European Journal of Medicinal Chemistry 45 (2010) 2949–2956

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

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

Original article

Synthesis and anticancer activity of some novel 2-substituted benzimidazole derivatives

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ARTICLE INFO

Article history:
Received 3 January 2010
Received in revised form
14 March 2010
Accepted 17 March 2010
Available online 25 March 2010

Keywords:
Oxothiazolidinylidene-methylbenzimidazole
Thioxothiazolylbenzimidazoles
Fluorobenzylidenecyano-methylbenzimidazoles
Cycloalkylidenecyano-methylbenzimidazoles
Oxothiazolidinylidenecyano-methyl benzimidazoles
Thiazolylidenecyano-methyl benzimidazoles
Thiazinylidenecyano-methylbenzimidazoles
Anticancer

ABSTRACT

In an effort to establish new candidates with improved anticancer activity, we report here the synthesis of various series of 2-substituted benzimidazoles: 2-[(4-oxothiazolidin-2-ylidene) methyl and (4-amino-2-thioxothiazol-5-yl) benzimidazoles (**2** and **3**, respectively); 2-[(4-fluorobenzylidene and cycloalkylidene) cyanomethyl] benzimidazoles (**4** and **5**, respectively), together with the synthesis of certain of 2-[(4- or 5-oxothiazolidin-2-ylidene, 4-substituted thiazolyl-2-ylidene and [1,3]thiazin-2-ylidene) cyanomethyl]benzimidazoles (**6**, **8**, **7** and **9**, respectively). Several of the synthesized products were subjected to in vitro anticancer screening that revealed that all the tested compounds exhibited anti-tumor activity against human hepatocellular carcinoma (HEPG2), human breast adenocarcinoma (MCF7) and human colon carcinoma (HCT 116) cell lines, with IC50's < 10 μg/ml.

1. Introduction

The benzimidazole ring system is an important pharmacophore in medicinal chemistry and modern drug discovery. 2-Substituted benzimidazoles have been known to act as potential anticancer [1–4]. For example, bis-benzimidazole derivatives [5–9] were remarkably active compounds in interfering with DNA topoisomerase I [5] and were also found to be cytotoxic against breast adenocarcinoma (MCF7) [7] and skin epidermoid carcinoma (A431) [7–9]. Also, Methyl-2-benzimidazole carbamate (carbendazim, FB642) is an anticancer agent that induces apoptosis of cancer cells [10,11].

Albendazole, a benzimidazole carbamate (methyl 5-propylthio-1H-benzimidazol-2-yl carbamate) with extensive clinical use as an anthelmintic drug, can also inhibit hepatocellular carcinoma cell proliferation under both in vitro and in vivo experimental conditions [12].

The next generation of rationally designed inhibitors, benzimidazole carboxamides, for example, 2-(4-hydroxyphenyl)benzimidazole-4-carboxamide (NU1085) [13] and 2-(4-oxadiazolylphenyl) analogue [14], Fig. 1, were vastly more potent as poly(ADP-ribose) polymerase inhibitor (PARP inhibitor) and enhanced the effects of chemotherapy and radiation therapy in vitro [13] and in vivo [14]. Also, the tricyclic benzimidazole (AG14361), Fig. 1, which is a PARP inhibitor, has been developed and used in vivo at non-toxic doses to augment the effect of the DNA-damaging agents irinotecan (topoisomerase I poisons), γ-irradiation or temozolomide (DNA alkylating agent) [15].

On the other hand, the antiviral activity of 5-chloro and 5,6-dichloro-2-substituted benzimidazole derivatives against several viruses such as influenza, human cytomegalovirus, hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency retrovirus (HIV-1) was also reported [16–18]. These compounds were also reported to possess anticancer activity against breast and

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doi:10.1016/j.ejmech.2010.03.022

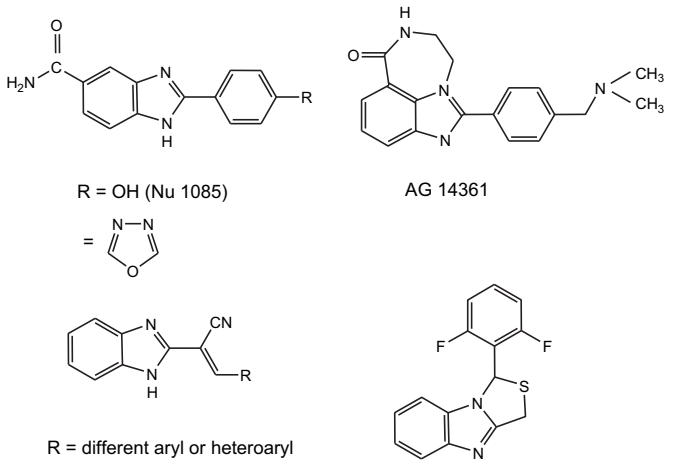


Fig. 1.

prostate cancer cell lines [19] or as potential topoisomerase II inhibitors [20]. Whereas 2-substituted benzimidazole-5-carboxylic acid and acid derivatives were widely used in the design of antineoplastic agents [21–23], hepatitis C virus polymerase inhibitors [24] and antimicrobial agents [25–27].

Careful literature survey revealed that 3-arylacrylonitriles with either heteroaryl [28–30] or benzimidazole [31,32] substituents in position 2 of the acrylonitrile, Fig. 1, have good cytotoxic activity on human cancer cells. In addition, it was observed that thiazolobenzimidazoles [33,34], Fig. 1, and thiazolylbenzimidazoles [35,36] exhibited strong anticancer activity.

As a part of an ongoing study of biologically active 2-substituted benzimidazole, the present work was directed to synthesize certain novel 2-substituted benzimidazole derivatives that comprise the aforementioned moieties in their framework in order to investigate their in vitro antitumor activities.

2. Chemistry

Prior to delving into the synthesis and biological evaluation of the target compounds (Schemes 1 and 2), we decided to investigate 5-chloro-2-cyanomethylbenzimidazole (**1a**) and 2-cyanomethylbenzimidazole-5-carboxylic acid (**1b**) by spectral studies. Although the 5-chloro derivative **1a** was reported [37,38], it was not fully characterized and no ^{1}H NMR spectral data has yet been reported. On the other hand, 2-cyanomethylbenzimidazole-5-carboxylic acid (**1b**) was not previously reported.

The ^{1}H NMR spectrum of **1a** revealed an exchangeable proton (NH) at δ 12.76 ppm, while compound **1b** showed two exchangeable protons (NH and COOH) at δ 12.48–12.82 ppm. The aromatic ring protons of compounds **1a** and **1b** displayed a characteristic set of three signals as follows: Ar-H (4) as singlet at δ 8.12 and 8.13 ppm, respectively, Ar-H (6) as doublet at δ 7.81 ppm (J = 8.6 Hz) and Ar-H (7) as doublet at δ 7.57 ppm (J = 8.6 Hz). In addition, the CH_2 group appeared as singlet at δ 4.58 and 4.59 ppm, respectively. It was noticed that this pattern was more or less the same in all the newly synthesized compounds. On the other hand, the IR spectra of compounds **1a** and **1b** showed the characteristic C≡N stretching absorption at ν 2200 and 2208 cm^{-1}, respectively, in addition to C=O absorption at ν 1674 cm^{-1} for compound **1b**. Further structural evidence stemmed from the mass spectrum of compound **1b**, which corroborates the spectral data and the proposed structure, giving a molecular ion peak with m/z 202.07.

Preparation of 2-[(4-oxothiazolidin-2-ylidene) methyl] benzimidazole-5-carboxylic acid (**2**) was accomplished by the reaction of **1b** with thioglycolic acid. Where as reacting **1b** with sulfur and the appropriate isothiocyanate, following the reaction conditions reported for the preparation of related compounds [39], afforded 2-(4-amino-3-substituted-2-thioxo-2,3-dihydrothiazol-5-yl) benzimidazole-5-carboxylic acid derivatives (**3a**,**b**). The structures of compounds **2** and **3a**,**b** were confirmed by IR and ^{1}H NMR spectra. The IR spectra proved as useful in tracing the disappearance of the C≡N stretching absorption of the parent compound **1b** and the appearance of a C=O stretching absorption in compound **2** and a C=S absorption together with NH_2 absorption in compounds **3a**,**b**. The ^{1}H NMR spectra of compounds **3a**,**b** displayed the disappearance of CH_2 absorption of the precursor **1b** and the presence of the expected benzimidazole protons signals together with other signals assigned to N-benzyl and N-phenyl groups, respectively. It is reported that compounds containing a fluoro substituent were highly active anticancer agents [20,34,40]. Therefore, 4-fluorobenzylidenecyanomethylbenzimidazoles (**4a**,**b**) were prepared via the treatment of **1a**,**b** with 4-fluorobenzaldehyde in dimethyl formamide following reported reaction conditions [32]. The ^{1}H NMR spectra showed significant absorption bands at δ 7.84 and 7.56 ppm corresponding to =CH proton of compounds **4a** and **4b** respectively.

In addition, 2-cycloalkylidenecyanomethylbenzimidazoles (**5a**, **b**) were prepared by reacting **1a**,**b** with cyclopentanone or cyclohexanone in the presence of ammonium acetate. The ^{1}H NMR spectra were consistent with the proposed structures.

On the other hand, the synthesis of 2-[(3-substituted-5-oxothiazolidin-2-ylidene) cyanomethyl]benzimidazole-5-carboxylic acid derivatives (**6a**,**b**), 2-[(4-aryl-3-substituted thiazolidin-2-ylidene) cyanomethyl] benzimidazoles (**7a**–**c**), 2-[(3-substituted-4-oxothiazolidin-2-ylidene)cyanomethyl] benzimidazoles (**8a**,**b**) and 2-[(3-phenyl[1,3]thiazin-2-ylidene)cyanomethyl] benzimidazoles (**9a**,**b**) was achieved as previously described for the preparation of analogous compounds [41]. Accordingly, treatment of **1a**,**b** with the appropriate arylisothiocyanate in dimethyl formamide in the presence of potassium hydroxide, produced the non isolated potassium salts of 2-[(N-substituted thiocarbamoyl)cyanomethyl]benzimidazole. Cyclization of the latter with chloroacetyl chloride, gave 2-[(3-substituted-5-oxothiazolidin-2-ylidene)cyanomethyl]benzimidazole -5-carboxylic acid derivatives (**6a**,**b**). Whereas cyclocondensation of the respective potassium salts with substituted phenacyl bromides yielded the corresponding benzimidazoles **7a**–**c**.

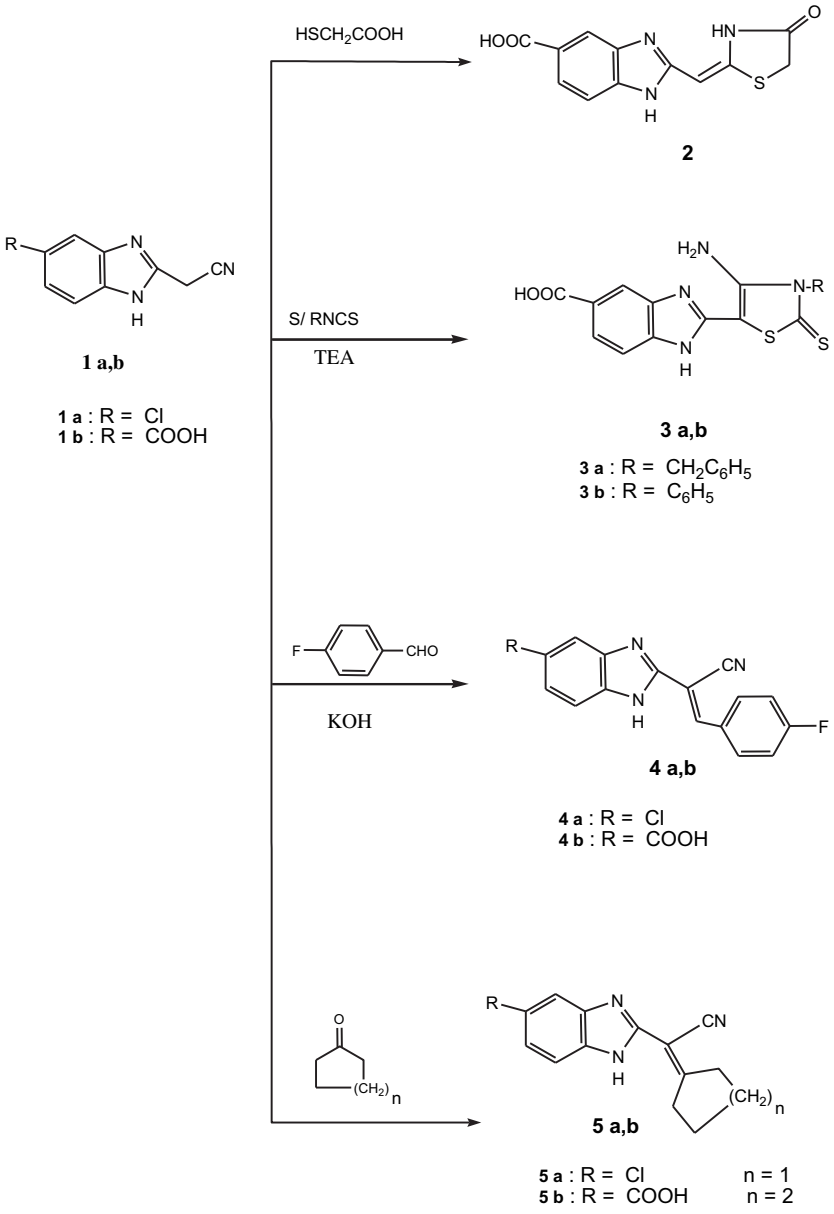
Finally, treatment of the respective potassium salt of 2-(thiocarbamoyl cyanomethyl)benzimidazole with ethyl bromoacetate or 1, 3-dibromopropane afforded 2-[(4-aryl-3-substituted thiazolidin-2-ylidene) cyanomethyl] benzimidazoles (**8a**,**b**) and 2-[(3-phenyl [1,3]thiazin-2-ylidene) cyanomethyl] benzimidazoles (**9a**,**b**).

The IR spectra of compounds **6**–**9** clearly showed the C≡N stretching in addition to the C=O stretching for compounds **6a**, **b** and **8a**,**b**. Whereas the ^{1}H NMR spectra displayed signals at δ 4.19–4.91 ppm corresponding to two thiazolidinone protons which were assigned to the structures **6a**,**b** and **8a**,**b** . Further, compounds **7a**–**c** showed a thiazole proton at δ 6.35–6.44 ppm and compounds **9a**,**b** exhibited a set of three aliphatic signals integrating for 6 protons (3× CH_2).

3. Results and discussion

3.1. Cytotoxic activity

The cytotoxicity of compounds **3a**, **4a**, **5b**, **6a**, **7c**, **8a** and **9b** was evaluated against three cell lines representing three common forms of human cancer i.e. human hepatocellular carcinoma cell line (HEPG2), human breast adenocarcinoma cell line (MCF7) and colon carcinoma cell line (HCT 116). For comparison purposes, the cytotoxicity of doxorubicin, a standard antitumor drug, was evaluated



Scheme 1.

under the same conditions. The IC50 and IC90 (dose of the compound which caused a 50% and 90% reduction of survival values) are shown in Tables 1 and 3 respectively. The results are represented graphically in Figs. 2–6.

All the tested compounds were found to possess potential antitumor activities against all of the tested tumor cell lines, with IC50's < 10 μg/ml (Table 1). Generally, all the tested compounds tended to be more active against HEPG2, than against other tumor cell lines. Compounds **3a** and **4a** showed the highest potency against HEPG2 while compounds **5b**, **8a** and **7c** were the most active against MCF7. Compounds **8a** and **7c** were moderately potent against HCT 116 (Tables 1 and 3).

The results in Table 2 demonstrate the sensitivity of individual cell lines. The 2-thiazolylbenzimidazole derivative (**3a**), benzylidene cyanomethylbenzimidazole (**4a**) and oxothiazolidin-2-ylidenecyanomethyl benzimidazole (**8a**) were the most potent compounds possessing broad spectrum of activity against all three cell lines. While some other compounds were not the most potent ones, their specific activity against particular cell lines makes them of interest for further development as anticancer agents.

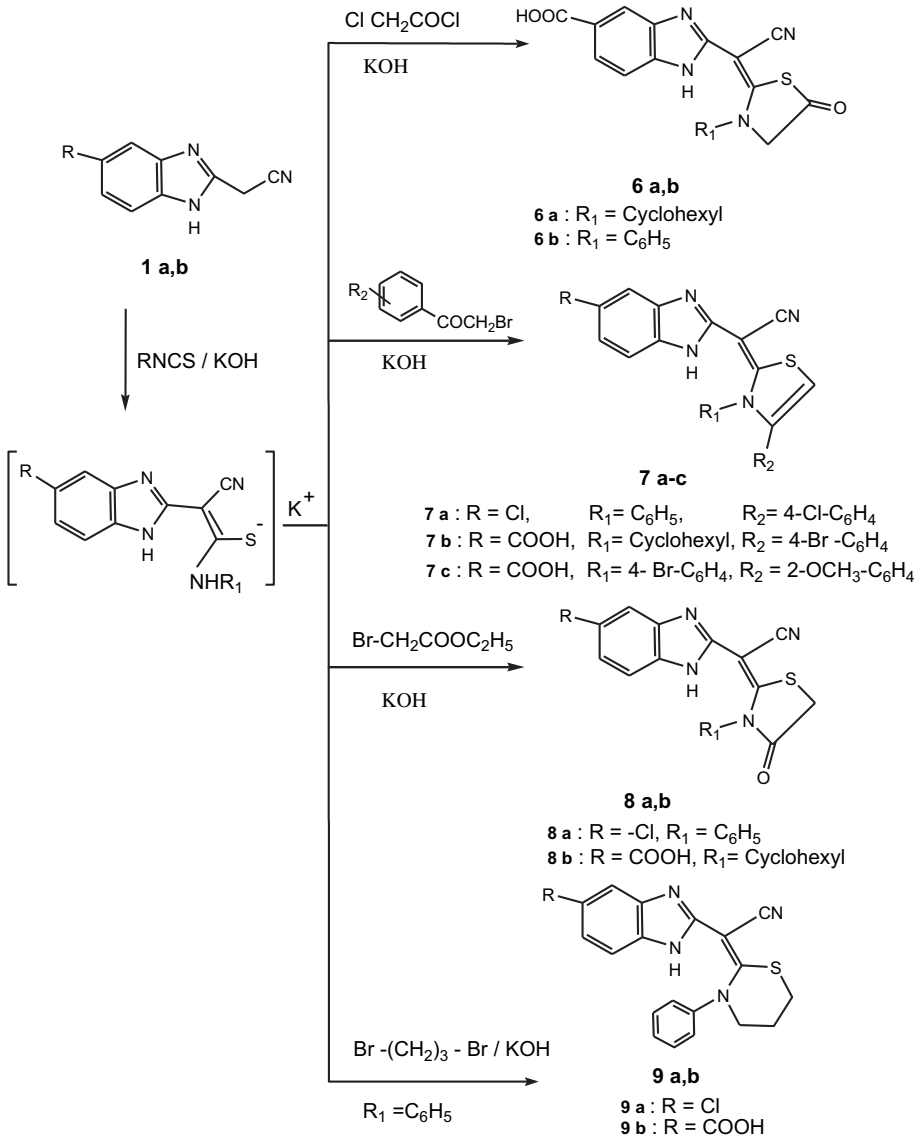
4. Conclusion

In summary, the synthesis and characterization of new series of 2-substituted benzimidazole derivatives having 5-chloro or 5-underivatized carboxylic acid group have been described. All the tested compounds displayed antitumor activity against HEPG2, MCF7 and HCT 116 and could therefore serve as lead chemical entities for further modification to render them clinically useful drug agents.

5. Experimental

5.1. Chemistry

Melting points were obtained on a Griffin apparatus and are uncorrected. Microanalyses for C, H and N were carried out at the



Scheme 2.

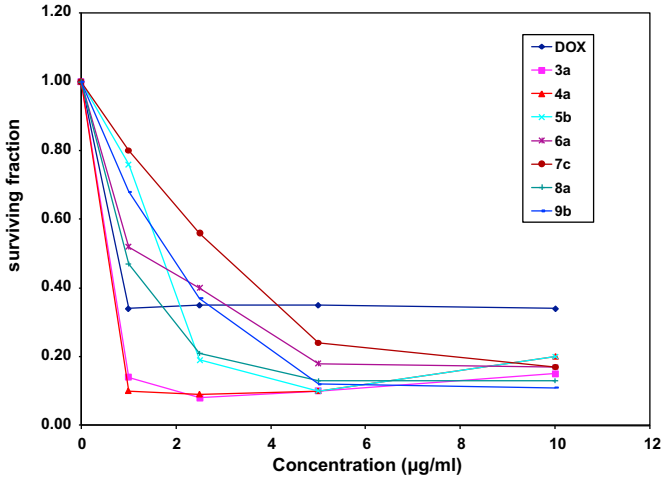


Fig. 2. Cytotoxicity of **3a**, **4a**, **5b**, **6a**, **7c**, **8a**, **9b** and doxorubicin against human hepatocellular carcinoma cell line (HEPG2).

microanalytical center, Cairo University. IR spectra were recorded on a Shimadzu 435 spectrometer, using KBr discs. ^{1}H NMR spectra were performed on a joel NMR FXQ-200 MHz spectrometer, using TMS as the internal standard. Mass spectra were recorded on a GCMP-QP1000 EX Mass spectrometer. Progress of the reactions were monitored by TLC using precoated aluminum sheet silica gel MERCK 60F 254 and was visualized by UV lamp.

*5.1.1. General procedure for the preparation of 2-cyanomethylbenzimidazoles (**1a**,**b**)*

Substituted 1,2-phenylenediamine (viz; 4-chloro-1,2-phenylenediamine, 1,2-diaminobenzoic acid) (10 mmol) and ethylcyanoacetate (17 g, 15 mmol) were placed in the reaction tube and heated at 170–180 °C for 20 min. The residue was broken up and extracted with ether. The residue was recrystallized from ethanol.

*5.1.1.1. 5-Chloro-2-cyanomethylbenzimidazole (**1a**).* Yield: 88%; mp: 102–104 °C; IR (cm^{-1}): 3263 (NH), 2200 (CN); ^{1}H NMR (DMSO-d_6): δ 4.58 (s, 2H, CH_2), 7.57 (d, 1H, J = 8.6 Hz, benzimidazole-C_7-H), 7.81 (d, 1H, J = 8.6 Hz, benzimidazole-C_6-H), 8.12 (s, 1H, benzimidazole-C_4-H), 12.76 (brs, 1H, NH, D_2O exchangeable).

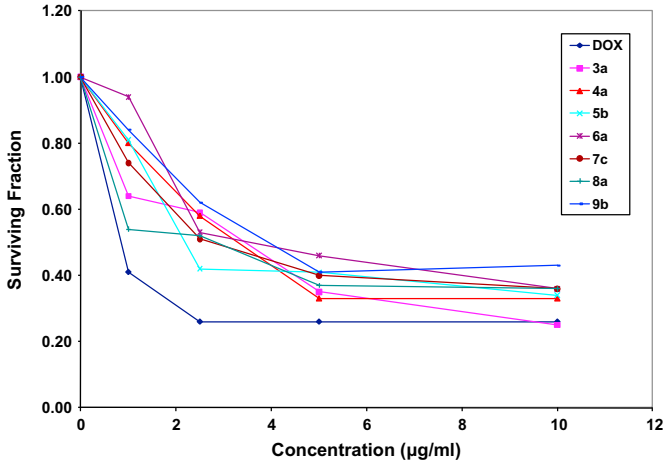


Fig. 3. Cytotoxicity of **3a**, **4a**, **5b**, **6a**, **7c**, **8a**, **9b** and doxorubicin against human breast adenocarcinoma cell line (MCF7).

*5.1.1.2. 2-Cyanomethylbenzimidazole-5-carboxylic acid (**1b**).* Yield: 92%; mp: 292–294 °C; IR (cm^{-1}): 3385–3089 (NH and COOH), 2208 (CN), 1674 (C=O); ^1H NMR (DMSO-d_6): δ 4.59 (s, 2H, CH_2), 7.57 (d, 1H, J = 8.6 Hz, benzimidazole-C_7-H), 7.81 (d, 1H, J = 8.6 Hz, benzimidazole-C_6-H), 8.13 (s, 1H, benzimidazole-C_4-H), 12.48–12.82 (brs, 2H, NH and COOH, D_2O exchangeable); MS: m/z 202.07 (M+, 100%); Anal. Calcd. for $C_{10}H_7N_3O_2$ (201.17): C, 59.70, H, 3.50, N, 20.88. Found: C, 59.74, H, 3.46, N, 20.96.

*5.1.2. 2-(4-Oxothiazolidin-2-ylidene)methylbenzimidazole-5-carboxylic acid (**2**)*

To a solution of 2-cyanomethylbenzimidazole-5-carboxylic acid (2 g, 10 mmol) in absolute ethanol (5 ml), thioglycolic acid (0.92 g, 10 mmol) was added. The reaction mixture was heated under reflux for 7 h then left to cool to room temperature. The separated crystalline product was filtered, washed with ethanol and crystallized from ethanol. Yield: 52%; mp: >300 °C; IR (cm^{-1}): 3388–3050 (NH and COOH), 1675, 1656 (2C=O); ^1H NMR (DMSO-d_6): δ 4.04 (s, 2H, CH_2), 7.03 (s, 1H, =CH), 7.57 (d, 1H, J = 8.5 Hz, benzimidazole-C_7-H), 7.81 (d, 1H, J = 8.5 Hz, benzimidazole-C_6-H), 8.47 (s, 1H, benzimidazole-C_4-H), 12.94–13.17 (brs, 2H, NH and COOH, D_2O exchangeable), 13.96 (brs, 1H, NH, D_2O exchangeable); Anal. Calcd. for $C_{12}H_9N_3O_3S$ (275.27): C, 52.35, H, 3.29, N, 15.26. Found: C, 52.46, H, 3.33, N, 15.31.

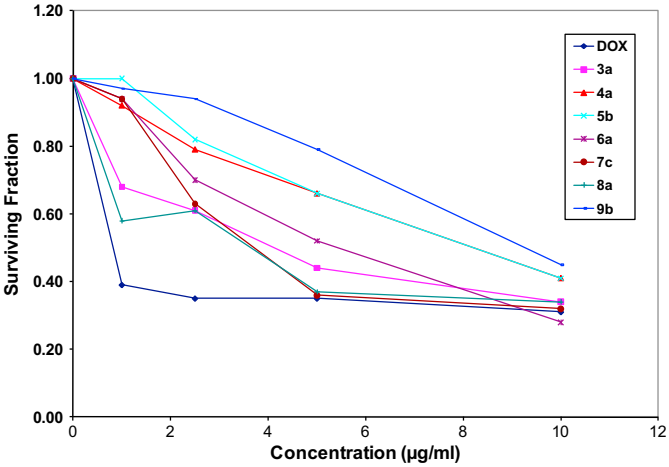


Fig. 4. Cytotoxicity of **3a**, **4a**, **5b**, **6a**, **7c**, **8a**, **9b** and doxorubicin against human colon carcinoma cell line (HCT 116).

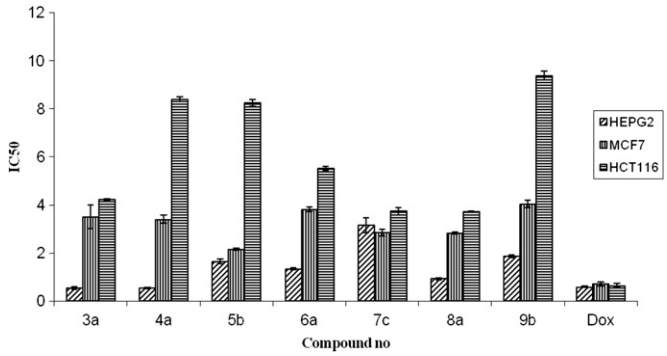


Fig. 5. IC50 values of compounds **3a**, **4a**, **5b**, **6a**, **7c**, **8a** and **9b** against HEPG2, MCF7 and HCT 116.

*5.1.3. General procedure for the preparation of compounds **3 a,b***

A mixture of 2-cyanomethylbenzimidazole-5-carboxylic acid (**1b**) (2.0 g, 10 mmol), finely divided sulfur (0.32 g, 10 mmol) and triethylamine (1.4 ml, 10 mmol) in absolute ethanol (15 ml) was stirred at room temperature for 30 min. The appropriate isothiocyanate (10 mmol) was gradually added and stirring was continued for 3 h during which a crystalline product separated out. The separated product was filtered, washed with ether, dried and crystallized from dimethyl formamide.

*5.1.3.1. 2-(4-Amino-3-benzyl-2-thioxo-2,3-dihydrothiazol-5-yl) benzimidazole-5-carboxylic acid (**3a**).* Yield: 51%; mp: >300 °C; IR (cm^{-1}): 3320–3063 (NH, NH_2), 2900 (CH aliph.), 1684 (C=O), 1280 (C=S); ^1H NMR (DMSO-d_6): δ 4.58 (s, 2H, CH_2–C_6H_5), 7.58 (d, 1H, J = 8.5 Hz, benzimidazole-C_7-H), 7.86 (d, 1H, J = 8.5 Hz, benzimidazole-C_6-H), 7.94 – 8.1 (m, 5H, Ar-H), 8.47 (s, 1H, benzimidazole-C_4-H), 12.94 (brs, 1H, NH, D_2O exchangeable), 13.95 (brs, 1H, COOH,, D_2O exchangeable); Anal. Calcd. for $C_{18}H_{14}N_4O_2S_2$ (382.44): C, 56.52, H, 3.68, N, 14.65. Found: C, 56.48, H, 3.70, N, 14.71.

*5.1.3.2. 2-[4-Amino-3-phenyl-2-thioxo-2,3-dihydrothiazol-5-yl)] benzimidazole-5-carboxylic acid (**3b**).* Yield: 48%; mp: >300 °C; IR (cm^{-1}): 3334–3066 (NH, NH2), 1692 (C=O), 1305 (C=S); ^1H NMR (DMSO-d_6): δ 7.09–7.19 (m, 3H, Ar-H), 7.47 (distorted d,1H, benzimidazole-C_7-H), 7.79 (m, 1H, benzimidazole-C_6-H), 7.97 (m, 2H, Ar-H), 8.03 (s, 1H, benzimidazole-C_4-H), 12.79–13.54 (brs, 2H, NH and COOH, D_2O exchangeable); MS: m/z 369 (M+, 0.42%); Anal. Calcd. for $C_{17}H_{12}N_4O_2S_2$ (368.42): C, 55.41, H, 3.28, N, 15.20. Found: C, 55.28, H, 3.30, N, 15.15.

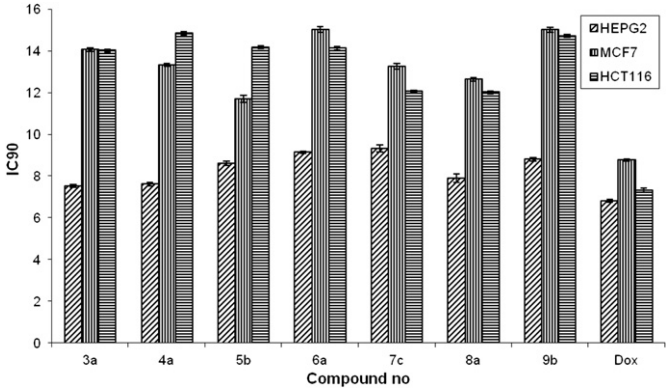


Fig. 6. IC90 values of compounds **3a**, **4a**, **5b**, **6a**, **7c**, **8a** and **9b** against HEPG2, MCF7 and HCT 116.

Table 1
IC50 values[a] of compounds **3a**, **4a**, **5b**, **6a**, **7c**, **8a** and **9b** against HEPG2, MCF7 and HCT 116.

Comp. no.	Cell line		
	HEPG2	MCF7	HCT 116
3a	0.55 ± 0.05	3.51 ± 0.50**	4.23 ± 0.04**
4a	0.55 ± 0.03	3.41 ± 0.17**	8.40 ± 0.10**
5b	1.65 ± 0.09**	2.15 ± 0.04**	8.25 ± 0.13**
6a	1.35 ± 0.05**	3.83 ± 0.09**	5.52 ± 0.09**
7c	3.16 ± 0.30**	2.85 ± 0.15**	3.75 ± 0.16**
8a	0.93 ± 0.04**	2.83 ± 0.03**	3.72 ± 0.03**
9b	1.87 ± 0.06**	4.04 ± 0.16**	9.39 ± 0.18**
Dox.	0.59 ± 0.03	0.72 ± 0.08	0.65 ± 0.09**
F-value	172.86	81.09	213.35
p-value	0.000*	0.000*	0.000*

Values were calculated from dose–response curves done in triplicate for each compound. Values were given ±standard deviation. HEPG2: human hepatocellular carcinoma, MCF7: human breast adenocarcinoma, HCT 116: human colon carcinoma cell lines, Dox.: Doxorubicin.
*There is a significant difference by using One Way ANOVA at $p < 0.05$.
**There is a significant difference from Dox by using Dunnett *t*-test at $p < 0.05$.
[a] IC50 (μg/ml): dose of the compound which caused a 50% reduction of survival.

*5.1.4. General procedure for the preparation of compounds **4 a**,**b***

A solution of the appropriate 2-cyanomethylbenzimidazole (**1a**,**b**) (10 mmol) and 4-fluorobenzaldehyde (1.8 g, 10 mmol) in dry dimethyl formamide (5 ml) was treated with 5 drops of 10% methanolic potassium hydroxide solution. The reaction mixture was heated under reflux for 2 h and then the crude product that precipitated was filtered off and crystallized from dimethyl formamide.

*5.1.4.1. 5-Chloro-2-[(4-fluorobenzylidene)cyanomethyl]benzimidazole (**4a**).* Yield: 91%; mp: >300 °C; IR (cm^{-1}): 2229 (C≡N), ^{1}H NMR (DMSO-d_6): δ 6.84–7.02 (m, 2H, benzimidazole-$C_{7,6}$-H), 7.25–7.48 (m, 4H, ArH), 7.84 (s, 1H, =CH), 7.98 (s, 1H, benzimidazole-C_4-H), 12.62 (brs, 1H, NH, D_2O exchangeable); MS: m/z 298 (M+, 7.26%); Anal. Calcd. for $C_{16}H_9ClFN_3$ (297.70): C, 64.54, H, 3.04, N, 14.11. Found: C, 64.61, H, 3.05, N, 14.15.

*5.1.4.2. 2-[(4-Fluorobenzylidene)cyanomethyl]benzimidazole-5-carboxylic acid (**4b**).* Yield: 95%; mp: 230–232 °C; IR (cm^{-1}): 2227 (C≡N), 1692 (C=O), ^{1}H NMR (DMSO-d_6): δ 6.79–7.27 (m, 2H, benzimidazole-$C_{7,6}$-H), 7.31 (d, 2H, J = 8.4 Hz, Ar-$C_{2,6}$-H), 7.44 (d, 2H, J = 8.4 Hz, Ar-$C_{3,5}$-H), 7.56 (s, 1H, =CH), 7.79 (s, 1H, benzimidazole-C_4-H), 12.79–13.67 (brs, 2H, NH and COOH, D_2O exchangeable); Anal. Calcd. for $C_{17}H_{10}FN_3O_2$ (307.27): C, 66.44, H, 3.28, N, 13.67. Found: C, 66.38, H, 3.25, N, 13.70.

*5.1.5. General procedures for the preparation of compounds (**5a**,**b**)*

To a solution of the appropriate 2-cyanomethylbenzimidazole (**1a**,**b**) (10 mmol) in absolute ethanol (10 ml), ammonium acetate (0.77 g, 10 mmol) and cyclopentanone or cyclohexanone (10 mmol) were added. The reaction mixture was heated under reflux for 6 h and left to cool to room temperature. The separated crystalline product was filtered off, dried and recrystallized from dimethyl formamide.

Table 2
Mean[a] and mean difference of IC50 values of compounds **3a**, **4a**, **5b**, **6a**, **7c**, **8a** and **9b** against HEPG2, MCF7 and HCT 116.

Comp. no.	Mean(I)	Mean difference (I – J)
3a	2.76 ± 0.56	2.11
4a	4.11 ± 1.14	3.46*
5b	4.01 ± 1.06	3.36*
6a	3.57 ± 0.60	2.91*
7c	3.25 ± 0.14	2.60
8a	2.49 ± 0.41	1.84
9b	5.09 ± 1.11	4.44*
Dox. (J)	0.65 ± 0.02	0

Values were given ±standard deviation. HEPG2: human hepatocellular carcinoma, MCF7: human breast adenocarcinoma, HCT 116: human colon carcinoma cell lines. Dox.: Doxorubicin. *There is a significant difference by using One Way ANOVA at $p < 0.05$.
[a] Mean: averaged IC50 values overall tested cancer cell lines.

Table 3
IC90 values of compounds[a] **3a**, **4a**, **5b**, **6a**, **7c**, **8a** and **9b** against HEPG2, MCF7 and HCT 116.

Comp. no	Cell line		
	HEPG2	MCF7	HCT 116
3a	7.53 ± 0.06	14.06 ± 0.09**	14.02 ± 0.07**
4a	7.62 ± 0.09	13.33 ± 0.08**	14.84 ± 0.08**
5b	8.61 ± 0.10**	11.70 ± 0.17	14.17 ± 0.06**
6a	9.15 ± 0.05**	15.02 ± 0.13**	14.13 ± 0.09**
7c	9.31 ± 0.17**	13.25 ± 0.13**	12.05 ± 0.06
8a	7.89 ± 0.21	12.63 ± 0.09	12.02 ± 0.07
9b	8.80 ± 0.08	15.01 ± 0.13**	14.72 ± 0.07**
Dox	6.82 ± 0.06	8.77 ± 0.06	7.32 ± 0.09
F-value	105.68	35.21	328.89
p-value	0.000*	0.000*	0.000*

Values were calculated from dose–response curves done in triplicate for each compound. Values were given ±standard deviation. HEPG2: human hepatocellular carcinoma, MCF7: human breast adenocarcinoma, HCT 116: human colon carcinoma cell lines, Dox.: Doxorubicin.
*There is a significant difference by using One Way ANOVA at $p < 0.05$.
**There is a significant difference from Dox by using Dunnett *t*-test at $p < 0.05$.
[a] IC90 (μg/ml): dose of the compound which caused a 90% reduction of survival.

*5.1.5.1. 5-Chloro-2-[(cyclopentylidene)cyanomethyl]benzimidazole (**5a**).* Yield: 48%; mp: 273–275 °C; IR (cm^{-1}): 3393 (NH), 2228 (C≡N); ^{1}H NMR (DMSO-d_6): δ 1.21–1.43 (m, 4H,cyclopentyl-$C_{3,4}$-H), 2.73, 2.89 (2m, each 2H, cyclopentyl-$C_{2,5}$-H), 7.56–7.74 (m, 2H, benzimidazole-$C_{6,7}$-H), 7.95 (s, 1H, benzimidazole-C_4-H), 12.89 (brs, 1H, NH, D_2O exchangeable); MS: m/z 258 (M+, 2.73%); Anal. Calcd. for $C_{14}H_{12}ClN_3$ (257.71): C, 65.24, H, 4.69, N, 16.30. Found: C, 65.41, H, 4.71, N, 16.36.

*5.1.5.2. 2-[(Cyclohexylidene)cyanomethyl]benzimidazole-5-carboxylic acid (**5b**).* Yield: 56%; mp: 202–204 °C; IR (cm^{-1}): 3273–3109 (NH and COOH), 2991, 2941 (CH aliph.), 2220 (C≡N), 1673 (C=O); ^{1}H NMR (DMSO-d_6): δ 1.66–1.84 (m, 6H, cyclohexyl-$C_{3,4,5}$-H), 2.73, 2.89 (2m, each 2H, $C_{2,6}$-H), 7.57 (d, 1H, J = 8.2 Hz, benzimidazole-C_7-H), 7.81 (d, 1H, J = 8.2 Hz, benzimidazole-C_6-H), 8.12 (s, 1H, benzimidazole-C_4-H), 12.76–13.97 (brs, 2H, NH and COOH); Anal. Calcd. for $C_{16}H_{15}N_3O_2$ (281.30): C, 68.31, H, 5.37, N, 14.93. Found: C, 68.06, H, 5.36, N, 14.97.

*5.1.6. General procedure for the preparation of compounds **6**–**9***

To a well-stirred and ice-cooled suspension of finely powdered potassium hydroxide (0.56 g, 10 mmol) and the appropriate 2-cyanomethylbenzimidazole (**1a**,**b**) (5 mmol) in dry dimethyl formamide (15 ml), the appropriate isothiocyanate (5 mmol) was added portion wise. After complete addition, stirring was continued at room temperature for 3 h. The reaction mixture was cooled to 0 °C, treated with the appropriate halogenated compound (5 mmol), stirred at room temperature for 24 h then poured onto ice/water. The resulting product was filtered off, dried and recrystallized from dimethyl formamide.

*5.1.6.1. 2-[(3-Cyclohexyl-5-oxothiazolidin-2-ylidene)cyanomethyl] benzimidazole-5-carboxylic acid (**6a**).* Yield: 63%; mp: 263–265 °C; IR (cm^{-1}): 2930, 2855 (CH aliph.), 2142 (C≡N), 1720, 1658 (2C=O); ^{1}H NMR (DMSO-d_6): δ 0.9–1.45 (m, 11H, cyclohexyl-H), 4.32 (s, 2H, thiazolidinone-C_4-H), 7.42 (d, 1H, J = 8.6 Hz, benzimidazole-C_7-H), 7.95 (distorted d, 1H, benzimidazole-C_6-H), 8.39 (s, 1H, benzimidazole-C_4-H), 12.70–13.34 (brs, 2H, NH and COOH, D_2O

exchangeable); Anal. Calcd. for $C_{19}H_{18}N_4O_3S$ (382.42): C, 59.66, H, 4.74, N, 14.65. Found: C, 59.79, H, 4.76, N, 14.70.

*5.1.6.2. 2-[(3-Phenyl-5-oxothiazolidin-2-ylidene)cyanomethyl] benzimidazole-5-carboxylic acid (**6b**).* Yield: 72%; mp: 296–298 °C; IR (cm^{-1}): 2900, 2870 (CH aliph.), 2230 (C≡N), 1720, 1660 (2C=O); ^1H NMR (DMSO-d_6): δ 4.44 (s, 2H, thiazolidinone-C_4-H), 7.44–7.64 (m, 5H, ArH), 7.84 (d, 1H, J = 8.4 Hz, benzimidazole-C_7-H), 7.93 (distorted d, 1H, benzimidazole-C_6-H), 8.30 (s, 1H, benzimidazole-C_4-H), MS: m/z 376 (M+, 0.7%); Anal. Calcd. for $C_{19}H_{12}N_4O_3S$ (376.38): C, 60.62, H, 3.21, N, 14.88. Found: C, 60.41, H, 3.09, N, 14.92.

*5.1.6.3. 5-Chloro-2-[(3-phenyl-4-(4-chlorophenyl)thiazol-2-ylidene) cyanomethyl]benzimidazole (**7a**).* Yield: 82%; mp: 198–200 °C; IR (cm^{-1}): 2225 (C≡N), ^1H NMR (DMSO-d_6): δ 6.35 (s, 1H, thiazole-C_5-H), 7.02–7.11 (m, 4H, ArH), 7.28–7.35 (m, 5H, ArH), 7.59–7.64 (m, 2H, benzimidazole-$C_{6,7}$-H), 8.01 (s, 1H, benzimidazole-C_4-H); Anal. Calcd. for $C_{24}H_{14}Cl_2N_4S$ (461.35): C, 62.47, H, 3.05, N, 12.14. Found: C, 62.29, H, 3.08, N, 12.17.

*5.1.6.4. 2-[(4-(4-Bromophenyl)-3-cyclohexylthiazol-2-ylidene) cyanomethyl]benzimidazole-5-carboxylic acid (**7b**).* Yield: 87%; mp: 233–235 °C; IR (cm^{-1}): 2220 (C≡N), 1695 (C=O); ^1H NMR (DMSO-d_6): δ 1.4–2.04 (m, 11H, cyclohexyl-H), 6.44 (s, 1H, thiazole-C_5-H), 7.60–8.18 (m, 6H, benzimidazole-$C_{7,6}$-H and 4 ArH), 8.32 (s, 1H, benzimidazole-C_4-H), 12.96–13.68 (brs, 2H, NH and COOH, D_2O exchangeable); Anal. Calcd. for $C_{25}H_{21}Br\,N_4O_2S$ (521.42): C, 57.58, H, 4.06, N, 10.74. Found: C, 57.74, H, 4.03, N, 10.71.

*5.1.6.5. 2-[3-(4-Bromophenyl)-4-(2-methoxyphenylthiazol-2-ylidene) cyanomethyl]benzimidazole-5-carboxylic acid (**7c**).* Yield: 91%; mp: 198–200 °C; IR (cm^{-1}): 3228 (C≡N), 1673 (C=O); ^1H NMR (DMSO-d_6): δ3.71 (s, 3H,OCH_3), 6.40 (s, 1H, thiazole-C_5-H), 6.84–6.89 (m, 2H, 2-methoxyphenyl-$C_{4,5}$-H), 7.09, 7.24 (2d, each 1H, J = 8.4 Hz, 2-methoxyphenyl-$C_{3,6}$-H), 7.45 (d, 2H, J = 8.7 Hz, 4-bromophenyl-$C_{2,6}$-H), 7.52 (d, 2H, J = 8.7 Hz, 4-bromopenyl-$C_{3,5}$-H), 7.61 (d, 1H, J = 8.4, benzimidazole-C_7-H), 7.79 (distorted d, 1H, benzimidazole-C_6-H), 7.95 (s, 1H, benzimidazole-C_4-H), 12.01–13.22 (brs, 2H, NH and COOH, D_2O exchangeable); MS: m/z 545 (M+, 7.5%); Anal. Calcd. for $C_{26}H_{17}Br\,N_4O_3S$ (545.40): C, 57.25, H, 3.14, N, 10.27. Found: C, 57.41, H, 3.15, N, 10.32.

*5.1.6.6. 5-Chloro-2-[(3-phenyl-4-oxothiazolidin-2-ylidene)cyanomethyl] benzimidazole (**8a**).* Yield: 88%; mp: 246–248 °C; IR (cm^{-1}): 2230 (C≡N), 1707 (C=O); ^1H NMR (DMSO-d_6): δ 4.19 (s, 2H,thiazolidinone-C_5-H), 6.91–7.12 (m, 2H, benzimidazole-$C_{7,6}$-H), 7.35–7.45 (m, 5H, ArH), 8.06 (s, 1H, benzimidazole-C_4-H), 10.96 (s, 1H, NH, D_2O exchangeable); Anal. Calcd. for $C_{18}H_{11}Cl\,N_4OS$ (366.81): C, 58.93, H, 3.02, N, 15.27. Found: C, 58.74, H, 3.03, N, 15.24.

*5.1.6.7. 2-[(3-Cyclohexyl-4-oxothiazolidin-2-ylidene)cyanomethyl] benzimidazole-5-carboxylic acid (**8b**).* Yield: 93%; mp < 300 °C; IR (cm^{-1}): 2925 (aliph. CH), 2223 (C≡N), 1717, 1670 (2C=O); ^1H NMR (DMSO-d_6): δ 1.11–1.40 (m, 6H, cyclohexyl-$C_{3,4,5}$-H), 1.80–1.90 (m, 4H, cyclohexyl-$C_{2,6}$-H), 4.18 (m, 1H, J = 7.2 Hz, cyclohexyl-C_1-H), 4.91 (d, 2H, J = 15.6 Hz, thiazolidinone-C_5-H), 7.62 (d, 1H, J = 6.9 Hz, benzimidazole-C_7-H), 7.83 (d, 1H, J = 6.9 Hz, benzimidazole-C_6-H), 8.18 (s, 1H, benzimidazole-C_4-H), 12.80 (brs, 1H, NH, D_2O exchangeable), 13.89 (brs, 1H, COOH, D_2O exchangeable); Anal. Calcd. for $C_{19}H_{18}N_4O_3S$ (382.42): C, 59.66, H, 4.74, N, 14.65. Found: C, 59.51, H, 4.76, N, 14.68.

*5.1.6.8. 5-Chloro-2-[(3-phenyl[1,3]thiazin-2-ylidene)cyanomethyl] benzimidazole (**9a**).* Yield: 78%; mp: 196–198 °C; IR (cm^{-1}): 2900 (aliph. CH), 2230 (C≡N); ^1H NMR (DMSO-d_6): δ 2.06 (brs, 2H, CH_2), 4.14 (t, 2H, J = 7.1 Hz, N-CH_2), 4.39 (s, 2H, S-CH_2), 6.2–6.9 (m, 5H, ArH), 7.20 (d,1H, J = 8.7 Hz, benzimidazole-C_7-H), 7.87 (d, 1H, J = 8.7 Hz, benzimidazole-C_6-H), 7.94 (s, 1H, benzimidazole-C_4-H), 12.80 (brs,1H, NH, D_2O exchangeable); Anal. Calcd. for $C_{19}H_{15}Cl\,N_4S$ (366.85): C, 62.20, H, 4.12, N, 15.27. Found: C, 62.48, H, 4.10, N, 15.31.

*5.1.6.9. 2-[(3-Phenyl[1,3]thiazin-2-ylidene)cyanomethyl]benzimidazole-5-carboxylic acid (**9b**).* Yield: 83%; mp: 210–212 °C; IR (cm^{-1}): 2919, 2853 (aliph. CH), 2228 (C≡N), 1711 (C=O); ^1H NMR (DMSO-d_6): δ 2.24 (brs, 2H, CH_2), 3.64 (t, 2H, J = 6.9 Hz, N-CH_2), 4.44 (brs, 2H, S-CH_2), 7.07–7.59 (m, 5H, ArH), 7.84–7.97 (m, 2H, benzimidazole-$C_{7,6}$-H), 8.29 (s, 1H, benzimidazole-C_4-H), 13.02 (brs, 2H, NH and COOH, D_2O exchangeable); MS: m/z 367 (M+, 0.41%); Anal. Calcd. for $C_{20}H_{16}N_4O_2S$ (376.42): C, 63.81, H, 4.28, N, 14.88. Found: C, 63.62, H, 4.31, N, 14.83.

5.1.7. Cytotoxic activity studies

Anticancer activity studies were done at Cairo University, National Cancer Institute, Cancer Biology Department, Pharmacology Unit.

Compounds **3a**, **4a**, **5b**, **6a**, **7c**, **8a** and **9b** were tested at concentrations between 1 and 10 μg/ml using SulfoRhodamine-B (SRB) assay for cytotoxic activity against the following tumor cell lines:

1. Liver carcinoma cell line (HEPG2)
2. Breast carcinoma cell line (MCF7)
3. Colon carcinoma cell line (HCT 116)

5.1.8. Measurement of potential cytotoxicity by SRB assay

Potential cytotoxicity of the compounds was tested using the method of Skehan et al. [42], as follows:

Cells were plated in 96 multiwell plate (104 cells/well) for 24 h before treatment with the compound(s) to allow attachment to the wall of the plate. Different concentrations of the compounds (0, 1, 2.5, 5 and 10 μg/ml) were added to the cell monolayer triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compound(s) for 48 h at 37 °C in atmosphere of 5% CO_2. After 48 h, cells were fixed, washed and stained with SulfoRhodamine-B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. Color intensity was measured in an ELISA reader.

The relation between surviving fraction and drug concentration is plotted to get the survival curve of each tumor cell line after the specified compound.

5.1.9. Statistical analysis

Differences between different treatment groups were analyzed using ANOVA followed by Dunnett t-test. P values of less than 0.05 were considered to represent a significant difference.

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