –OCH_3 and δ 7.33–6.90 ppm for aromatic protons.

Thioureas were confirmed by the absence of characteristic IR absorption peak at 2000–2200 cm^{-1} –SCN group. Bands at 1600–1610 cm^{-1} confirmed formation of thioureas –NH–CS–NH–. ^{1}H NMR spectra elaborated the up field shift in resonance of proton attached to chiral carbon atom from δ 4.90–4.65 ppm to δ 6.00–5.00 ppm. Further occurrence of two broad peaks at δ 8.31 and 6.23 ppm corresponding two –NH groups substantiated the formation of thioureas. ^{13}C NMR confirmed conversion of –SCN to –NH–CS–NH– by a peak at δ 181–179 ppm corresponding to >C═S. The mass spectra showed M^+ peak at respective molecular weights of the compounds. Some of them were subjected to HRMS to obtain respective molecular weights.

Optically active novel 2-aminobenzothiazoles showed IR absorption band at 3309, 2867, 1629 cm^{-1}. ^{1}H NMR spectra revealed all the corresponding peaks at δ 1.25–1.00 ppm, –CH_3, δ 4.85–4.60 ppm, proton of chiral carbon, broad peak at δ 6.90–5.99 ppm, –NH. Aromatic proton showed peaks at δ 7.30–7.00 ppm. ^{13}C NMR gave valuable information to confirm cyclisation of substituted thioureas to respective substituted 2-aminobenzothiazoles with characteristic peak at δ 165–155 ppm for C-2. Further HRMS gave all the M^+ ion peaks corresponding to molecular weight of confirmed novel compounds.

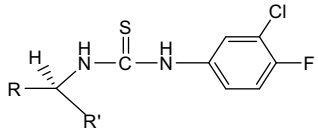
2.2. Preliminary cytotoxicity and time course MTT assay

In vitro cytotoxicity of synthesized compounds was assessed by standard MTT bioassay in different cancer cells at 24 h of drug exposure. In EAC cells the optically active thioureas were found to be more effective when compared with benzothiazole derivatives. To assess the efficacy and to select the promising compounds human cancer cells (MCF-7 and HeLa cells) were used.

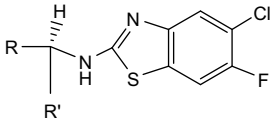


Scheme 1. Synthetic pathway of novel optically active thioureas and their derivatives, 2-aminobenzothiazoles.

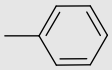
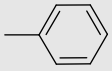
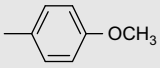
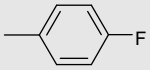
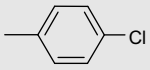
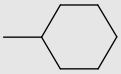
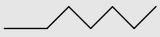
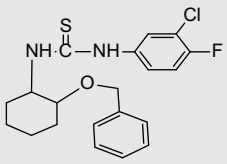
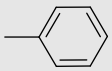
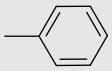
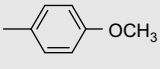
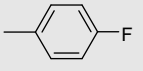
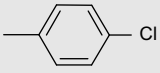
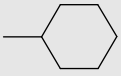
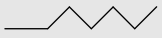
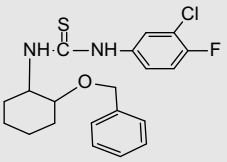
Table 1
Characteristics and preliminary cytotoxicity screening of synthesized compounds by MTT assay at 24 h of drug exposure.



IV a-g



V a-g

Compounds	R	R′	IC_{50} (μM)		
			EAC	MCF-7	HeLa
IVa		$-CH_3$	23.56	26.08	66.21
IVb		$-CH_2CH_3$	80.25	>100 μM	NT[a]
IVc		$-CH_3$	45.64	31.39	77.53
IVd		$-CH_3$	29.94	34.15	71.43
IVe		$-CH_3$	17.32	23.23	42.99
IVf		$-CH_3$	15.11	25.04	45.75
IVg		$-CH_3$	25.48	25.71	49.09
IVh			10.63	20.56	33.81
Va		$-CH_3$	36.64	42.73	58.93
Vb		$-CH_2CH_3$	32.22	39.30	53.26
Vc		$-CH_3$	75.44	>100 μM	NT[a]
Vd		$-CH_3$	35.35	42.32	61.93
Ve		$-CH_3$	25.47	33.00	69.69
Vf		$-CH_3$	29.78	32.90	72.54
Vg		$-CH_3$	24.62	29.80	48.01
Vh			20.63	40.56	60.81
Doxorubicin			4.18	2.62	3.24

The known numbers of cells (1.0×10^4) were incubated for 24 h in a 5% CO_2 incubator at 37 °C in the presence of different concentrations of test compounds. After 24 h of drug incubation the MTT solution was added and supernatant was discarded and 100 μl DMSO was added in each well and absorbance was recorded at 540 nm by ELISA reader.
Concentration required to inhibit the 50% cell growth. Experiment was performed in triplicate.
[a] NT: Not tested.

In MCF-7 cells, the IC_{50} values for benzothiazole and thiourea derivatives were in the range of 29.80–42.73 and 20.56–34.15 μM, respectively (Table 1). Similarly in HeLa cells, the IC_{50} values for benzothiazole and thiourea derivatives were found in the range of 48.01–72.54 and 33.81–77.53 μM, respectively (Table 1). In MCF-7 cells, the compounds **Vc** and **IVb** did not show 50% inhibition even at a concentration of 100 μM; hence they were not evaluated in HeLa cells. Present study reveals that among the human cancer cell lines tested, MCF-7 cells are more sensitive to all the tested compounds than HeLa cells. Many anticancer drugs are effective against MCF-7 and HeLa cells by causing apoptosis through the expression of caspase-3, generating reactive oxygen species (ROS) and damaging DNA [14]. Cisplatin causes cytotoxicity in MCF-7 and HeLa cells by a similar mechanism [15] Chemotherapeutic agents such as doxorubicin, mitoxantrone and bleomycin cause cytotoxicity by generating ROS [16]. Hence like other cytotoxic drugs (doxorubicin, mitoxantrone, bleomycin and cisplatin), the synthesized compounds may act as effective anticancer drugs by similar mechanism. Previous studies have shown that strong electronegative atom substitution such as chloro/bromo at the para position of the aromatic ring increases the lipophilicity of molecules and is responsible for enhanced cytotoxicity in MTT model [17]. Similar substitutions are present in the compounds **IVe**, **IVf**, **IVh** and **Vg**. We have also observed enhanced cytotoxicity in these molecules. Hence these molecules were taken up to assess the cytotoxic potency at different intervals in MCF-7 cells.

In the MTT time course study, the selected compounds (**IVe**, **IVf**, **IVh** and **Vg**) showed dose-dependent and time-dependent activities. The previous study reported that the most potent fluorinated benzothiazole, 5F 203, produced apoptosis and DNA damage in MCF-7 cells, which is characteristic of cytotoxic activity [18,19] In our present study also MTT assays revealed substantial cytotoxicity in MCF-7 cells with increasing exposure to drug concentration, the IC_{50} values of promising compounds at 48 and 72 h were significantly reduced as compared with 24 h values (Table 2).

2.3. Effect on DNA damage

A number of cytotoxic compounds (cisplatin, doxorubicin, etc.) act as anticancer drugs by causing DNA damage and subsequently

Table 2
Cytotoxic activity of promising compounds in MCF-7 cells at different time points of drug exposure by MTT assay.

Compounds	IC50 (μm)		
	24 h	48 h	72 h
IVh	20.56	12.80	8.61
IVe	23.23	16.27	11.40
IVf	25.04	18.91	13.98
Vg	29.80	24.84	15.00

The IC_{50} values of promising compounds at 48 h and 72 h were significantly reduced in comparison with 24 h values. The most promising compound (IVh) causes 1.66 and 2.5 time reduction in IC_{50} at 48 h and 72 h.

inducing apoptosis in cancerous cells. To determine the DNA damaging activity of synthesized compounds, alkaline comet assay was performed in MCF-7 cells. Comet assay is a rapid and inexpensive method for measuring DNA single-strand breaks (SSBs). It also has an advantage over the other DNA damage-detecting methods, such as sister chromatid exchange, alkali elution, and micronucleus assay, because of its high sensitivity [20]. 24 h treatment with **IVe** and **IVf** produced a dose-dependent increase in tail moment. The damage produced by **IVf** was quite prominent as at 50 μM it produced apoptotic bodies and tail moment above 100. Elongated tail length and reduced DNA content in head are sufficient indicators of DNA damage, and higher degrees of damage would result in greater number of smaller fragments, ending up with longer tails. The SSB in DNA results from a number of different reaction types including base and nucleotide excision repairs, direct scission of the DNA backbone by chemical or radical attack, strand breakage following binding of intercalating agents and alkali-labile DNA adducts [21–23]. Both the studied compounds significantly increased the tail moment and tail length in MCF-7 cells. Previous studies have shown that cytotoxicity of benzothiazoles is mediated via activation of the AhR signaling pathway in sensitive MCF-7 cells [18,24,25]. The activation of AhR pathway leads to generation of reactive electrophilic species by inducing CYP1A1 expression. The generated highly reactive intermediates cause DNA damage, ultimately resulting in cell death by activation of apoptotic machinery [8,26]. Hence our compounds may be acting by similar mechanism(s) which, however, need to be confirmed by gene expression studies. (Tables 3 and 4).

3. Conclusion

In the present study, we have described the syntheses and in vitro cytotoxicity screening of optically active thioureas and their derivatives, 2-aminobenzothiazoles. Various aromatic, alicylic and aliphatic substitutions were considered for synthesis to draw the structure–activity relationship studies in relation to cytotoxic activity. An interesting observation made during cytotoxic study

Table 3
The DNA damaging activity of **IVe** in MCF-7 cells at 24 h of drug exposure by comet assay.[a]

Compounds	Tail moment	OTM	Tail length	Tail DNA
Control	7.18 ± 1.05	8.83 ± 0.83	65.56 ± 3.23	9.75 ± 1.16
5 μM	8.31 ± 0.98	10.23 ± 0.72	71.89 ± 4.16	11.21 ± 0.94
10 μM	18.62 ± 2.58	22.46 ± 5.67	102.56 ± 6.42[d]	16.90 ± 1.23[c]
25 μM	22.00 ± 3.10[b]	25.71 ± 7.29	110.70 ± 7.51[d]	18.80 ± 1.38[d]
50 μM	64.04 ± 5.27[d]	37.12 ± 2.57[d]	205.23 ± 7.09[d]	29.89 ± 1.62[d]
DOX 100 nm	47.67 ± 2.74[d]	36.55 ± 1.61[d]	149.04 ± 4.61[d]	31.14 ± 1.15[d]

[a] All values are means ± SEM.
[b] $P < 0.05$ compared to control.
[c] $P < 0.01$ compared to control.
[d] $P < 0.001$ compared to control.

Table 4
The DNA damaging activity of IVf in MCF-7 cells at 24 h of drug exposure by comet assay.[a]

Compounds	Tail moment	OTM	Tail length	Tail DNA
Control	7.18 ± 1.05	8.83 ± 0.83	65.56 ± 3.23	9.75 ± 1.16
5 μM	18.91 ± 1.45	13.31 ± 0.59	130.02 ± 4.47[b]	14.11 ± 0.75
10 μM	35.24 ± 2.03[b]	18.53 ± 0.99	202.92 ± 6.35[c]	17.12 ± 0.72[b]
25 μM	43.45 ± 2.47[c]	22.13 ± 1.34	210.31 ± 7.12[c]	20.41 ± 0.72[c]
50 μM	121.88 ± 15.55[c]	146.48 ± 26.28[c]	319.90 ± 33.40[c]	37.63 ± 2.68[c]
DO × 100 nm	47.67 ± 2.74[c]	36.55 ± 1.61	149.04 ± 4.61[c]	31.14 ± 1.15[c]

[a] All values are means ± SEM.
[b] $P < 0.01$ compared to control.
[c] $P < 0.001$ compared to control.

was that the intermediate thioureas (**IVe**, **IVf** and **IVh**) were more active than corresponding title compounds. The promising compounds, **IVe** and **IVf** showed significant DNA damage in MCF-7cells. The DNA damaging activity of the molecules might be due to the generation of ROS/highly reactive intermediates. Further studies are in progress to elucidate the molecular mechanism responsible for their cytotoxicity and DNA damaging effect.

4. Experimental section

4.1. Chemistry

^{1}H NMR spectra were measured at 300 MHz with a JEOL GSX 270FT NMR spectrometer. Chemical shifts were measured relative to internal standard TMS (δ: 0). ^{13}C NMR spectra were recorded at 67.8 MHz on the same instrument with internal TMS (δ: 0, $CDCl_3$). IR spectra were recorded on an UNICAM series FT-instrument. Mass spectra were recorded on AEI MS 902 or VG ZAB-E-instruments. Microanalyses were performed by MEDAC Ltd., Surrey. Melting points were determined on Gallen Kamp capillary melting point apparatus and are uncorrected. Optical rotations were measured in chloroform solution using a Bellingham and Stanley ADP 220 polarimeter. Flash chromatography was performed using Fluka silica gel 60 (230–400 mesh). Thin layer chromatography was carried out using pre-coated aluminum plates (Merck Kieselghur 60 F_{254}) which were visualized under UV light and then with either phosphomolybdic acid or basic aqueous potassium permanganate as appropriate. All anhydrous reactions were carried out under argon or nitrogen. Anhydrous transfers were done with standard syringe techniques: all glasswares were pre-dried overnight. Dichloromethane was distilled from calcium hydride and stored over 4 Å molecular sieves.

4.1.1. General procedure for the synthesis of optically active substituted isothiocyanates (***IIa–h***, *Scheme 1) [13]*

To the solution of thiophosgene (0.01 mol) in CH_2Cl_2, optically active substituted primary amine (**Ia–h**) (0.01 mol) was added quickly at room temperature under stirring. Then a 25 ml saturated solution of $NaHCO_3$ was added slowly and stirred for 3 h. The CH_2Cl_2 layer was dried over anhydrous sodium sulphate and removed under reduced pressure. The residue was purified by flash chromatography using silica gel and petroleum ether:ether (40:1).

4.1.2. General procedure for the synthesis of optically active substituted thioureas (***IVa–h***, *Scheme 1)*

To a solution of **II** (0.01 mol) in dry MeOH (10 ml), an equimolar quantity of aromatic primary amine (0.01 mol) was added with stirring. The reaction mixture was heated on a steam bath at 70 °C for about 1 h and then the solvent was distilled off. The solid residue that separated was washed with water and dried at

40 °C. The resulting compounds were purified over silica gel using $CHCl_3$:EtOAc (7:3) to yield pure optically active thiourea derivatives.

*4.1.3. General procedure for the synthesis of optically active substituted 2-aminobenzothiazoles (**Va–h**, Scheme 1)*

Optically active substituted thioureas (0.01 mol) were dissolved in chloroform (15 ml), the reaction mixture was cooled in an ice bath and then bromine:chloroform (1:9) mixture was added drop wise. The reaction was monitored by TLC and after an hour, was poured on to crushed ice. The solid that separated was filtered, dried in each case and then purified by column chromatography using silica gel. It was eluted with $CHCl_3$:EtOAc (7:3) and the eluents on evaporation and crystallization yielded pure optically active substituted 2-aminobenzothiazoles.

*4.1.3.1. 1-(3-Chloro-4-fluorophenyl)-3-((R)-1-phenylethyl)thiourea (**IVa**).* This compound was prepared as per the above-mentioned procedure purified and isolated as pale yellow liquid: yield 64%; mp 110 °C $[\alpha]_D^{20} = +4.2$(c1.20 $CHCl_3$); IR (KBr) ν_{max} 3130, 2928, 2831, 2120, 1610 cm^{-1}; ^1H NMR ($CDCl_3$) δ ppm; 7.70 (br s, 1H, NH), 7.62–7.29 (m, 5H, Ar-H), 6.25 (br s, 1H, NH), 4.65 (m, 1H), 3.62 (m, 1H), 3.44 (m, 2H), 1.76–1.25 (m, 8H); ^{13}C NMR ($CDCl_3$) δ ppm; 138.5, 132.0, 129.0, 128.0, 128.2, 80.9, 60.5, 32.0, 31.3, 30.0, 23.7, 23.4.

*4.1.3.2. 1-(3-Chloro-4-fluorophenyl)-3-((R)-1-phenylpropyl)thiourea (**IVb**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid; yield 60%; mp 210–211 °C; $[\alpha]_D^{20} = -50.4$ (c1.30 $CHCl_3$); IR (KBr) ν_{max} 3224, 3041, 2967, 1600, 1542 cm^{-1}; ^1H NMR ($CDCl_3$) δ ppm; 7.77 (br s, 1H, NH) 7.38–7.28, (m, 3H, Ar-H), 7.15–7.07, (m, 5H, Ar-H), 6.30 (br s, 1H, NH), 5.26 (1H), 1.09 (m, 2H), 0.92 (t,3H, $J = 7.25$ Hz, $J = 7.23$ Hz); ^{13}C NMR ($CDCl_3$) δ ppm; 185.5, 155.2, 140.6, 133.1, 129.0, 128.0, 127.7, 126.5, 125.3, 125.2, 117.7, 60.6, 29.8, 10.5.

*4.1.3.3. 1-(3-Chloro-4-fluorophenyl)-3-((R)-1-(4-methoxyphenyl)ethyl) thiourea (**IVc**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid; yield 68%; mp 29–30 °C; IR (KBr) ν_{max} 3326, 2969, 1637, 1598, 1563, 1496 cm^{-1}; ^1H NMR ($CDCl_3$) δ ppm; 7.30–7.27 (m, 3H, Ar-H), 7.14–6.82 (m, 4H, Ar-H), 6.31 (br s, 1H, NH), 5.50 (b, 1H), 3.84 (s, 3H), 1.54 (d, 3H), 3.43; ^{13}C NMR ($CDCl_3$) δ ppm; 180.1, 160.0, 158.3, 155.0, 143.5, 133.2, 130.0, 127.7, 125.2, 118.2, 113.0, 55.3, 21.7; HRMS (EI) *m/z* calcd for $C_{16}H_{16}ClFN_2OS$: 339.0729; found: 339.0730.

*4.1.3.4. 1-(3-Chloro-4-fluorophenyl)-3-((R)-1-(4-fluorophenyl)ethyl) thiourea (**IVd**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid; yield 70%; mp 55–56 °C; $[\alpha]_D^{20} = -52.2$ (c1.18 $CHCl_3$); IR (KBr) ν_{max} 3224, 3033, 2969, 1602, 1535 cm^{-1}; ^1H NMR ($CDCl_3$) δ ppm; 8.31 (br s, 1H, NH), 7.31–7.00 (m, 7H, Ar-H), 6.23 (br s,1H, NH), 5.56 (br s, 1H), 1.55 (d, 3H, $J = 6.7$ Hz); ^{13}C NMR ($CDCl_3$) δ ppm; 179.9, 163.8, 160.5, 158.4, 137.7, 127.7, 125.1, 117.8, 117.5, 115.5, 53.7, 21.6; DIPMS 327.0 (M^+).

*4.1.3.5. 1-(3-Chloro-4-fluorophenyl)-3-((R)-1-(4-chlorophenyl)ethyl) thiourea (**IVe**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid; yield 55%; mp 58–60 °C; $[\alpha]_D^{20} = -77.2$ (c1.23 $CHCl_3$); IR (KBr) ν_{max} 3218, 3035, 1598, 1542, 1496 cm^{-1}; ^1H NMR ($CDCl_3$) δ ppm; 8.20 (br s, 1H, NH), 7.33–7.28 (m, 3H, Ar-H), 7.23–7.09 (m, 4H, Ar-H), 6.18 (br s, 1H, NH), 5.57 (1H), 1.51 (d, 3H, $J = 6.7$ Hz); ^{13}C NMR ($CDCl_3$) δ ppm; 180.1, 158.5, 155.2, 140.5, 133.5, 129.0, 127.5, 125.3, 125.2, 117.8, 117.5, 53.7, 21.5; DIPMS 343.0 (M^+).

*4.1.3.6. 1-(3-Chloro-4-fluorophenyl)-3-((S)-1-cyclohexylethyl)thiourea (**IVf**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid; yield 75%; mp 74–75 °C; $[\alpha]_D^{20} = -45.7$ (c1.05 $CHCl_3$); IR (KBr) ν_{max} 3237, 3052, 2933, 2850, 1608, 1544 cm^{-1}; ^1H NMR ($CDCl_3$) δ ppm; 8.25 (br s, 1H, NH), 7.33–7.14 (m, 3H, Ar-H), 5.78 (br s, 1H, NH), 4.37 (br s, 1H), 1.77–1.05 (m, 14H); ^{13}C NMR ($CDCl_3$) δ ppm; 180.0, 158.4, 155.0, 133.0, 127.8, 125.3, 117.6, 55.7, 42.9, 29.2, 29.0, 26.3, 26.1, 26.0, 17.2; DIPMS 315.1 (M^+).

*4.1.3.7. 1-(3-Chloro-4-fluorophenyl)-3-((R)-octan-2-yl)thiourea (**IVg**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid; yield 74%; mp 60–61 °C; $[\alpha]_D^{20} = -41.1$ (c1.24 $CHCl_3$); IR (KBr) ν_{max} 3224, 3045, 2927, 2856, 1600, 1544 cm^{-1}; ^1H NMR ($CDCl_3$) δ ppm; 8.45 (br s, 1H, NH), 7.35–7.18 (m, 3H, Ar-H), 5.75 (br s, 1H, NH), 4.47 (br s, 1H), 1.31–1.24 (m, 10H), 1.21 (d, 3H, $J = 6.4$ Hz), 0.91 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 179.7, 158.4, 133.0, 127.8, 125.3, 122.2, 117.5, 51.5, 36.4, 31.6, 29.0, 25.8, 22.5, 20.3, 14.0 ppm; DIPMS 317.1 (M^+).

*4.1.3.8. 1-(2-(Benzyloxy)cyclohexyl)-3-(3-chloro-4-fluorophenyl)thiourea (**IVh**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid: yield 61%; mp 112–113 °C; $[\alpha]_D^{20} = +75.4$ (c1.18 $CHCl_3$); IR (KBr) ν_{max} 3232, 3035, 2944, 1594, 1556 cm^{-1}; ^1H NMR ($CDCl_3$) δ ppm; 9.39 (br s, 1H, NH), 7.37–7.21 (m, 3H, Ar-H), 7.79–7.95 (m, 5H, Ar-H), 6.25 (br s, 1H, NH), 4.76 (d, 3H, $J = 6.0$ Hz, 1H), 4.44 (d, 3H, $J = 6.2$ Hz, 1H), 3.43 (m, 2H), 2.36–1.27 (m, 8H); HRMS (EI) *m/z* calculated for $C_{20}H_{22}ClFN_2OS$: 393.1198; found: 393.1198.

*4.1.3.9. 5-Chloro-6-fluoro-N-((R)-1-phenylethyl)benzo[d]thiazol-2-amine (**Va**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid; yield 55%; mp 109–110 °C; $[\alpha]_D^{20} = +11.2$ (c1.06 $CHCl_3$); IR (KBr) ν_{max} 3303, 2975, 1629, 1563 cm^{-1}; ^1H NMR (DMSO-d_6) δ ppm; 7.62–7.04 (m, 7H, Ar-H) 6.88, (br s, 1H, NH), 4.89, (m, 1H), 1.41 (d, 3H, $J = 6.9$ Hz); ^{13}C NMR (DMSO-d_6) δ ppm; 156.2, 155.3, 154.8, 145.0, 137.7, 129.0, 127.4, 126.4, 120.5, 117.9, 116.7, 49.5, 23.2; HRMS (EI) *m/z* calcd for $C_{15}H_{12}ClFN_2S$: 307.0467; found: 307.0462.

*4.1.3.10. 5-Chloro-6-fluoro-N-((R)-1-phenylpropyl)benzo[d]thiazol-2-amine (**Vb**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid; yield 40%; mp 159–160 °C; $[\alpha]_D^{20} = +10.5$ (c1.04 $CHCl_3$); IR (KBr) ν_{max} 3153, 3070, 2929, 1619, 1519 cm^{-1}; ^1H NMR (DMSO-d_6) δ ppm; 9.20 (br s, 1H, NH), 7.90–7.32, (2H, Ar-H), 7.29–7.01, (5H, Ar-H), 4.90, (1H), 1.98, (m, 2H), 0.99, (3H); ^{13}C NMR (DMSO-d_6) δ ppm; 167.0, 153.7, 142.5, 130.6, 128.5, 127.3, 126.6, 117.7, 116.7, 114.4, 110.0, 60.3, 29.7, 10.9; ^{19}F NMR δ −124.3, 126.6 ppm; HRMS (EI) *m/z* calcd for $C_{16}H_{14}ClFN_2S$: 321.0623; found: 321.0618.

*4.1.3.11. 5-Chloro-6-fluoro-N-((R)-1-(4-methoxyphenyl)ethyl)benzo[d] thiazol-2-amine (**Vc**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid; yield 38%; mp 119–120 °C; $[\alpha]_D^{20} = +11.9$ (c1.00 $CHCl_3$); IR (KBr) ν_{max} 3421, 3153, 3077, 2850, 1627, 1525 cm^{-1}; ^1H NMR (DMSO-d_6) δ ppm; 7.82–7.22 (6H, Ar-H), 5.70 (br s, 1H, NH), 4.63 (br s, 1H), 3.98 (s, 3H, OCH_3), 1.62 (d, 3H, $J = 6.8$ Hz, 3H); HRMS (EI) *m/z* calcd for C16H14ClFN$_2$OS: 336.0499; found: 336.0478.

*4.1.3.12. 5-Chloro-6-fluoro-N-((R)-1-(4-fluorophenyl)ethyl)benzo[d] thiazol-2-amine (**Vd**).* The compound was prepared as per the general procedure mentioned above and isolated as colourless solid; yield 45%; mp 124–125 °C; $[\alpha]_D^{20} = +20.8$ (c1.25 $CHCl_3$); IR (KBr) ν_{max} 3405, 2915, 1627, 1511 cm^{-1}; ^1H NMR (DMSO-d_6) δ ppm; 7.88–6.96

(6H, Ar-H), 6.85 (br s, 1H, NH), 4.84 (m, 1H), 1.64 (d, 3H, $J = 6.6$ Hz, 3H); ^{13}C NMR (DMSO-d_6) δ ppm; 155.5, 136.5, 135.0, 130.0, 129.0, 128.0, 127.9, 120.1, 118.0, 116.9, 116.3, 116.0, 115.6, 111.1, 49.0, 23.5; HRMS (EI) m/z calcd for $C_{15}H_{11}ClF_2N_2S$: 325.0372; found: 325.0381.

*4.1.3.13. 5-Chloro-*N*-((R)-1-(4-chlorophenyl)ethyl)-6-fluorobenzo[*d*]thiazol-2-amine (**Ve**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid; yield 35%; mp 149–150 °C; $[\alpha]_D^{20} = +14.9$ (c1.00 $CHCl_3$); IR (KBr) ν_{max} 3421, 3153, 3077, 2852, 1623, 1525 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ ppm; 7.82–7.22 (6H, Ar-H), 5.70, (br s, 1H, NH), 4.63, (br s, 1H), 1.62, (d, 3H, $J = 6.7$ Hz, 3H); HRMS (EI) m/z calcd for $C_{15}H_{11}Cl_2FN_2S$: 341.0077; found: 341.0078.

*4.1.3.14. 5-Chloro-*N*-((R)-1-cyclohexylethyl)-6-fluorobenzo[*d*]thiazol-2-amine (**Vf**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid; yield 51%; mp 134–135 °C; $[\alpha]_D^{20} = +26.4$ (c1.06 $CHCl_3$); IR (KBr) ν_{max} 3424, 3158, 3091, 2927, 2852, 1629 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ ppm; 9.50 (br s, 1H, NH), 7.78 (1H, Ar-H), 7.54 (1H, Ar-H), 3.90 (1H), 1.79–1.50 (m, 3H), 1.25 (d, 3H, $J = 6.7$ Hz, 3H), 1.23–0.99 (m, 8H); ^{13}C NMR (DMSO-d_6) δ ppm; 166.7, 145.6, 129.3, 115.9, 114.6, 111.3, 110.5, 56.0, 28.6, 25.2, 25.5, 17.0,16.9; ^{19}F NMR δ ppm; 126.3; HRMS (EI) m/z calcd for $C_{15}H_{18}ClFN_2S$: 313.0936; found: 313.0940.

*4.1.3.15. 5-Chloro-6-fluoro-*N*-(R)-(octan-2-yl)benzo[*d*]thiazol-2-amine (**Vg**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid; yield 35%; mp 119–120 °C; $[\alpha]_D^{20} = +41.4$ (c1.11 $CHCl_3$)IR (KBr) ν_{max} 3154, 3068, 2925, 1627, 1523, 1471 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ ppm; 8.04 (br s, 1H, NH), 7.37 (s, 1H, Ar-H), 6.93 (s, 1H, Ar-H), 5.54 (m, 1H), 2.97 (3H), 1.53–0.77 (m, 13H); ^{13}C NMR(DMSO-d_6) δ ppm; 155.6, 137.8, 120.7, 120.5, 118.9, 117.9, 116.6, 52.1, 40.5, 39.6, 32.1, 29.5, 26.3, 21.1, 14.5; HRMS (EI) m/z calcd for $C_{15}H_{20}ClFN_2S$: 315.1093; found: 315.1085.

4.1.3.16. N*-(1R,2S)-2-(Benzyloxycyclohexyl)-5-chloro-6-fluorobenzo[*d*]thiazol-2-amine (**Vh**).* The compound was prepared as per the general procedure mentioned above purified and isolated as colourless solid: yield 41%; mp 102–103 °C; $[\alpha]_D^{20} = +70.4$ (c1.19 $CHCl_3$); IR (KBr) ν_{max} 3232, 3035, 2944, 1594, 1556 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ ppm; 9.39 (br s, 1H, NH), 7.82–7.22 (m, 2H, Ar-H), 7.19 (m, 5H, Ar-H), 4.70 (d, 3H, $J = 4.7$ Hz, 1H), 4.40 (d, 3H, $J = 4.6$ Hz, 1H), 3.42 (m, 2H), 2.36–1.27 (m, 8H); ^{13}C NMR (DMSO-d_6) δ ppm; 168.6, 150.8, 146.7, 137.5, 128.9, 127.8, 124.6, 122.6, 117.6, 107.6, 75.1, 72.5, 56.6, 29.1, 28.5, 23.3, 22.1; HRMS (EI) m/z calculated for $C_{20}H_{20}ClFN_2OS$: 390.1198; found: 390.1194.

4.2. Pharmacology

4.2.1. Cell lines

Human cancer cell lines, MCF-7 and HeLa cells, procured from National Centre for Cell Sciences, Pune, India, were cultured in MEM medium supplemented with 10% FBS, 1% L glutamine and 50 μg/ml gentamicin sulphate in a CO_2 incubator in a humidified atmosphere of 5% CO_2 and 95% air. The EAC cells were maintained for 12–14 days in the peritoneal cavity of Swiss albino mice. The tumor cell cultures were started from mouse Ehrlich Ascites with at least one passage in vitro prior to use.

4.2.2. In vitro cytotoxic activities (MTT assay)

In vitro cytotoxicity was determined using a standard MTT assay [27] with protocol appropriate for the individual test system. In brief, exponentially growing cells were plated in 96-well plates (10^4 cells/well in 100 μl of medium) and incubated for 24 h for attachment. Test compounds were prepared prior to the experiment by dissolving in 0.1% DMSO and diluted with medium. The cells were then exposed to different concentrations of the drugs (1–100 μM) in the volume of 100 μl/well. Cells in the control wells received the same volume of medium containing 0.1% DMSO. After 24 h, the medium was removed and cell cultures were incubated with 100 μl MTT reagent (1 mg/ml) for 4 h at 37 °C. The formazan produced by the viable cells was solubilized by addition of 100 μl DMSO. The suspension was placed on micro-vibrator for 5 min and absorbance was recorded at 540 nm by the ELISA reader. The experiment was performed in triplicate. The percentage cytotoxicity was calculated using the formula

$$\%\ \text{Cytotoxicity} = \frac{(\text{Control abs} - \text{Blank abs}) - (\text{Test abs} - \text{Blank abs})}{(\text{Control abs} - \text{Blank abs})} \times 100$$

For MTT time course study, MCF-7 cells (5×10^3cells/well) seeded in 96-well plates were exposed to different concentrations of test compounds (1–50 μM). The percentage cytotoxicity and IC_{50} values were determined at 24, 48 and 72 h of drug incubation.

4.2.3. DNA damaging activity (alkaline comet assay)

The effect of promising compounds on DNA was assessed by comet assay as described by protocol [28] with slight modifications. Briefly, after the drug treatment, cells were harvested from each culture flask and counted by trypan blue exclusion method. About 20,000 cells in 50 μl medium was suspended in 150 μl of low melting agarose (0.75%) and layered onto slides pre-coated with an earlier layer of agarose (1.5%). Slides were kept in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris; pH 10–10.5; 1% Triton X-100 and 10% DMSO) overnight at 4 °C in dark, these were subjected to unwinding under alkaline conditions (pH 13) for 1 h to allow DNA supercoils to relax and express DNA single-strand breaks and alkali-labile sites. Electrophoresis was then carried out under highly alkaline (pH 13) conditions for 20 min at 16 V and 300 mA. After neutralization, staining was done using ethidium bromide. Two slides were prepared for each concentration. About 50 cells were captured per slide using fluorescent microscope and the images were analyzed using Komet 5.5 software (Kinetic imaging systems, UK). A variety of objective measurements like Head DNA, Tail DNA, and Olive Tail Moment (OTM) were made. Since the Tail moment has been regarded as one of the best indices of DNA damage among the other parameters, it was calculated as follows:

$$\text{Tail moment} = \frac{\text{Tail length} \times \%\text{Tail DNA}}{100}$$

Acknowledgement

One of the authors, S.N. Manjula, is grateful to the Department of Science and Technology, Goverment of India for providing financial support to carry out this project (Research grant no. SSR/WOS-A/LS-37/2007) and also to Dr. B.S. Satish Rao, Professor and Head,

Department of Radiobiology, Manipal Life Sciences Centre, Manipal University, Manipal, India for extending cooperation in the accomplishment of the work.

References

[1] N. Aydemir, R. Bilaloglu, Mutat. Res. 537 (2003) 43–51.
[2] C. Mortimer, G. Wells, J.P. Crochard, E.L. Stone, T.D. Bradshaw, M.G.F. Stevens, A.D. Westwell, J. Med. Chem. 49 (2006) 179–185.
[3] M. Yoshida, I. Hayakawa, N. Hayashi, T. Agatsuma, Y. Oda, F. Tanzawa, S. Iwasaki, K. Koyama, H. Furukawa, S. Kurakata, Y. Sugano, Bioorg. Med. Chem. Lett. 15 (2005) 3328–3332.
[4] P. Vicini, A. Geronikaki, M. Incerti, B. Busonera, G. Poni, C.A. Cabras, P.L. Colla, Bioorg. Med. Chem. 11 (2003) 4785–4789.
[5] C. Sanmartin, M. Echeverria, B. Mendıvil, L. Cordeu, E. Cubedo, J. Garcia-Foncillas, M. Fontc, J.A. Palop, Bioorg. Med. Chem. 13 (2005) 2031–2044.
[6] G. Hallur, A. Jimeno, S. Dalrymple, T. Zhu, M.K. Jung, M. Hidalgo, J.T. Isaacs, S. Sukumar, E. Hamel, S.R. Khan, J. Med. Chem. 49 (2006) 2357–2360.
[7] E. Moreau, S. Fortin, M. Desjardins, J.L.C. Rousseau, E.C. Petitclerc, R.C. Gaudreault, Bioorg. Med. Chem. 13 (2005) 6703–6712.
[8] T.D. Bradshaw, A.D. Westwell, Curr. Med. Chem. 11 (2004) 1241–1253.
[9] A. Esteves-Souza, K. Pissinate, G.M. Nascimento, N.F. Grynberg, E. Aurea, Bioorg. Med. Chem. 14 (2006) 492–499.
[10] S.J. Choi, H.J. Park, S.K. Lee, S.W. Kim, G. Han, H.Y.P. Choo, Bioorg. Med. Chem. 14 (2006) 1229–1235.
[11] T.K. Venkatachalam, C. Mao, F.M. Ucku, Bioorg. Med. Chem. 12 (2004) 4275–4284.
[12] P.T. Lind, J.M. Morin, R.J. Noreen, R.J Ternansky, WO 9303022. Through CA 119: 160110, 1993.
[13] T. Ishikawa, J. Org. Chem. 65 (2000) 7774–7778.
[14] C.O. Leong, M. Gaskell, E.A. Martin, R.T. Heydon, P.B. Farmer, M.C. Bibby, P.A. Cooper, J.A. Double, T.D. Bradshaw, M.F. Stevens, Br. J. Cancer 88 (2003) 470–477.
[15] S. Osbild, L. Brault, E. Battaglia, D. Bagrel, Anticancer Res. 26 (2006) 3595–3600.
[16] H. Mizutani, Yakugaku Zasshi 127 (2007) 1837–1842.
[17] H.P. Hari, D. Umashankar, J.Q. Wilson, K. Masami, S. Hiroshi, R.D. Jonathan, Eur. J. Med. Chem. 43 (2008) 1–7.
[18] P.A. Loaiza, V. Trapani, C. Hose, S.S. Singh, J.B. Trepel, M.F.G. Stevens, T.D. Bradshaw, E.A. Sausville, Mol. Pharmacol. 61 (2002) 13–19.
[19] V. Trapani, V. Patel, C.O. Leong, H.P. Ciolino, G.C. Yeh, C. Hose, J.B. Trepel, M.F.G. Stevens, E.A. Sausville, P.A. Loaiza, Br. J. Cancer 88 (2003) 599–605.
[20] R.F. Lee, S. Steinert, Mutat. Res. 544 (2003) 43–64.
[21] N. Kopjar, V.V. Garaj, I. Milas, Teratog. Carcinog. Mutagen. 22 (2002) 13–30.
[22] P. Vernole, B. Tedechi, D. Caporossi, M. Maccarrone, G. Melino, P.M. Annicchiarico, Mutagenesis 13 (1998) 209–215.
[23] E. Harvathova, D. Slamenova, L. Hlincikova, T.K. Mandal, A. Gabelova, A.R. Collins, Mutat. Res. 409 (1998) 163–171.
[24] E. Kashiyama, I. Hutchinson, M.S. Chua, S.F. Stinson, L.R. Phillips, G. Kaur, E.A. Sausville, T.D. Bradshaw, A.D. Westwell, M.F.G. Stevens, J. Med. Chem. 42 (1999) 4172–4184.
[25] M.S. Chua, E. Kashiyama, T.D. Bradshaw, S.F. Stinson, E.J. Brantley, E.A. Sausville, M.F.G. Stevens, Cancer Res. 60 (2000) 5196–5203.
[26] R. Dubey, P.K. Shrivastava, P.K. Basniwal, B.S. Moorthy, Mini Rev. Med. Chem. 6 (2006) 33–37.
[27] T. Mossman, J. Immunol. Methods 65 (1983) 55–63.
[28] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, Exp. Cell Res. 175 (1988) 184–191.