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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Design, synthesis, and biological evaluation of new 4-thiazolidinone derivatives substituted with benzimidazole ring as potential chemotherapeutic agents

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Abstract In search of novel antiviral and anticancer agents with promising pharmacotoxicological profile, a study was initiated to synthesize new 2-thioxo-4-thiazolidinones as well as 2-phenylimino-4-thiazolidinones substituted with benzimidazole ring system. The compounds were screened primarily for their antiviral as well as anticancer activities. The synthesis of some novel 5-substituted thiazolidinones was also described. None of the tested compounds showed inhibitory activity against Hepatitis C virus replication. Two 2-phenylimino-4-thiazolidinone derivatives (**9a** and **10**) exhibited significant antiproliferative activity against human colon carcinoma cell line HCT 116 and human hepatocellular carcinoma HEPG2 cell line, respectively. Results also indicated that six thiazolidinone derivatives (**5a**, **5d**, **5e**, **5f**, **5h**, and **9d**) showed moderate antiproliferative activity against human breast adenocarcinoma cell line MCF7 in comparison to the standard drug Doxorubicin. Moreover, a docked pose of the most potent three cytotoxic compounds **5a**, **5h**, and **9d** against MCF7 was obtained bound to Human *N*-acetyl transferase1 NAT1 binding pocket by molecular operating environment module.

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Introduction

The life threatening infections caused by viruses have increased vigorously, especially in immunocompromised patients. The high mortality associated with Hepatitis C infection and cancer urgently calls for new, refined therapies, and molecular targets to be directed for the aid of treating hepatitis infection and cancer. Chronic infection with Hepatitis C virus represents a major public health threat. The current treatment Interferon alpha (INF-α), apart from being costly and having complicated polypeptide structure with poor pharmacokinetic profile, shows successful relapse rate in <50 % of patients (De Francesco and Carfi, 2007).

Cancer has surpassed heart diseases as the leading cause of death in the U.S (Edward and Elizabeth, 2005). For most types of cancer, chemotherapeutic treatments have achieved limited success to date, which highlight the need for more efficacious and better-tolerated therapy. Improved understanding of cancer biology at the molecular level has led to a shift in treatment paradigm from the traditional cytotoxics toward more rationally designed targeted therapies.

Breast cancer is the most common cancer in the United Kingdom and worldwide; more than a million women are diagnosed with breast cancer every year, accounting for a tenth of all new cancers and 23 % of all female cancer cases (http://info.cancerresearchuk.org). Breast cancer is a heterogeneous disease and different breast cancer subtypes can be identified based on gene expression profiling. Current breast cancer therapies, based on Selective Estrogen

Receptor Modulators (SERMs), such as tamoxifen or aromatase inhibitors, can be unsuccessful due to intrinsic or acquired resistance to these therapies (Weinshilboum, 2008).

After profiling gene expression, identification of some unexpected overexpressed genes in breast cancer cells has emerged new targets for drug discovery. Human *N*-acetyl transferase 1 (NAT1) is one such protein which has emerged as a new diagnostic marker or drug target for breast cancer. Furthermore, specific activity of human NAT1 in a range of breast cancer cell lines (MCF7, T47D, MDA-MB-231, and ZR-75-1), which have been proven to be good models for studying molecular pathways in estrogen receptor (ER) breast tumors, were analyzed (Zhu *et al.*, 2006). Among ER-positive lines, the human breast adenocarcinoma MCF7 is explored, in which NAT1 activity ranged from 1.8 ± 0.4 nmol/min/mg (Wakefield *et al.*, 2006).

In the course of an initial high throughput screening of a small molecule compound library, the lead compound (**A**) was discovered as a selective Hepatitis C virus (HCV) NS5B inhibitor (IC_{50} = 0.2 μM) (Fig. 1).

Comprehensive SAR study was reported on this lead compound, we have combined the information previously reported (Powers *et al.*, 2006) into a cohesive picture of possible structural features required for the inhibition of NS5B, which could be the presence of lipophilic substituent on the 5-arylidene moiety, the presence of olefin moiety for the covalent binding to Cys^{366} of the target NS5B protein and free rotation for the group attached to the N-3 of 4-thiazolidinone ring (Fig. 2).

The incorporation of benzimidazole nucleus, a biologically accepted pharmacophore, in the 4-thiazolidinone molecule has made it a versatile heterocycle possessing wide spectrum of biological activity. Benzimidazole ring is one of the important fused heterocyclic system that constitutes a part of countless derivatives of biological relevance such as antiviral (Evers *et al.*, 2004; Middleton *et al.*, 2004; Hirashima *et al.*, 2006; Li *et al.*, 2006), anticancer (Kumar *et al.*, 2002; Hranjec *et al.*, 2010; Refaat, 2010), antimicrobial (Ayhan-Kilcigil *et al.*, 1999; Gulgun and Nurten, 2003), and antifungal activity (Pawar *et al.*, 2004).

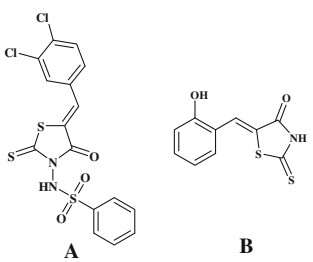


Fig. 1 Structures of the lead compounds **a** and **b**

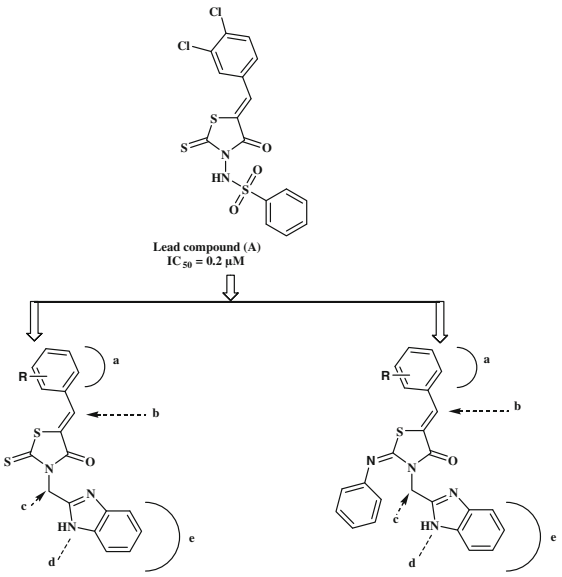


Fig. 2 Features similarities between the lead and the target compounds

The aim of the present work was to design and synthesize novel heterocycles structurally related to 4-thiazolidinones derivatives with potential anti-HCV activity. The optimization strategy of the lead compound (**A**) depends on the following approaches: Combination of two heterocyclic systems (4-thiazolidinone and benzimidazole) having diverse biological activities in one hybrid molecule which might provide distinguished efficacy and accessible synergistic activity. Modifications of the aryl functionality in position **5** of the 4-thiazolidinone moiety. The type of substituent on the phenyl ring appears to play an important role in activity. This behavior depends on the nature and position of substituent as well as the biological target. In this work, we try to study the influence of versatile electron donating, electron withdrawing, lipophilic, and hydrophilic substituent groups. The isosteric replacement of 2-thioxo group by 2-phenylimino in the 4-thiazolidinone core.

During the anti-HCV testing, some of the newly synthesized compounds showed cytotoxic activity on the tested cell line. This directed us to explore the cytotoxic activity of these compounds on three different cell lines. Most of the tested compounds exhibited antiproliferative activity against human breast adenocarcinoma cell line MCF7. In this work, a theoretical docking study was performed on the potent synthesized compounds to illustrate the preferred binding modes of the thiazolidine ring with the target protein NAT1. The docking study was performed by MOE software (MOE Chemical Computing Group Inc; Montreal). The rationale behind this concept was based on the finding of several specific inhibitors for NAT1 that were based on the structure of rhodanine. These were

identified through a screen of a library of 5000 compounds against five different recombinant NATs from prokaryote and eukaryote sources (Russell *et al.*, 2009). One of these, (*Z*)-5-(2′-hydroxybenzylidene)-2-thioxothiazolidin-4-one (**B**), demonstrated potent inhibition of human NAT1 ($IC_{50} = 1.1$ μM). Docking studies suggested that this compound interacted with the hydrophobic pocket of the active site of the enzyme. The crystal structure of this compound has suggested that it may interact with the adjacent molecules forming several weak bonds such as phi and hydrogen bonds (Barreiro *et al.*, 2007). Also, based on literature, it was found that 2-substituted benzimidazoles are active against the human breast adenocarcinoma cell line MCF7 (where NAT1 enzyme is expressed) (Hranjec *et al.*, 2010).

Results and discussion

Chemistry

The synthetic strategies to obtain the target compounds are depicted in Schemes 1 and 2. The key starting material 2-aminomethyl benzimidazole dihydrochloride salt (**2**) was prepared, according to Cescon and Day's (Cescon and Day, 1962) method. Treating **2** with bis (carboxymethyl) trithiocarbonate in the presence of sodium carbonate afforded the desired rhodanine **3**. This strategy is based on the method of Holmberg (1910) for preparing N-substituted rhodanines. It involves the direct cyclization of a wide variety of amines with bis (carboxymethyl) trithiocarbonate in the presence of sodium carbonate to afford the desired rhodanine heterocycle in one step (Strube, 1959; Bulic *et al.*, 2007). Cyclization proceeded successfully when an aqueous suspension of the previous mixture was heated in water on a boiling water bath for 5 h. The same product was obtained in the same yield when the reaction mixture was refluxed in isopropanol for 18 h. It was found that coupling of diazonium salts of the appropriate amine in acetic acid with the 3-[(1H-benzo[d]imidazol-2-yl) methyl]-2-thioxothiazolidin-4-one (**3**) in dioxane, at the nucleophilic C-5 active methylene yielded the corresponding 5-arylazo derivatives (**4a–c**).

Knoevenagel condensation of rhodanine **3** with aromatic aldehydes at the nucleophilic C-5 active methylene was accomplished using sodium acetate in refluxing acetic acid to afford **5a–j**. Based on literature precedence the isomeric ratio of products was presumed to be mainly *Z* in all cases (Sing *et al.*, 2001). It was reported that the thermodynamically stable *Z*-isomer predominated with a ratio of Z:E isomers 10:1 after recrystallization for all arylidene rhodanines. The ratio of the two geometrical stereoisomers was readily quantified by ^{1}H NMR as previously reported in the literature (Ohishi *et al.*, 1990). The methylene proton of the Z-isomer was more downfield (7.9 ppm) than that of the E-isomer (7.4 ppm) due to the interaction with the carbonyl group at the 4-position (Sing *et al.*, 2001).

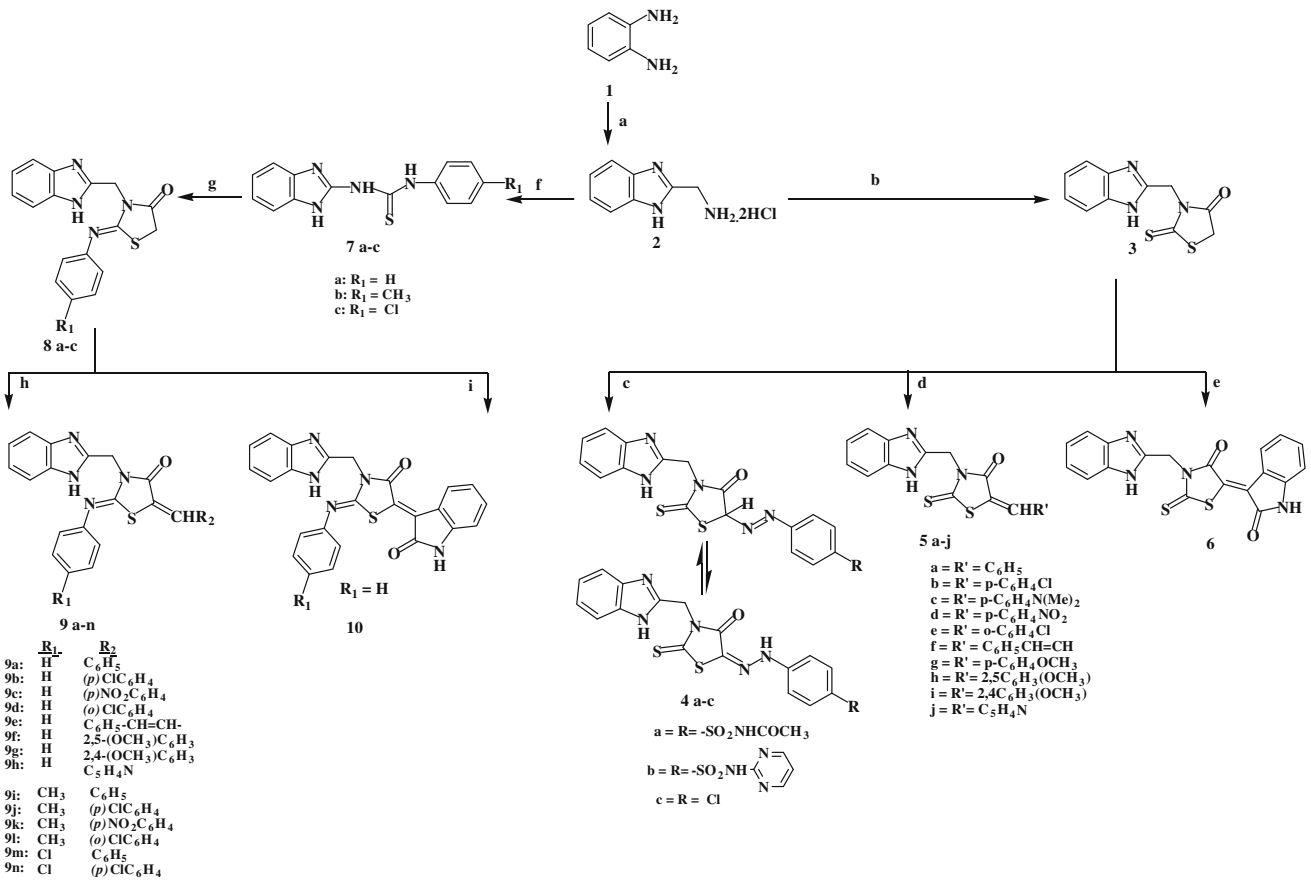
On the other hand, Knoevenagel condensation of rhodanine with aromatic ketones proceeded in the presence of ammonium chloride or ammonium acetate with heating (Sing *et al.*, 2001). Refluxing 3-[(1H-benzo[d]imidazol-2-yl) methyl]-2-thioxothiazolidin-4-one (**3**), isatin, ammonium chloride, and ammonium hydroxide in ethanol for 12 h yielded **6**, where isatin condenses only with one of its carbonyl groups at the nucleophilic C-5 active methylene of rhodanine nucleus giving the required product in a good yield.

Compounds **7a–c** were obtained in good yields through an addition of 2-amino methyl benzimidazole dihydrochloride salt (**2**) to the selected aryl isothiocyanate in ethanol at room temperature (Husain *et al.*, 1982; Rida *et al.*, 1986). Potassium carbonate was added to liberate the free base of **2** during reaction.

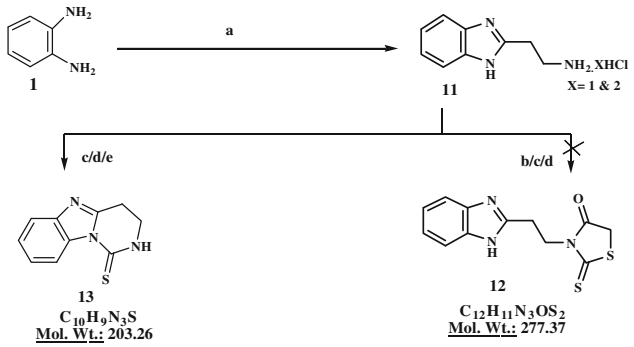
3-[(1H-Benzo[d]imidazol-2-yl) methyl]-(4-substituted phenylimino) thiazolidin-4-ones (**8a–c**) were prepared by refluxing 1-[(1H-benzo[d]imidazol-2-yl) methyl]-3-(4-substituted phenyl) thiourea (**7a–c**) with equimolar amount of ethyl bromoacetate in ethanol (Husain *et al.*, 1982; Rida *et al.*, 1986; Soliman, 1981). The reaction mixture was cooled and the product was separated without addition of sodium acetate. The ring closure of compounds **7a–c** with ethyl bromoacetate was confirmed as being oriented toward the formation of structures **8a–c** in form **I** rather than form **II** based on what previously mentioned in literature (St. Laurent *et al.*, 2004) (Fig. 3).

Several aldehydes have been condensed with pseudothiohydantoins (**8a–c**) by heating under reflux with sodium acetate in acetic acid at 80 °C for several hours to yield the corresponding 3-[(1H-benzo[d]imidazol-2-yl) methyl]-2-(4-substituted phenylimino)-5-arylidenethiazolidin-4-ones (**9a–n**). Knoevenagel condensation was accomplished by reaction of 3-[(1H-benzo[d]imidazol-2-yl) methyl]-2-(phenylimino) thiazolidin-4-one (**8a**) with isatin, ammonium chloride, and ammonium hydroxide in ethanol for several hours to afford **10**. This was achieved in accordance with the reported general procedure for aldol condensation of ketones with 4-thiazolidinones (Brown, 1961).

Scheme 2 starts with the preparation of 2-*β*-aminoethyl benzimidazole hydrochloride (**11**) as described by Cescon and Day (1962). Trials to cyclize 2-*β*-aminoethyl benzimidazole hydrochloride (**11**) to the corresponding rhodanine (**12**) by heating under reflux with bis (carboxymethyl) trithiocarbonate in water for 5 h, according to Holmberg (1910) method, were unsuccessful and no product could be separated (method **B**). In another trial for the synthesis of the target rhodanine (**12**), refluxing of the above mentioned



Scheme 1 (*a*) glycine (1.5 equiv), 5.5 N HCl, reflux 30 h; (*b*) bis (carboxymethyl) trithiocarbonate (1.0 equiv), Na_2CO_3, H_2O, reflux 5 h; (*c*) 1ry amine in acetic acid (1.0 equiv), $NaNO_2$ in water, dioxane, stir at 5 C; (*d*) R'CHO (1.2 equiv), NaOAc, acetic acid, reflux 3-4 h; (*e*, *i*) Isatin (1.2 equiv.), NH4Cl, NH4OH, ethanol, reflux 12 h; (*f*), K_2CO_3, ethanol, rt (overnight); (*g*) $BrCH_2COOEt$, ethanol, reflux 20–30 h; (*h*) R_2CHO (1.2 equiv), NaOAc, acetic acid, reflux 12–15 h



Scheme 2 (*a*) β-alanine (1.5 equiv), 5.5 N HCl, reflux 24 h; (*b*) $HOOCCH_2SC(=S)SCH_2COOH$ (1.0 equiv), Na_2CO_3, H_2O, reflux 5 h; (*c*) $HOOCCH_2SC(=S)SCH_2COOH$ (1.0 equiv), i-PrOH, reflux 18 h; (*d*) CS_2, $BrCH_2COOEt$, $(Et)_3N$, MeOH, rt; (*e*) CS_2, EtOH, rt

mixture in isopropanol for 18 h was proposed (Bulic *et al.*, 2007). The spectral data showed that such conditions did not impact cyclization as expected, and unexpected product was obtained (method **C**). Also, many attempts have been done to synthesize the rhodanine (**12**) by stirring a mixture of 2-β-aminoethyl benzimidazole hydrochloride (**11**), carbon disulfide, ethyl bromoacetate, and triethylamine in methanol at room temperature (Lee and Sim, 2000). Unfortunately, the infrared (IR) spectrum of the product lacked the strong absorption band of the C=O function, indicating that the reaction proceeded via another unexpected pathway (method **D**). It gave the same product as that obtained from the previously mentioned method. However, during our attempts to synthesize 3-[2-(1H-benzo[d]imidazol-2-yl) ethyl]-2-thioxothiazolidin-4-one (**12**) either by condensing 2-β-Aminoethyl benzimidazole hydrochloride (**11**) with bis (carboxymethyl) trithiocarbonate in the presence of sodium carbonate (method **C**), or with carbon disulfide and ethyl bromoacetate in the presence of triethylamine (method **D**), a nitrogenous product was isolated. Elemental analysis and the molecular ion peak showed this product to have the molecular formula $C_{10}H_9N_3S$. Its IR (cm^{-1}) spectrum revealed a broad band of medium intensity centered at 3412 (N–H), while the expected sharp band of (C=O) did not appear.

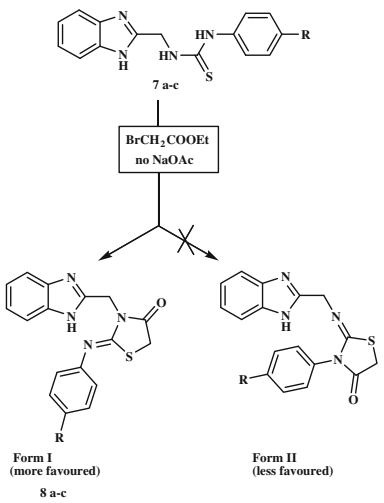


Fig. 3 Possible cyclization modes of 1-[(1H-benzo[d]imidazol-2-yl) methyl]-3-(4-substituted phenyl) thiourea (**7a–c**) with ethyl bromoacetate

The ^{1}H NMR (ppm) showed two triplets allocated for two methylene groups (2 × CH_2) two doublets for the benzimidazole $C_{4,7}$-Hs, two doublet of doublet for the benzimidazole $C_{5,6}$-Hs at the expected range of δ–ppm scale and a D_2O exchangeable singlet for the NH function at 10.32. The properties of this compound are therefore in accordance with the proposed structure (**13**) namely; 3, 4-dihydropyrimido [1, 6-a] benzimidazole-1(2H)–thione.

Unequivocally, the same compound was obtained via condensing 2-β-Aminoethyl benzimidazole hydrochloride (**11**) and carbon disulfide in ethanol the molecular formula of which is $C_{10}H_9N_3S$ (method **E**). In addition, the physical constant (mp) and IR spectral data of compound **13** conicided with those of that compound, which has been prepared according to a reported procedure (Singh *et al.*, 1986).

Biological studies

Antiviral activity

Qualitative in vitro anti-HCV screening

All the newly synthesized compounds were tested for their cytotoxicity prior to anti-HCV evaluation. Compounds **4a**, **4b**, **5c**, **5d**, **5g**, **5h**, **6**, **9b**, **9j**, and **10** proved to be safe to human blood (didn't show hemolysis at reasonable concentrations). Thus, they were selected for the anti-HCV assay. Others were found not suitable for further processing.

Compounds **4a**, **4b**, **5c**, **5d**, **5g**, **5h**, **6**, **9b**, **9j**, and **10** were investigated for their in vitro action as anti-HCV using the hepatocellular carcinoma HEPG2 cell line infected with the hepatitis-C virus. Monitoring of the HCV viremia pre-and post-antiviral therapy through the detection of viral (+)- and/or (−)-RNA strands by the use of qualitative RT-PCR has become the most frequently used, reliable, and sensitive technique. Recently, it has been reported that the detection of the (−)-strand HCV-RNA by the RT-PCR is a very important tool for understanding the life cycle of the HCV. It provides a reliable marker for the diagnosis of HCV and monitors the viral response to antiviral therapy (El-Awady *et al.*, 1999). Based on these facts, the adopted method in the present study contributes to the simultaneous detection of the (+)- and/or (–)-HCV-RNA strands in HEPG2 hepatoma cells infected with HCV. Inhibition of viral replication was detected by amplification of viral RNA segments by the RT-PCR technique both in the cultivated infected cells alone (as a positive control) and at the specified dose for each test compound as shown in Table 1 at optimal temperature. The test compound is considered to be active when it is capable of inhibiting the viral replication inside the HCV-infected HEPG2 cells, as evidenced by the disappearance of the (+)- and/or (–)-strands viral RNA-amplified products detected by the RT-PCR (compared to positive control).

Unfortunately, the ten tested compounds, **4a**, **4b**, **5c**, **5d**, **5g**, **5h**, **6**, **9b**, **9j**, and **10**, did not show any inhibition of HCV replication at the specified doses as shown in Fig. 4, as all of them showed the appearance of both (+)- and (−)-strands of HCV RNA at the doses used.

Anticancer activity

Single dose assay

According to the National Cancer Institute- Cairo, structures are generally selected for screening based on their ability to add diversity to the NCI small compound

Table 1 Results of the neutral red uptake assay of the thiazolidinone derivatives

Compound ID	IC_{50} (μM)	Used dose (μM)
4a	15.26	0.30
4b	30.55	0.611
5c	14.76	0.295
5d	39.52	0.078
5g	54.28	0.108
5h	38.95	0.077
6	55.75	1.116
9b	32.38	0.647
9j	19.71	0.394
10	15.61	0.312

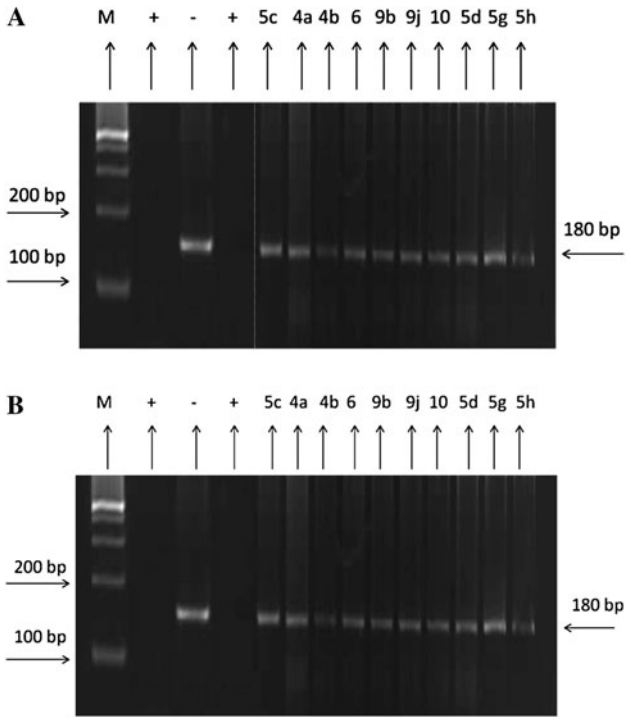


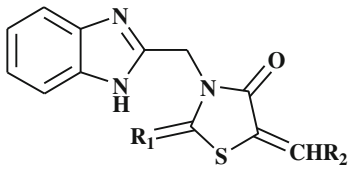
Fig. 4 RT-PCR amplification products on gel electrophoresis: **a** represents the PCR product of HCV RNA (+) strands isolated from HEPG2 cells for the different samples. **b** represents the PCR product of HCV RNA (+) strands isolated from peripheral blood mononuclear cells (PBMC) of infected patients for the different samples. In both cases identical results were obtained for the (−) strand RNA

collection. The NCI standards aim to study the influence of versatile electron donating, electron withdrawing, lipophilic, and hydrophilic substituent groups on the biological activity.

The cytotoxicity of 12 representative compounds **5e**, **5f**, **5g**, **5h**, **5i**, **5j**, **9a**, **9c**, **9d**, **9g**, **9h,** and **10** was evaluated against three cell lines representing three common forms of human cancer, i.e., human breast adenocarcinoma cell line MCF7, colon carcinoma cell line HCT 116, and human hepatocellular carcinoma cell line HEPG2 by the method of Skehan (Skehan *et al.*, 1990) at 100-μM concentration.

Regarding the structures of the tested compounds we can notice that rhodanine derivatives (**5e**, **5f**, **5g**, **5h**, and **5j**) showed higher activity compared to pseudothiohydantoin congeners (**9a**, **9c**, **9d**, **9g**, **9h**, and **10**) (Table 2). All the tested compounds were found to possess potential antitumor activities against all the tested tumor cell lines with growth percentages less than 25 % at 100 μM concentration. In general, all the compounds tended to be more active against MCF7, than against other tumor cell lines. Also, study of the results presented in Table 2 indicated that compounds **5e**, **5f**, **5g**, **5h**, **5j**, and **9d** showed the least growth percentages against MCF7, while compounds **9a** and **10** were the most active against HCT 116 and HEPG2 cell lines, respectively. Thus, they are likely to be interesting candidates for advanced five dose assay on these cell lines.

Table 2 Growth percentages of 100 μM of thiazolidinones derivatives against three cell lines in primary anticancer study



Compound	R_1	R_2	Growth percentage			Most sensitive cell line
			Breast MCF7	Colon HCT 116	Liver HEPG2	
5e	S	*(o)*ClC_6H_4	−4.32	27.6	9.07	Breast
5f	S	C_6H_5–CH=CH–	−3.93	21.22	14.18	Breast
5g	S	*(p)*$OCH_3C_6H_4$	−5.75	16.19	11.83	Breast
5h	S	2,5-$(OCH_3)C_6H_3$	−2.27	20.8	18.56	Breast
5i	S	2,4-$(OCH_3)C_6H_3$	0.34	19.2	17.8	Breast
5j	S	C_5H_4N	−3.83	16.7	22.8	Breast
9a	N–Ar	C_6H_5	−0.69	7	24.4	Breast
9c	N–Ar	*(p)*$NO_2C_6H_4$	−0.79	17.98	24	Breast
9d	N–Ar	*(o)*ClC_6H_4	−1.74	13.8	18.9	Breast
9 g	N–Ar	2,4-$(OCH_3)C_6H_3$	−1.19	15.52	18.39	Breast
9 h	N–Ar	C_5H_4N	−1.39	22.86	19.73	Breast
10	N–Ar	Isatin nucleus	7.07	16.2	6.8	Liver
Positive control	–	–	379.92	336.08	341.27	–

Five dose assay

Potential cytotoxicity of compounds **5e**, **5f**, **5g**, **5h**, **5j**, and **9d** as well as compounds **5a**, **5d**, and **6** against MCF7-breast cancer cell line, compound **9a** against HCT 116-colon cancer cell line and compound **10** against HEPG2-liver cancer cell line, was tested by the method of Skehan *et al.*, (1990). The relation between surviving fraction and drug concentration was plotted to get the survival curve for each compound (Fig. 5). Also, the IC_{50} (dose of the compound which caused a 50 % reduction of survival values) for each compound was determined and shown in Table 3 against the standard antitumor Doxorubicin (Dox.). The results are also represented graphically in Figs. 6 and 7.

Molecular modeling

As a reference to our modeling and docking study, the complex of human NAT1 enzyme coupled with (*Z*)-5-(2′-hydroxybenzylidene)-2-thioxothiazolidin-4-one (**B**) as inhibitor was used. The aryl substituent of which was predicted to extend into a hydrophobic pocket, with contact residues around the groove being Val^{93}, Phe^{125}, Val^{216}, and Phe^{287}. This provides a possible explanation for the preference for lipophilic substituents. Stabilization also appeared to be provided by two hydrogen bonding interactions between Arg^{127} and both the carbonyl and 2′-hydroxy substituent of rhodanine and the hydroxyl group of the Thr^{289} (Russell *et al.*, 2009).

The lowest energy conformations for the most potent inhibitors **5a**, **5h,** and **9d** showed relatively similar binding modes using the deposited crystal structure of human NAT1 (2PQT). They performed proper hydrophobic interactions with amino acids of the hydrophobic pocket at the binding site, namely, Phe^{125}, Val^{216}, and Phe^{287}, which confirms the preference of the lipophilic substituents on the aryl moiety as it was predicted by our reference compound (**B**).

Molecular modeling studies of our target compound **5a** indicated that imidazole-N_3 and the carbonyl oxygen contributed three hydrogen bonding interactions with NH of Arg^{127}, further stabilization occurs through an additional hydrogen bonding interaction between the imidazole-NH and the hydroxyl group of Phe^{287} residue of the target protein (Fig. 8).

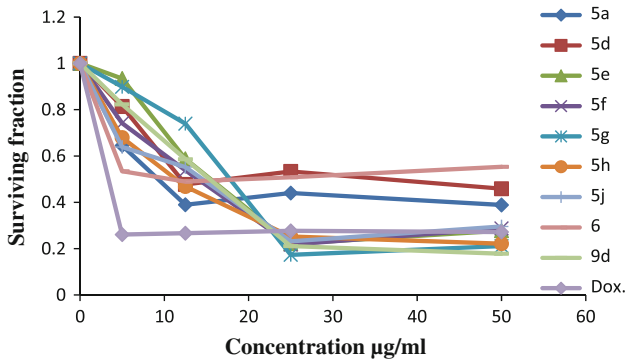


Fig. 5 Surviving fraction and dose effect of **5a**, **5d**, **5e**, **5f**, **5g**, **5h**, **5j**, **6**, **9d**, and Doxorubicin against human breast adenocarcinoma cell line MCF7

Table 3 IC_{50} values of thiazolidinones **5a**, **5d**, **5e**, **5f**, **5g**, **5h**, **5j**, **6**, **9a**, **9d**, **10,** and doxorubicin as determined by SRB assay

ID	IC_{50} (μM)*	Cell line
5a	35.05 ± 0.005	MCF7
5d	39.35 ± 0.010	MCF7
5e	39.64 ± 0.009	MCF7
5f	37.35 ± 0.010	MCF7
5g	46.66 ± 0.018	MCF7
5h	27.7 ± 0.010	MCF7
5j	40.85 ± 0.010	MCF7
6	35.41 ± 0.25	MCF7
9a	7.3 ± 0.013	HCT 116
9d	34.83 ± 0.08	MCF7
10	5.91 ± 0.011	HEPG2
Dox.	1.28 ± 0.08	MCF7
	1.19 ± 0.09	HCT 116
	1.08 ± 0.03	HEPG2

* Values were calculated from dose response curves done in triplicate for each compound. Values were given ± standard deviation, *MCF7* human breast adenocarcinoma, *HCT 116* human colon carcinoma, *HEPG2* human hepatocellular carcinoma, *Dox*. Doxorubicin

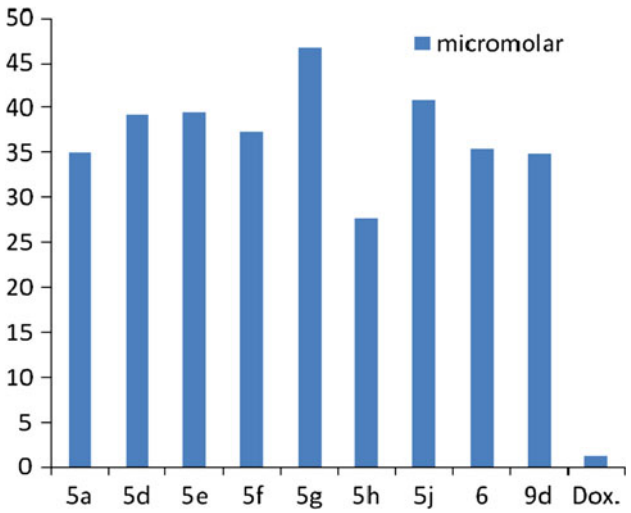


Fig. 6 IC_{50} values of compounds **5a**, **5d**, **5e**, **5f**, **5g**, **5h**, **5j**, **6**, **9d**, and Doxorubicin against MCF7 cell line

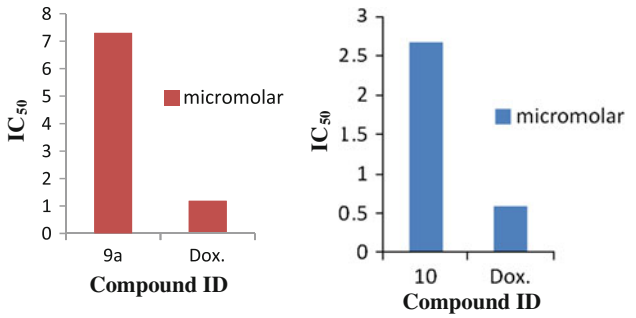


Fig. 7 IC_{50} values of compounds **9a**, **10,** and Doxorubicin against HCT 116 and HEPG2 cell lines, respectively

In compound **5h**, carrying two methoxy groups at the 2nd and 5th positions affects the conformational configuration leading to extending the ligand away from the conserved residues at the binding site. As a result, **5h** changed its overall conformation to avoid clashes with the protein binding backbone. However, it showed hydrogen bond interaction at the 5-OCH_3 with Tyr^{208} residue and better lipophilic recognition within the binding pocket due to the hydrophobic aromatic interactions with the surrounding residues Arg^{127}, Tyr^{129}, Lys^{188}, and Ile^{189} (Fig. 9).

The pseudothiohydantoin derivative **9d** showed restricted flexibility due to the *ortho*-chloro group in addition to the bulkiness of the phenylimino group at the C-2 of the thiazolidinone ring, which keeps the carbonyl group unexposed for any hydrogen bond interactions. However, the presence of the lipophilic *ortho*-chloro group encouraged more hydrophobic interactions with the surrounding residues Val^{93}, Ser^{102}, and Ile^{106}. This in addition to the

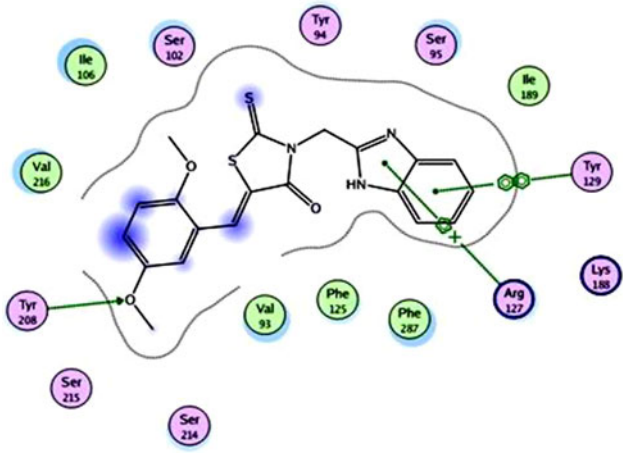


Fig. 9 Binding mode for compound **5h** docked and minimized in the NAT1 binding pocket by MOE software

interactions with Arg^{127}, Tyr^{129}, Lys^{188}, and Ile^{189}, similarly as in case of compound **5h** (Fig. 10).

Although both compounds **5a** and **5j** are 2-thioxo-4-thiazolidinone derivatives, yet they displayed contradicting activity. This could be explained by studying the molecular electrostatic potential (MEP) of both compounds to explain this difference in activity. The molecular electrostatic potential surface of compound **5a** (relatively NAT1 inhibitor) showed three intense red (negative) regions in comparison to compound (**B**), while compound **5j** (relatively inactive toward NAT1) showed 4 intense red regions. This extra region may lead to non-favorable interactions with the receptor (Fig. 11).

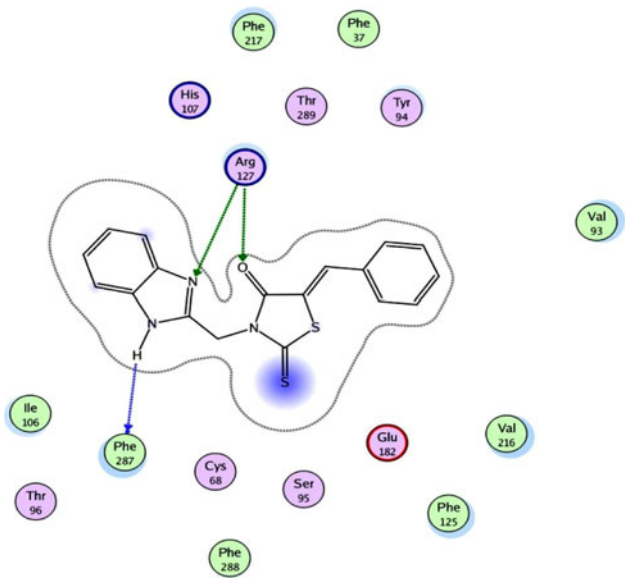


Fig. 8 Binding mode for compound **5a** docked and minimized in the NAT1 binding pocket by MOE software

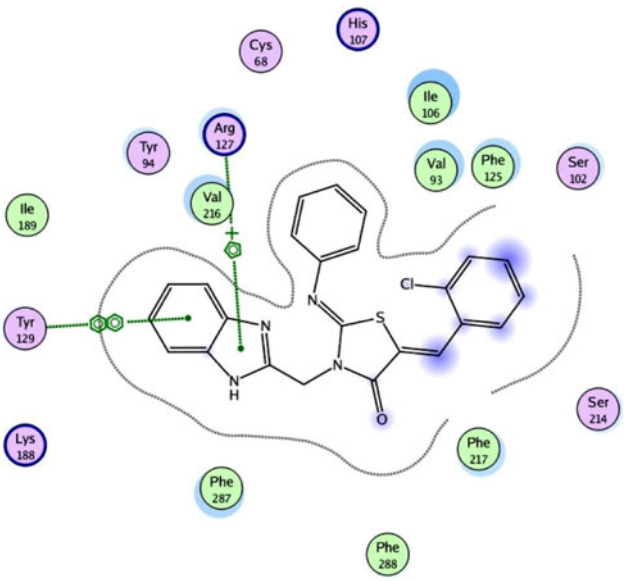


Fig. 10 Binding mode for compound **9d** docked and minimized in the NAT1 binding pocket by MOE software

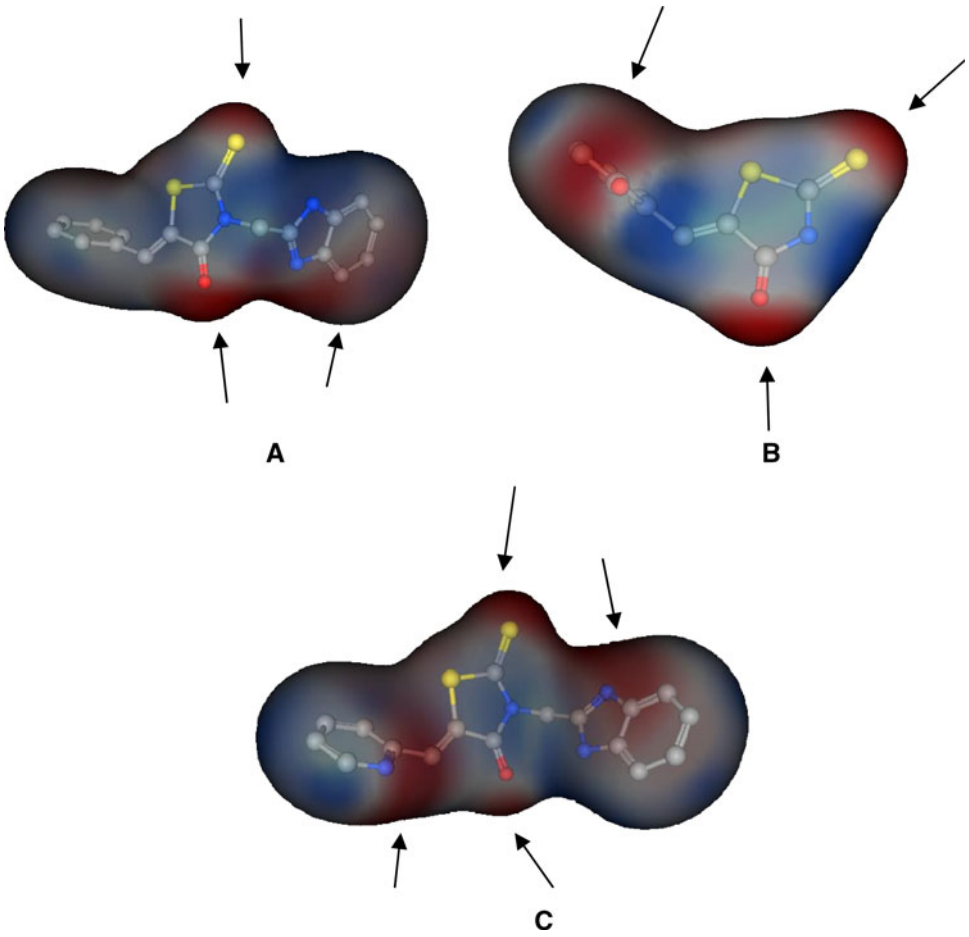


Fig. 11 Molecular electrostatic potential on a Gaussian surface of compounds **a 5a**, **b B**, **c 5j**. *Red* is negative and *blue* is positive

Conclusion

In the present study, a series of new 2-thioxo-4-thiazolidinones as well as 2-phenylimino-4-thiazolidinones substituted with benzimidazole ring was synthesized and screened for their antiviral and anticancer activities. The ten tested compounds did not show any inhibition of HCV replication at the specified doses when investigated for their in vitro action as anti-HCV using the hepatocellular carcinoma HEPG2 cell line infected with the hepatitis-C virus. On the other hand, thiazolidinone derivatives (**5a**, **5d**, **5e**, **5f**, **5h,** and **9d**) showed moderate antiproliferative activity ($IC_{50} = 40$ μM or less) against human breast adenocarcinoma cell line MCF7 in comparison to the standard Doxorubicin ($IC_{50} = 1.28$ μM). Regarding the structures of the tested compounds, we can notice that rhodanine derivative (**5h)** with electron donating groups (methoxy group at 2nd and 5th positions) showed higher activity ($IC_{50} = 27.7$ μM) compared to the unsubstituted congener **5a**. Activity was reduced upon substitution with electron withdrawing groups, e.g., compounds **5d** and **5e**, substituted with nitro and chloro groups, respectively. A decrease in activity is noticed as the position of methoxy group changes as going from the 2nd and 5th to the 4th position, e.g., compound **5 g** has an $IC_{50} = 46.66$ μM; while, compounds **9a** and **10** exhibited significant antiproliferative activity against colon carcinoma cell line HCT 116 and human hepatocellular carcinoma cell line HEPG2, respectively.

The overall outcome of the molecular docking study revealed that the three active compounds **5a**, **5h,** and **9d** showed relatively similar binding modes in compare to the lead compound (**B**). Benzimidazole ring also contributed in H-bonding interactions, for example **5a**. Also, Compound **5h** showed hydrogen bonding through its 5-OCH_3 group with Tyr^{208}, which may provide an explanation for the relatively high potency of **5h** over compound **9d**.

Experimental

Chemistry

Melting points were determined in open-glass capillaries on Griffin melting point apparatus and were uncorrected. The (IR) spectra were recorded on Perkin-Elmer 1430 IR spectrophotometer using the KBr palate technique. ^{1}H and ^{13}C-NMR spectra were run on a Varian Gemini 200,

300 MHz and Joel 500 MHz spectrophotometers using tetramethylsilane (TMS) as the internal standard and DMSO-d_6 as the solvent (Chemical shifts in δ, ppm). Splitting patterns were designated as follows: s: singlet; d: doublet; m: multiplet. The mass spectra were scanned on a Finnigan mass spectrometer model SSQ/7000 (70 e.V) and on a gas chromatograph/mass spectrometer Shimadzu GCMS/Qp2010 Plus operating at 70 eV. Microanalyses were performed at the Microanalytical Unit, Faculty of Science, Cairo University, Egypt and the found values were within ±0.4 % of the theoretical values.

3-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-2-thioxothiazolidin-4-one (**3**)

An equimolar mixture of 2-aminomethyl benzimidazole dihydrochloride (**2**) (1.09 g, 5 mmol) and sodium carbonate (0.53 g, 5 mmol) in 10 ml of water or isopropanol was heated on a water bath with stirring for 15 min. To the clear solution, a hot solution of bis (carboxymethyl) trithiocarbonate (1.13 g, 5 mmol) in 15 ml water or isopropanol is added at once. The mixture was heated on a water bath with stirring for 15 min; within 10 min a yellow precipitate was formed. Heating and stirring were continued for 5 h for aqueous solution or 18 h for isopropanol solution. The precipitate was removed by suction filtration. The solid was transferred to a flask containing 10 ml water, was heated on a water bath to 70–75 °C, while the lumps were crushed by a glass rod to obtain a homogenous mixture. The mixture was then filtered with suction when hot and the product was washed by rinsing it with small amount of hot water, dried, and crystallized from ethanol, yield: 58.1 %, mp: 220–222 °C. IR ν (cm^{-1}): 3452 (N–H), 1738 (C=O), 1614 (C=N), 1440, 1332, 1271, 1194 (NCS amide I, II, III, IV bands respectively). ^{1}H NMR (500 MHz), δ: 4.37 (s, 2H, CH_2–C=O), 5.23 (s, 2H, CH_2–N), 7.09–7.13 (m, 2H, benzimidazole-$C_{5,6}$-Hs), 7.4–7.42 (d, 1H, benzimidazole-C_4-H, $J = 10$ Hz), 7.48–7.5 (d, 1H, benzimidazole-C_7-H, $J = 10$ Hz), 12.4 (s, 1H, NH, D_2O exchange). Anal. calcd for $C_{11}H_9N_3OS_2$ (263.33): C, 50.17; H, 3.44; N, 15.96. Found C, 49.61; H, 4.04; N, 15.37.

General procedure for the synthesis of compounds **4a–c**

To an ice-cooled solution of the appropriate aromatic amine (3 mmol) in glacial acetic acid (6 ml) a solution of sodium nitrite (0.21 g, 3 mmol) in water (2 ml) was added. The mixture was stirred for 10 min at 0–5 °C. The resulted clear diazonium salt, over a period of 10 min to a stirred, ice cooled solution of 3-[(1H-benzo[d]imidazol-2-yl) methyl]-2-thioxothiazolidin-4-one (**3**) (0.78 g, 3 mmol) in dioxane (13 ml) was added. Stirring was continued for 4 h and the reaction was monitored by TLC technique. Upon dilution, a heavy precipitated product was obtained, filtered, washed with water, and purified by crystallization from proper solvents to give **4a–c**; yields (80–90 %).

3-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-5-(sulfacetamido phenylazo)-2 thioxothiazolidin-4-one (**4a**)

This compound was obtained according to the general procedure and crystallized from acetic acid as a yellow crystal, mp: 284–286°C. IR ν (cm^{-1}) 3311 (N–H, benzimidazole), 3219 (N–H, sulfonamide), 1721 (C=O, rhodanine), 1693 (C=O, acetyl group), 1620 (C=N), and 1217 (C=S). ^{1}H NMR (300 MHz), δ: 1.92 (s, 3H, CO–CH_3), 5.45 (s, 2H, CH_2–N), 7.14–7.18 (m, 2H, benzimidazole-$C_{5,6}$-Hs), 7.42–7.51 (m, 4H, sulfonamide Ar–Hs), 7.84–7.92 (m, 2H, benzimidazole-$C_{4,7}$-Hs), 11.32 and 11.94 (s, 1H, diazo-hydrazo-NH, D_2O exchange), 11.59 (s, 1H, sulfacetamide-NH, D_2O exchange), 12.52 (s, 1H, benzimidazole-NH, D_2O exchange). Anal. calcd for $C_{19}H_{16}N_6O_4S_3.½H_2O$ (497.56): N, 16.88; S, 19.29. Found N, 16.16; S, 18.41.

3-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-5-[(2-sulfadiazinyl) phenylazo]-2-thioxothiazolidin-4-one (**4b**)

This compound was obtained according to the general procedure and crystallized from Ethanol/H_2O as a brownish yellow crystal, mp: 177–178°C. IR ν (cm^{-1}) 3038 (N–H), 1697 (C=O), 1580 (C=N), and 1160 (C=S). ^{1}H NMR (300 MHz), δ: 5.45 (s, 2H, CH_2–N), 7.06–7.09 (m, 2H, benzimidazole-$C_{5,6}$-Hs), 7.60–7.64 (m, 4H, sulfonamide Ar–Hs), 7.87–8.18 (m, 2H, benzimidazole-$C_{4,7}$-Hs), 8.46–8.52 (d, 1H, pyrimidine-C_5-H), 8.53 and 8.55 (2s, 2H, pyrimidine-$C_{4,6}$-Hs), 11.52 and 11.98 (s, 1H, diazo-hydrazo-NH, D_2O exchange), 12 (s, 1H, sulfacetamide-NH, D_2O exchange), 12.52 (s, 1H, benzimidazole-NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 423 (6.3) ($M^{+\cdot}$+1), 524 (4.0) (M^+), 331 (5.5), 297 (5.3), 255 (13.5), 192 (17.7), 170 (30.4), 162 (15.9), 149 (8.4), 144 (7.0), 128 (13.4), 118 (18.1), 98 (8.6), 96 (38.8), 77 (18.1), 64 (100). Anal. calcd for $C_{21}H_{16}ClN_8O_3S_3$ (524.6): C, 48.08; H, 3.07. Found C, 48.03; H, 3.74.

3-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-5-[4-(chlorophenylazo)]-2-thioxothiazolidin-4-one (**4c**)

This compound was obtained according to the general procedure and crystallized from dioxane/petroleum ether as an orange crystal, mp: 168–171°C. IR ν (cm^{-1}) 3219 (NH), 1699 (C=O), 1603 (C=N), and 1126 (C=S). ^{1}H NMR

(300 MHz), δ: 5.45 (s, 2H, CH_2–N), 7.13–7.19 (m, 2H, benzimidazole-$C_{5,6}$–Hs), 7.27–7.44 (m, 4H, sulfonamide Ar–Hs), 7.47–7.53 (m, 2H, benzimidazole-$C_{4,7}$-Hs), 11.07 and 11.40 (s, 1H, diazo-hydrazo-NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 402 (2.1) ($M^{+\cdot}+1$), 401 (4.0) ($M^{+\cdot}$), 199 (7.1), 189 (30.3), 173 (9.2), 163 (6.7), 146 (7.1), 131 (100.0), 126 (32.6), 99 (14.0), 90 (9.3), 77 (11.8), 63 (5.5). Anal. calcd for $C_{17}H_{12}ClN_5OS_2$ (401.89): C, 50.81; H, 3.01; N, 17.43. Found C, 50.08; H, 2.57; N, 16.59.

General procedure for the synthesis of compounds ***5a–j***

To a mixture of 3-[(1H-benzo[d]imidazol-2-yl) methyl]-2-thioxothiazolidin-4-one (**3**) (1 mmol) and the appropriate aldehyde (1.2 mmol) in glacial acetic acid (10 ml), a slight excess of anhydrous sodium acetate (1.5 mmol) was added. The reaction mixture was refluxed for 3–4 h; the reactions were monitored by thin-layer chromatography technique. After completion of the reaction, the reaction mixture was cooled and diluted with water (10 ml). The products precipitated **5a–5j** were filtered, washed with water; dried, and crystallized from ethanol.

(*Z*)-3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-benzylidene-2-thioxothiazolidin-4-one (**5a**)

This compound was obtained according to the general procedure as a golden yellow crystal, yield: 71 %, mp: 228–230 °C. IR ν (cm^{-1}): 3440 (NH), 1703 (C=O), 1559 (C=N). ^{1}H NMR (300 MHz), δ: 5.45 (s, 2H, CH_2–N), 7.12–7.17 (m, 2H, benzimidazole-$C_{5,6}$-Hs), 7.44–7.46 (d, 1H, phenyl-C_4-H, $J = 6$ Hz), 7.52–7.62 (m, 4H, phenyl-$C_{2,3,5,6}$-Hs), 7.69–7.7 (d, 1H, bezimidazole-C_4-H, $J = 3$ Hz), 7.72 (d, 1H, benzimidazole-C_7-H, $J = 1.8$ Hz), 7.92 (s, 1H, C=CH vinylic H), 12.49 (s, 1H, NH, D_2O exchange). ^{13}C-NMR, δ: 51.53 (CH_2–N), 131.21–142.9 (Ar–Cs of benzimidazole and phenyl rings), 157.51 (C=CH), 176.16 (C=O) and 202.92 (C=S). MS, *m/z* (rel. Abund. %): 353 (2.0) ($M^{+\cdot}+2$), 352 (4.3) ($M^{+\cdot}+1$), 351 (15.7) ($M^{+\cdot}$), 189 (45), 134 (100), 131 (52.8), 118 (10.4), 90 (14), 77 (13). Anal. calcd for $C_{18}H_{13}N_3OS_2$ (351.44): C, 61.52; H, 3.73; N, 11.96. Found C, 60.69; H, 3.71; N, 11.75.

(*Z*)-3-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-5-(4-chlorobenzylidene)-2-thioxothiazolidin-4-one (**5b**)

This compound was obtained according to the general procedure as a yellow crystal, yield: 78 %, mp: 255–256 °C. IR ν (cm^{-1}): 3433 (NH), 1724 (C=O), 1604 (C=N). ^{1}H NMR (300 MHz), δ: 5.45 (s, 2H, CH_2–N), 7.13–7.16 (m, 2H, benzimidazole-$C_{5,6}$-Hs), 7.49 (m, 2H, benzimidazole-$C_{4,7}$-Hs), 7.64–7.67 (d, 2H, phenyl-$C_{2,6}$-Hs, $J = 9$), 7.72–7.75 (d, 2H, phenyl-$C_{3,5}$-Hs, $J = 9$), 7.92 (s, 1H, C=CH vinylic H), 12.5 (s, 1H, NH, D_2O exchange). Anal. calcd for $C_{18}H_{12}ClN_3OS_2$ (385.89): C, 56.02; H, 3.13; N, 10.89. Found C, 55.19; H, 3.33; N, 1057.

(*Z*) 3-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-5-(4-dimethyl aminobenzylidene)-2-thioxothiazolidin-4-one (**5c**)

This compound was obtained according to the general procedure as a reddish orange crystal, yield: 81.2 %, mp: 258–60 °C. IR ν (cm^{-1}): 3433 (NH), 1689 (C=O), 1615-1614 (C=N). ^{1}H NMR (300 MHz), δ: 3.04–3.06 (s, 6H, *p*-$N(CH_3)_2$), 5.42 (s, 2H, CH_2–N), 7.85–7.88 (m, 2H, benzimidazole-$C_{5,6}$-Hs), 7.12–7.15 (m, 2H, benzimidazole-$C_{4,7}$-Hs), 7.48–7.55 (m, 2H, phenyl-Ar–Hs), 7.72 (s, 1H, C=CH vinylic H), 12.49 (s, 1H, NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 396 (11.7) ($M^{+\cdot}+2$), 395 (21) ($M^{+\cdot}+1$), 394 (54.5) ($M^{+\cdot}$), 264 (7.3), 206 (28), 177 (100.0), 131(14.9), 104 (14). Anal. calcd for $C_{20}H_{18}N_4OS_2.1½H_2O$ (421.51): C, 56.93; H, 4.98; N, 13.28. Found C, 56.53; H, 4.68; N, 13.12.

(*Z*)-3-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-5-(4-nitrobenzylidene)-2-thioxothiazolidin-4-one (**5d**)

This compound was obtained according to the general procedure as a golden yellow crystal, yield: 88.38 %, mp: 242–244 °C. IR ν (cm^{-1}): 3394 (NH), 1732 (C=O), 1605 (C=N). ^{1}H NMR (300 MHz), δ: 5.45 (s, 2H, CH_2–N), 7.13–7.16 (m, 2H, benzimidazole-$C_{5,6}$-Hs), 7.5 (m, 2H, benzimidazole-$C_{4,7}$-Hs), 7.65–7.67 (d, 2H, phenyl-$C_{2,6}$-Hs, $J = 6$), 7.73–7.75 (d, 2H, phenyl-$C_{3,5}$-Hs, $J = 6$), 7.92 (s, 1H, C=CH vinylic H), 12.49 (s, 1H, NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 398 (3.9) ($M^{+\cdot}+2$), 397 (4.2) ($M^{+\cdot}+1$), 396 (15.2) ($M^{+\cdot}$), 189 (69.4), 131 (100), 118 (27.1), 89 (41.2), 63 (19.0). Anal. calcd for $C_{18}H_{12}N_4O_3S_2$(396.44): S, 16.18. Found S, 15.50.

(*Z*)-3-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-5-(2-chlorobenzylidene)-2-thioxothiazolidin-4-one (**5e**)

This compound was obtained according to the general procedure as a golden yellow crystal, yield: 93.5 %, mp: 250–252 °C. IR ν (cm^{-1}): 3437 (NH), 1712 (C=O), 1596 (C=N). ^{1}H NMR (300 MHz), δ: 5.45 (s, 2H, CH_2–N), 7.13–7.16 (m, 2H, benzimidazole-$C_{5,6}$-Hs), 7.45-7.47 (m, 2H, benzimidazole-$C_{4,7}$-Hs), 7.67–7.8 (m, 4H, phenyl-$C_{3,4,5,6}$-Hs), 7.92 (s, 1H, C=CH vinylic H), 12.49 (s, 1H, NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 387 (44.4) ($M^{+\cdot}+2$), 386 (31.6) ($M^{+\cdot}+1$), 385 (100) ($M^{+\cdot}$), 352 (35.7), 189 (86.3), 131 (83.4), 118 (31.1), 77 (48). Anal. calcd for $C_{18}H_{12}ClN_3OS_2.½H_2O$ (394.89): C, 54.69; H, 3.29; N, 10.63. Found C, 54.65; H, 3.712; N, 10.93.

(*Z*)-3-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-5-(3-phenyl allylidene)-2-thioxothiazolidin-4-one (**5f**)

This compound was obtained according to the general procedure as a yellow crystal, yield 87.5 %, mp 248–50 °C. IR *v* (cm^{-1}): 3437 (NH), 1704 (C=O), 1578 (C=N). ^{1}H NMR (200 MHz), *δ*: 5.45 (s, 2H, CH_2–N), 7.21–7.77 (m, 11 H, benzimidazole, phenyl Hs and 2Hs of conjugated system), 12.5 (broad signal, 2H, NH and 1H of conjugated system). MS, *m*/*z* (rel. Abund. %): 379 (2.9) ($M^{+\cdot}$+2), 378 (5.4) ($M^{+\cdot}$+1), 377 (16.5) ($M^{+\cdot}$), 189 (76.9), 160 (75.5), 131 (100), 115 (75.7), 77 (37.0). Anal. calcd for $C_{20}H_{15}N_3OS_2$ (377.48): N, 11.13. Found N, 10.8.

(*Z*)-3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-(4-methoxybenzylidene)-2-thioxothiazolidin-4-one (**5g**)

This compound was obtained according to the general procedure as a golden yellow crystal, yield: 97.11%, mp: 250–252 °C. IR *v* (cm^{-1}): 3438 (NH), 1708 (C=O), 1590 (C=N). ^{1}H NMR (300 MHz), *δ*: 3.82 (s, 3H, C_4-OCH_3), 5.45 (s, 2H, CH_2–N), 7.12–7.16 (m, 2H, benzimidazole-$C_{5,6}$-Hs), 7.02-7.04 (d, 2H, phenyl-$C_{3,5}$-Hs, $J = 6$), 7.5-7.52 (m, 2H, benzimidazole-$C_{4,7}$-Hs), 7.60-7.62 (d, 2H, phenyl-$C_{2,6}$-Hs, $J = 6$), 7.9 (s, 1H, C=CH vinylic H), 12.5 (s, 1H, NH, D_2O exchange). Anal. calcd for $C_{19}H_{15}N_3O_2S_2.1½H_2O$ (408.47): C, 55.81; H, 4.40; N, 10.28; S, 15.66. Found C, 55.70; H, 3.75; N, 10.21; S, 16.00.

(*Z*)-3-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-5-(2,5-dimethoxybenzylidene)-2-thioxothiazolidin-4-one (**5h**)

This compound was obtained according to the general procedure as a golden yellow crystal, yield: 97.32 %, mp: 244–246°C. IR *v* (cm^{-1}): 3441 (NH), 1714 (C=O), 1584 (C=N). ^{1}H NMR (300 MHz), *δ*: 3.79–3.88 (2s, 6H, phenyl-$C_{2,5}$-OCH_3), 5.45 (s, 2H, CH_2–N), 6.99–7.9 (m, 7H, Ar–Hs), 7.96 (s, 1H, C=CH vinylic H), 12.5 (s, 1H, NH, D_2O exchange). ^{13}C-NMR, *δ*: 41.9 (CH_2–N), 55.57 (C_5-OCH_3), 56.03 (C_2-OCH_3), 113.25–128.76 (Ar–Cs of benzimidazole and phenyl rings), 147.95 (C=CH), 152.47 (2-OCH_3), 153.21 (5-OCH_3), 166.61 (C=O) and 193.65 (C=S). MS, *m*/*z* (rel. Abund. %): 413 (27.5) ($M^{+\cdot}$+2), 412 (26.3) ($M^{+\cdot}$+1), 411 (100) ($M^{+\cdot}$), 378 (44.7), 189 (78.1), 131 (25.9), 77 (16.4). Anal. calcd for $C_{20}H_{17}N_3O_3S_2$ (411.49): N, 10.21. Found N, 9.96.

(*Z*)-3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-(2,4-dimethoxybenzylidene)-2-thioxothiazolidin-4-one (**5i**)

This compound was obtained according to the general procedure as a yellow crystal, yield 85.15 %, mp 248–250 °C. IR *v* (cm^{-1}): 3434 (NH), 1700 (C=O), 1576 (C=N). ^{1}H NMR (500 MHz), *δ*: 3.8–3.82 (2 s, 6H, phenyl-$C_{2,4}$-OCH_3), 5.71 (s, 2H, CH_2–N), 6.32 (s, 1H, phenyl-C_3-H), 6.43-6.45 (d, 1H, phenyl-C_5-H, $J = 10$), 7.06–7.08 (d, 1H, phenyl-C_6-H, $J = 10$), 7.18-7.2 (dd, 2H, benzimidazole-$C_{5,6}$-Hs), 7.55-7.57 (2d, 2H, benzimidazole-$C_{4,7}$-Hs), 7.8 (s, 1H, C=CH vinylic H). MS, *m*/*z* (rel. Abund. %): 412 (5.7) ($M^{+\cdot}$+1), 411 (10.7) ($M^{+\cdot}$), 189 (100), 179 (57.0), 151 (39.5), 131 (52.9), 77 (19.9). Anal. calcd for $C_{20}H_{17}N_3O_3S_2$ (411.49): N, 10.21. Found N, 9.99.

(*Z*)-3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-[(pyridin-2-yl)methylene]-2-thioxothiazolidin-4-one (**5j**)

This compound was obtained according to the general procedure as a yellow crystal, yield: 90.9 %, mp: 254–256°C. IR *v* (cm^{-1}): 3377 (NH), 1709 (C=O), 1615 (C=N). ^{1}H NMR (300 MHz), *δ*: 5.67 (s, 2H, CH_2–N), 7.23–7.57 (m, 8H, benzimidazole and pyridine-Hs), 7.9 (s, 1H, C=CH vinylic H), 12.49 (s, 1H, NH, D_2O exchange). MS, *m*/*z* (rel. Abund. %): 354 (1.7) ($M^{+\cdot}$+2), 353 (5.1) ($M^{+\cdot}$+1), 352 (14.5) ($M^{+\cdot}$), 319 (7.6), 221 (31.4), 189 (22.5), 135 (100.0), 91 (12.6), 77 (23.9). Anal. calcd for $C_{17}H_{12}N_4OS_2$ (352.43): N, 15.90. Found N, 15.29.

3-[3-((1*H*-Benzo[*d*]imidazol-2-yl) methyl)-4-oxo-2-thioxothiazolidin-5-ylidene] indolin-2-one (**6**)

To a solution of 3-[(1H-benzo[d]imidazol-2-yl) methyl]-2-thioxothiazolidin-4-one (**3**) (0.26 g, 1 mmol) and isatin (0.17 g, 1.2 mmol) in ethanol (12 ml), a solution of ammonium chloride (0.07 g, 1.5 mmol) in concentrated ammonium hydroxide (0.05 ml, 1.5 mmol) was added. The reaction mixture was refluxed for 12 h, cooled, and the product separated out was filtered, washed with ethanol, dried, and crystallized from aqueous DMF, yield: 75.94 %, mp: >300 °C. IR *v* (cm^{-1}): 3228 (N–H), 1713 (C=O amide carbonyl of rhodanine), 1692 (C=O amide carbonyl of isatin), 1617 (C=N). ^{1}H NMR (200 MHz), *δ*: 5.52 (s, 2H, CH_2–N), 6.99–8.83 (m, 8H, Ar–Hs), 11.38 (s, 1H, isatin-NH), 12.55 (s, 1H, benzimidazole-NH). Anal. calcd for $C_{19}H_{12}N_4O_2S_2.½\ H_2O$ (401.45): C, 56.79; H, 3.23; N, 13.94. Found C, 56.15; H, 3.19; N, 13.46.

General procedure for the synthesis of compounds **7a–c**

A mixture of 2-aminomethyl benzimidazole dihydrochloride (**2**) (0.65 g, 3 mmol), the selected aryl isothiocyanate derivative (3 mmol) and potassium carbonate (0.41 g, 3 mmol) in ethanol (7 ml) was stirred at room temperature overnight. The reaction mixture was poured into (15 ml) ice water and the precipitated product was filtered, washed with cold water, dried, and crystallized from the appropriate solvent.

1-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-3-phenyl thiourea (**7a**)

This compound was obtained according to the general procedure and crystallized from ethanol as a white crystal, yield: 66.1 %, mp: 184–186 °C [reported 181–183°C] [30]. IR *v* (cm^{-1}): 3367 (NH), 3283 (NH), 1598 (C=N), 1365 (C=S of thiourea).

1-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-3-(4-methyl phenyl) thiourea (**7b**)

This compound was obtained according to the general procedure and crystallized from ethanol as a white crystal, yield: 87.83 %, mp: 204–206 °C (reported 187–188 °C) [31]. IR *v* (cm^{-1}): 3289 (NH), 3227 (NH), 1616 (C=N), 1238 (C=S of thiourea).

1-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-3-(4-chlorophenyl) thiourea (**7c**)

This compound was obtained according to the general procedure and crystallized from acetonitrile as a white crystal, yield: 86.49 %, mp: 200–202 °C (reported 195–197 °C–[30]. IR *v* (cm^{-1}): 3271 (NH), 3087 (NH), 1617 (C=N), 1333 (C=S of thiourea).

General procedure for the synthesis of compounds **8a–c**

A mixture of disubstituted thiourea (**7a–c**) (2 mmol) and ethyl bromoacetate (0.22 ml, 2 mmol) in ethanol (10 ml) was heated under reflux for 20–30 h. The reaction mixture was concentrated under reduced pressure and then cooled.

3-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-2-(phenylimino)thiazolidin-4-one (**8a**)

The precipitated product was filtered, washed with ethanol, dried, and recrystallized from ethanol, yield; 79 %, mp: 270–272 °C. IR *v* (cm^{-1}): 3439 (N–H), 1728 (C=O), 1624 (C=N), 1586 (C=C). ^{1}H NMR (300 MHz), δ: 4.21 (s, 2H, CH_2-C=O), 5.37 (s, 2H, CH_2–N), 6.80-6.81 (d, 1H, phenyl-C_2-H, $J = 1.8$ Hz), 6.83 (d, 1H, phenyl-C_6-H, $J = 1.8$ Hz),7.07–7.12 (m, 1H, phenyl-C_4-H), 7.29–7.35 (m, 2H, benzimidazole-$C_{5,6}$-Hs), 7.44-7.46 (d, 1H, benzimidazole-C_7-H, J = 3.3 Hz), 7.46–7.48 (d, 1H, benzimidazole-C_4-H, $J = 3.3$ Hz), 7.68–7.78 (m, 2H, phenyl-$C_{3,5}$-Hs). MS, *m/z* (rel. Abund. %): 322 (11.0) ($M^{+\cdot}$), 188 (7.5), 187 (7.1), 159 (14.2), 157 (35.0), 156 (19.7), 155 (100), 154 (41.3), 129 (5.5), 128 (14.6), 127 (24.8), 126 (11.4), 118 (13.8), 104 (10.2), 103 (5.5), 102 (6.7), 101 (15.7), 99 (28.7), 98 (10.6), 92 (9.8), 91 (10.6), 92 (9.8), 91 (10.6), 90 (16.1), 89 (10.6), 82 (11.4), 80 (7.1), 77 (35.0), 75 (21.3), 74 (13.4), 73 (16.9), 52 (15.7), 51 (25.2).

3-[(1*H*-benzo[*d*]imidazol-2-yl) methyl]-2-(4-methyl phenylimino) thiazolidin-4-one (**8b**)

The reaction mixture was poured into (15 ml) ice water. The precipitated product was filtered, washed by water, dried, and crystallized from toluene, yield: 74.4 %, mp: 224–226°C. IR *v* (cm^{-1}): 2937 (NH), 1726 (C=O), 1620 (C=N).^{1}H NMR (300 MHz), δ: 2.25 (s, 3H, phenyl-CH_3), 4.14 (s, 2H, CH_2-CO), 5.14 (s, 2H, CH_2–N), 6.73–6.89 (2d, 2H, phenyl-$C_{3,5}$-Hs), 7.11–7.27 (m, 4H, phenyl-$C_{2,6}$-Hs and benzimidazole-$C_{5,6}$-Hs), 7.36–7.54 (m, 2H, benzimidazole-$C_{4,7}$-Hs), 12.36 (s, 1H, NH, D_2O-exchange).

3-[(1*H*-Benzo[*d*]imidazol-2-yl) methyl]-2-(4-chlorophenylimino) thiazolidin-4-one (**8c**)

The precipitated product was triturated with petroleum ether, filtered, and dried. The product was used without further purification for the next step, yield: 89.8 %, mp: 260–261°C. IR *v* (cm^{-1}): 2930 (NH), 1722 (C=O), 1634 (C=N).

General procedure for the synthesis of compounds **9a–n**

To a solution of 1-[(1*H*-benzo[*d*]imidazol-2-yl) methyl]-2-(4-substituted phenylimino) thiazolidin-4-ones (**8**) (0.32 g, 1 mmol) and the appropriate aldehyde (1.2 mmol) in glacial acetic acid (10 ml) a slight excess of anhydrous sodium acetate (0.12 g, 1.5 mmol) was added. The reaction mixture was refluxed for 10 to 12 h; the reaction was monitored by TLC technique. After completion of the reaction, the reaction mixture was cooled and diluted with water (10 ml). The product formed was filtered, washed with water, dried, and crystallized from the appropriate solvent to give **9a–n**.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-benzylidene-2-(phenylimino) thiazolidin-4-one (**9a**)

This compound was obtained according to the general procedure and crystallized from ethanol as a golden yellow crystal, yield: 97.5 %, mp: 280–282°C. IR *v* (cm^{-1}): 3416 (NH), 1710 (C=O), 1633 (C=C), 1589 (C=N). ^{1}H NMR (200 MHz), δ: 5.35 (s, 2H, CH_2–N), 6.82–7.52 (m, 14H, Ar–Hs), 7.8 (s, 1H, C=CH vinylic H), 12.5 (s, 1H, NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 412 (1.2) ($M^{+\cdot}$+2), 411 (2.0) ($M^{+\cdot}$+1), 410 (7.1) ($M^{+\cdot}$), 305 (6.2), 275 (8.1), 263 (5.2), 247 (29.9), 236 (5.9), 220 (5.3), 161 (5.7), 145 (8.7), 134 (100), 118 (17.8), 104 (14.5), 90 (17.6), 89 (16.1), 77 (48.3) and 65 (7.0). Anal. calcd for $C_{24}H_{18}N_4OS$ (410.49): N, 13.65. Found N, 13.91.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-(4-chlorobenzylidene)-2-(phenylimino) thiazolidin-4-one (**9b**)

This compound was obtained according to the general procedure and crystallized from aqueous DMF as a yellow crystal, yield: 78.8 %, mp: 254–256°C. IR *v* (cm^{-1}): 3375 (NH), 1711 (C=O), 1635 (C=C), 1590 (C=N). ^{1}H NMR (200 MHz), *δ*: 5.32 (s, 2H, CH_2–N), 6.98-8.0 (m, 13H, Ar–Hs), 7.9 (s, 1H, C=CH vinylic H), 12.46 (s, 1H, NH, D_2O exchange). Anal. calcd for $C_{24}H_{17}ClN_4OS$ (444.94): N, 12.59; S, 7.21. Found N, 11.85; S, 6.60.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-(4-nitrobenzylidene)-2-(phenylimino) thiazolidin-4-one (**9c**)

This compound was obtained according to the general procedure and crystallized from acetic acid as a yellow crystal, yield: 72.5 %, mp: 300–332°C. IR *v* (cm^{-1}): 3397 (NH), 1713 (C=O), 1631 (C=C), 1588 (C=N). ^{1}H NMR (200 MHz), *δ*: 5.36 (s, 2H, CH_2–N), 6.97–8.32 (m, 13H, Ar–Hs), 7.97 (s, 1H, C=CH vinylic H), 12.63 (s, 1H, NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 457 (5.2) ($M^{+\cdot}$+2), 456 (9.6) ($M^{+\cdot}$+1), 455 (28.7) ($M^{+\cdot}$), 248 (33.5), 173 (12.5), 131 (90.4) and 77 (100.0). Anal. calcd for $C_{24}H_{17}N_5O_3S$(455.49): C, 63.29; H, 3.76; N, 15.38; S, 7.04. Found C, 63.06; H, 3.744; N, 14.9; S, 6.08.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-(2-chlorobenzylidene)-2-(phenylimino) thiazolidin-4-one (**9d**)

This compound was obtained according to the general procedure and crystallized from ethanol as a yellow crystal, yield: 79.5 %, mp: 258–260 °C. IR *v* (cm^{-1}): 3416 (NH), 1721 (C=O), 1635 (C=C), 1589 (C=N). ^{1}H NMR (200 MHz), *δ*: 5.34 (s, 2H, CH_2–N), 6.94-7.51 (m, 13H, Ar–Hs), 7.98 (s, 1H, C=CH vinylic H), 12.52 (s, 1H, NH, D_2O exchange). ^{13}C-NMR, *δ*: 40.76 (CH_2–N), 131.42-120.48 (Ar–Cs of benzimidazole and phenyl rings), 134 (C=CH), 147 (C=N-benzimidazole), 148.85 (C–N-phenylimino), 148.93 (C=N-phenylimino) and 165.11 (C=O). Anal. calcd for $C_{24}H_{17}ClN_4OS$ (444.94): C, 64.79; H, 3.85; N, 12.59. Found C, 65.61; H, 4.099; N, 12.40.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-(-3-phenylallylidene)-2-(phenylimino)thiazolidin-4-one (**9e**)

This compound was obtained according to the general procedure and crystallized from dioxane/ethanol as a yellow crystal, yield: 71.1 %, mp: 276–278°C. IR *v* (cm^{-1}): 3375 (NH), 1707 (C=O), 1635 (C=C), 1588 (C=N). ^{1}H NMR (500 MHz), *δ*: 5.68 (s, 2H, CH_2–N), 6.60–7.57 (m, 12H, Ar–Hs), 7.75 (s, 1H, C=CH vinylic H), 12.52 (s, 1H, NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 438 (9.0) ($M^{+\cdot}$+2), 437 (30.6) ($M^{+\cdot}$+1), 436 (100.0) ($M^{+\cdot}$), 248 (89.3), 207 (35.0), 160 (99.4) and 77 (96.7). Anal. calcd for $C_{26}H_{20}N_4OS$ (436.53): C, 71.54; H, 4.62; N, 12.83; S, 7.35. Found C, 70.97; H, 4.212; N, 12.46; S, 7.078.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-(2,5-dimethoxybenzylidene)-2-(phenylimino) thiazolidin-4-one (**9f**)

This compound was obtained according to the general procedure and crystallized from ethanol as a yellow crystal, yield: 70.5 %, mp: 238-240°C. IR *v* (cm^{-1}): 3416 (NH), 1710 (C=O), 1636 (C=C), 1591 (C=N). ^{1}H NMR (200 MHz), *δ*: 3.71 (s, 3H, OCH_3 at C_4), 3.84 (s, 3H, OCH_3 at C_2), 5.32 (s, 2H, CH_2–N), 6.97–7.57 (m, 12H, Ar–Hs), 7.96 (s, 1H, C=CH vinylic H), 12.51 (s, 1H, NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 472 (6.0) ($M^{+\cdot}$+2), 471 (18.5) ($M^{+\cdot}$+1), 470 (59.9) ($M^{+\cdot}$), 248 (61.1), 194 (88.7), 131 (53.0) and 77 (100.0). Anal. calcd for $C_{26}H_{22}N_4O_3S.1½H_2O$ (497.54) : C, 62.70; H, 5.02; N, 11.25; S, 6.43. Found C, 62.29; H, 4.391; N, 11.65; S, 6.197.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-(2,4-dimethoxybenzylidene)-2-(phenylimino) thiazolidin-4-one (**9g**)

This compound was obtained according to the general procedure and crystallized from ethanol as a yellow crystal, yield: 74.9 %, mp: 236–238 °C. IR *v* (cm^{-1}): 3410 (NH), 1707 (C=O), 1633 (C=C), 1590 (C=N). ^{1}H NMR (200 MHz), *δ*: 3.72–3.8 (2s, 6H, OCH_3 at $C_{2,5}$), 5.34 (s, 2H, CH_2–N), 6.94–7.54 (m, 12H, Ar–Hs), 7.95 (s, 1H, C=CH vinylic H), 12.51 (s, 1H, NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 472 (5.4) ($M^{+\cdot}$+2), 471 (15.4) ($M^{+\cdot}$+1), 470 (57.8) ($M^{+\cdot}$), 248 (58.6), 194 (100.0), 131 (41.1) and 77 (87.5). Anal. calcd for $C_{26}H_{22}N_4O_3S.½H_2O$ (479.54): C, 65.06; H, 4.79; N, 11.67. Found C, 64.53; H, 5.36; N, 11.61.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-2-(phenylimino)-5-[(pyridin-2-yl) methylene] thiazolidin-4-one (**9h**)

This compound was obtained according to the general procedure and crystallized from ethanol as a green crystal,

yield: 96.2%, mp: 268–270 °C. IR ν (cm^{-1}): 3385 (NH), 1707 (C=O), 1625 (C=C), 1586 (C=N). ^{1}H NMR (300 MHz), δ: 5.30 (s, 2H, CH_2–N), 6.90–7.50 (m, 13H, Ar–Hs), 7.94 (s, 1H, C=CH vinylic H), 12.59 (s, 1H, NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 413 (3.6) ($M^{+\cdot}$+2), 412 (9.1) ($M^{+\cdot}$+1), 411 (24.0) ($M^{+\cdot}$), 280 (7.80), 248 (24.6), 163 (21.3), 145 (23.11), 135 (100.0), 118 (26.9), 104 (22.2), 91 (18.7) and 77 (41.2). Anal. calcd for $C_{23}H_{17}N_5OS$ (411.48): N, 17.02. Found N, 16.90.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-benzylidene-2-(4-methyl phenylimino) thiazolidin-4-one (**9i**)

This compound was obtained according to the general procedure and crystallized from ethanol as a yellow crystal, yield: 66.1 %, mp: 270–272 °C. IR ν (cm^{-1}): 3390 (NH), 1709 (C=O), 1628 (C=C), 1581 (C=N). ^{1}H NMR (300 MHz), δ: 2.29 (s, 3H, phenyl-CH_3), 5.31 (s, 2H, CH_2–N), 6.82–7.59 (m, 13H, Ar–Hs), 7.82 (s, 1H, =CH vinylic-H), 12.49 (s, 1H, NH, D_2O exchange). Anal. calcd for $C_{25}H_{20}N_4OS.½H_2O$ (433.52): C, 69.20; H, 4.84; N, 12.91; S, 7.38. Found C, 68.47; H, 4.245; N, 12.99; S, 7.02.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-(4-chlorobenzylidene)-2-(4-methyl phenylimino) thiazolidin-4-one (**9j**)

This compound was obtained according to the general procedure and crystallized from ethanol as a yellow crystal, yield: 54.7 %, mp: 288–290 °C. IR ν (cm^{-1}): 3354 (NH), 1711 (C=O), 1639 (C=C), 1605 (C=N).^{1}H NMR (300 MHz), δ: 2.29 (s, 3H, phenyl-CH_3), 5.31 (s, 2H, CH_2–N), 6.82–7.59 (m, 13H, Ar–Hs), 7.82 (s, 1H, =CH vinylic-H), 12.49 (s, 1H, NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 460 (6.2) ($M^{+\cdot}$+2), 459 (4.6) ($M^{+\cdot}$+1), 458 (9.6) ($M^{+\cdot}$), 261 (19.3), 170 (24.9), 168 (56.0), 131 (100.0), 118 (24.1), 104 (17.5), 91 (17.7) and 77 (16.9). Anal. calcd for $C_{25}H_{19}ClN_4OS.½H_2O$ (467.96): C, 64.10; H, 4.27; N, 11.96; S, 6.83. Found C, 63.33; H, 3.84; N, 12.21; S, 6.29.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-(4-nitrobenzylidene)-2-(4-methyl phenylimino) thiazolidin-4-one (**9k**)

This compound was obtained according to the general procedure and crystallized from acetic acid as a yellow crystal, yield: 69.7 %, mp: >300 °C. IR ν (cm^{-1}): 3396 (NH), 1710 (C=O), 1637 (C=C). ^{1}H NMR (200 MHz), δ: 2.3 (s, 3H, phenyl-CH_3), 5.3 (s, 2H, CH_2–N), 6.95-8 (m, 13H, Ar–Hs), 7.9 (s, 1H, C=CH vinylic H), 12.5 (s, 1H, NH, D_2O exchange). Anal. calcd for $C_{25}H_{19}N_5O_3S$ (469.52): C, 63.95; H, 4.08; N, 14.92. Found C, 62.83; H, 3.55; N, 14.74.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-(2-chlorobenzylidene)-2-(4-methyl phenylimino) thiazolidin-4-one (**9l**)

This compound was obtained according to the general procedure and crystallized from ethanol as a yellow crystal, yield: 70.2 %, mp: 223–226°C. IR ν (cm^{-1}): 3406 (NH), 1708 (C=O), 1637 (C=C), 1605 (C=N). ^{1}H NMR (200 MHz), δ: 2.29 (s, 3H, phenyl-CH_3), 5.3 (s, 2H, CH_2–N), 6.8-7.8 (m, 13H, Ar–Hs), 7.85 (s, 1H, C=CH vinylic H), 12.51 (s, 1H, NH, D_2O exchange). Anal. calcd for $C_{25}H_{19}ClN_4OS.½H_2O$ (467.96): C, 64.10; H, 4.27; N, 11.96. Found C, 63.42; H, 4.61; N, 11.68.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-benzylidene-2-(4-chlorophenylimino)- thiazolidin-4-one (**9m**)

This compound was obtained according to the general procedure and crystallized from ethanol as a yellow crystal, yield: 63.6 %, mp: 218–221 °C. IR ν (cm^{-1}): 3410 (NH), 1718 (C=O), 1630 (C=C), 1590 (C=N). ^{1}H NMR (500 MHz), δ: 5.68 (s, 2H, CH_2–N), 6.60-7.57 (m, 13H, Ar–Hs), 7.75 (s, 1H, C=CH vinylic H), 12.52 (s, 1H, NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 446 (1.7) ($M^{+\cdot}$+2), 445 (2.9) ($M^{+\cdot}$+1), 444 (3.3) ($M^{+\cdot}$), 133 (100.0), 104 (10.0), 90 (15.4) and 77 (17.1). Anal. calcd for $C_{24}H_{17}ClN_4OS.½H_2O$ (453.94): C, 63.44; H, 3.96; N, 12.33. Found C, 63.10; H, 4.19; N, 12.41.

3-[(1*H*-Benzo[*d*]imidazol-2-yl)methyl]-5-(4-chlorobenzylidene)-2-(4-chlorophenylimino)-thiazolidin-4-one (**9n**)

This compound was obtained according to the general procedure and crystallized from ethanol as a yellow crystal, yield: 87.6 %, mp: 276–277 °C. IR ν (cm^{-1}): 3383 (NH), 1710 (C=O), 1628 (C=C), 1583 (C=N). ^{1}H NMR (500 MHz), δ: 5.58 (s, 2H, CH_2–N), 6.6-7.57 (m, 12H, Ar–Hs), 7.75 (s, 1H, C=CH vinylic H), 12.5 (s, 1H, NH, D_2O exchange). Anal. calcd for $C_{24}H_{16}Cl_2N_4OS$ (479.38): C, 60.13; H, 3.36; N, 11.69. Found C, 59.91; H, 3.74; N, 11.69.

3-[3-((1*H*-Benzo[*d*]imidazol-2-yl) methyl)-4-oxo-2-(phenylimino) thiazolidin-5-ylidene] indolin-2-one (**10**)

To a solution of 3-[(1*H*-benzo[*d*]imidazol-2-yl) methyl]-2-(phenylimino) thiazolidin-4-one (**8a**) (0.32 g, 1 mmol) and isatin (0.17 g, 1.2 mmol) in ethanol (12 ml), a solution of ammonium chloride (0.07 g, 1.5 mmol) in concentrated ammonium hydroxide (0.05 ml, 1.5 mmol) was added. The reaction mixture was refluxed for 12 h, cooled and the product precipitated was filtered, washed with ethanol, dried, and crystallized from aqueous DMF, yield: 53.21 %

mp: >300 °C. IR ν (cm^{-1}): 3228 (N–H); 1713 (C=O amide carbonyl of rhodanine), 1692 (C=O amide carbonyl of isatin), 1617 (C=N). ^{1}H NMR (300 MHz), δ: 5.12 (s, 2H, CH_2–N), 6.77–7.61 (m, 13H, Ar–Hs), 10.54 (s, 1H, isatin-NH, D_2O exchange), 12.49 (s, 1H, benzimidazole-NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 453 (4.9) ($M^{+\cdot}$+2), 452 (8.1) ($M^{+\cdot}$+1), 451 (28.1) ($M^{+\cdot}$), 333 (14.9), 248 (52.6), 175 131 (100.0) and 77 (73.9). Anal. calcd for $C_{25}H_{17}N_5O_2S_2.2H_2O$ (487.50): C, 61.53; H, 4.30; N, 14.35. Found C, 61.65; H, 4.54; N, 14.42.

3,4-Dihydropyrimido [1, 6-*a*] benzimidazole-1(2*H*)–thione (**13**)

Method C

A mixture of 2-aminoethyl benzimidazole hydrochloride (**11**) (0.53 g, 2.5 mmol) and sodium carbonate (0.53 g, 5 mmol) in 10 ml isopropanol was stirred under reflux. The mixture was heated on a water bath for 15 min, and to the clear solution, a hot solution of bis (carboxymethyl) trithiocarbonate (0.56 g, 2.5 mmol) in 15 ml isopropanol was added at once. A yellowish orange precipitate was formed. Heating and stirring were continued for 18 h. The precipitate was removed by suction filtration. The solid was transferred to a flask containing 5 ml water and heated on a water bath to 70–75 °C, while the lumps were crushed by a glass rod to obtain a homogenous mixture. The mixture was filtered with suction when hot, cleaned by rinsing it with small amount of hot water, dried as possible, and crystallized from ethanol, yield: 23.64 %, mp: 216–218 °C.

Method D

A mixture of 2-aminoethyl benzimidazole hydrochloride (**11**) (0.43 g, 2 mmol), carbon disulfide (0.12 ml, 2 mmol), ethyl bromoacetate (0.22 ml, 2 mmol) and triethylamine (0.83 ml, 6 mmol) in 8 ml methanol, was stirred at room temperature overnight. The precipitated product was filtered, washed with methanol, dried (yield 0.38 g, 93.59%), and crystallized from ethanol; m.p. 216–218 °C. The products obtained by method (**C**) and method (**D**) are one and the same compound. The identity of the product was confirmed by TLC technique, mp, and IR spectra. IR ν (cm^{-1}) : 3441 (N–H), 1614 (C=N), 1111 (C=S of thiourea). ^{1}H NMR (500 MHz), δ: 3.19–3.2 (t, 2H, CH_2-CH_2–N, $J = 6.9$ Hz), 3.50-3.53 (t, 2H, CH_2–N, $J = 6.85$ Hz), 7.28–7.29 (d, 1H, benzimidazole-C_4-H, $J = 3.8$ Hz), 7.29–7.30 (d, 1H, benzimidazole-C_7-H, $J = 3.8$ Hz), 7.59–7.60 (dd, 1H, benzimidazole-C_5-H, $J = 3.85$, 5.75 Hz), 8.76–8.78 (dd, 1H, benzimidazole-C_6-H, $J = 3$, 6 Hz), 10.32 (s, 1H, NH, D_2O exchange). MS, *m/z* (rel. Abund. %): 205 (3.8) ($M^{+\cdot}$+2), 204 (8) ($M^{+\cdot}$+1), 203 (53) ($M^{+\cdot}$), 145 (100), 131 (83.1), 118 (15.7), 104 (24.0), 90 (36.7), 77 (37.3), 63 (55.9) and 51 (41.4). Anal. calcd for $C_{10}H_9N_3S$ (203.26): C, 59.09; H, 4.46; N, 20.9.

Method E

A mixture of 2-aminoethyl benzimidazole hydrochloride (**11**) (0.43 g, 2 mmol) and carbon disulfide (0.12 ml, 2 mmol) in 8 ml methanol was stirred at room temperature for an overnight. The precipitated product was filtered, washed with methanol dried (yield 0.38 g, 93.59 %), and crystallized from ethanol; m.p. 216–218 °C. IR ν (cm^{-1}): 3444 (NH), 1615 (C=N), 1110 (C=S).

Biological studies

Antiviral activity

Neutral red uptake assay to measure cytotoxicity

Isolation of lymphocytes from whole human blood by means of gradient separation by Ficoll-PagueTM Plus (MP Biomedicals, France), 10×10^4 lymphocyte cells were seeded per well in 96-well plates and the plates were incubated in RPMI media containing different concentrations of the test compounds **4a**, **4b**, **5c**, **5d**, **5g**, **5h**, **6**, **9b**, **9j**, and **10** for 24, 48, 72, and 96 h.

The fraction of viable lymphocyte cells was measured by the neutral red assay

The neutral red assay is based on the initial protocol described by Borenfreund (Borenfreund and Puerner, 1984) and determines the accumulation of the neutral red dye in the lysosomes of viable cells (Fotakis and Timbrell, 2006).

Following exposure of different concentrations of each test chemicals to cells were incubated for 3 h with neutral red dye (40 μg/ml) dissolved in culture media RPMI. Cells were then washed using Phosphate Buffered Saline (PBS) and the addition of 1 ml of elution medium (ethanol/glacial acetic acid/water 50 %/1 %/49 %) followed by gentle shaking for 10 min so that complete dissolution was achieved. Aliquots of the resulting solutions were transferred to 96-well plates and absorbance at 490 nm was recorded using microliter plate reader spectrophotometer (Biotek, U.S.A).

The simplest estimate of IC_{50} is to plot *x–y* and fit the data with a straight line (linear regression).

Cell culture

HEPG2 cells were washed twice in RPMI1640 media supplemented with 200 μM L-glutamine and 25 μM

HEPES buffer; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (all chemicals and media, Cambrex). The cells were suspended at 2×10^5 cells/ml in RPMI culture media (RPMI supplemented media, 10% fetal bovine serum (FBS); Gibco-BRL). The cells were left to adhere on the polystyrene 6-well plates for 24 h in an incubator (37 °C, 5 % CO_2, 95 % humidity). The cells were washed twice from debris and dead cells using RPMI supplemented media.

Cytotoxicity assay was performed on HEPG2 cell line by the previously described method.

Qualitative in vitro anti-HCV screening

PBMC and HEPG2 cell cultures were prepared as discussed in EL-Hawash (EL-Hawash *et al.*, 2006), then infected with 2% HCV-infected serum in RPMI culture medium containing 8 % FBS. Each of the tested compounds was added at the specified doses. Positive and negative control cultures were included. After 96 h of incubation at 37 °C, 5 % CO_2, and 95 % humidity, a second dose of the test compound was added. The cells were incubated for a further 96 h followed by total RNA extraction. The positive strand and its replicating form (negative strand) were detected by RT-PCR using HCV specific primers to the 59-untranslated region of the virus.

RNA extraction and RT-PCR of HCV RNA

Total RNA was extracted from HEPG2 HCV-infected cells by the method described by El-Awady (El-Awady *et al.*, 1999). Briefly, culture cells were mixed with 200 µl of 4 M guanidinium isothiocyanate containing 25 mM sodium citrate, 0.5 % sarcosyl, 0.1 M β-mercaptoethanol, and 100 µl sodium acetate. The lysed cells were mixed with an equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1, pH 4). After vortexing of the sample, the mixture was centrifuged at 14 K rpm for 10 min at 4 °C. The aqueous layer was collected and mixed with an equal volume of isopropanol. After incubation at −20 °C overnight, RNA was precipitated by centrifugation at 14 K rpm for 30 min at 4 °C and the precipitated RNA was washed twice using 70 % ethanol. The complimentary DNA (cDNA) and the first PCR reaction of the nested PCR detection system for the HCV RNA was performed in a 50-µl volume single-step reaction using the Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 10 µM from each of the RT downstream primer, PCR forward primer, and reverse primer P2. The thermal cycling protocol was manipulated as follows: 30 min at 42 °C for cDNA synthesis followed by 5 min at 95 °C and 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. The nested PCR amplification was performed in 50-µl reaction mixture containing 0.2 mM from each dNTP, 10 µM from each of the reverse nested primer and the forward nested primer, two units of taq DNA polymerase (Promega, Madison, WI, USA) and 10 µl from the RT-PCR reaction product in a 1× buffer supplied by the Vendor. A fragment of 174 bp length was identified in positive samples.

Anticancer activity

Cell lines and culture

The human MCF7, HCT 116, and HEPG2 cell lines were obtained from the American Type Culture Collection (ATCC, USA). The MCF7 cells were maintained in L-glutamine containing Eagle's MEM (Sigma, Germany), supplemented with $NaHCO_3$ (2.2 g/l), sodium pyruvate (110 mg/l), gentamycin (50 mg/l), and 10 % fetal calf serum (FCS; Gibco, Germany) using 75 cm^2-culture flasks in a humidified atmosphere (95 % air/5 % CO_2) at 37 °C.

Measurement of potential cytotoxicity by sulforhodamine-B (SRB) assay

Single dose assay

The tested compounds **5e**, **5f**, **5g**, **5h**, **5i**, **5j**, **9a**, **9c**, **9d**, **9g**, **9h**, and **10** were submitted and evaluated at a single concentration (100 µM). Primary anticancer assays were performed according to the procedure of Skehan (Skehan *et al.*, 1990), which is described below. The compounds were added at a single concentration and the cell culture was incubated for 48 h. End point determinations were made with a protein binding dye, sulforhodamine B (SRB). Results for each test compound were reported as the percent of growth of the treated cells when compared to the untreated control cells. Since the compounds were dissolved in DMSO, it was used as vehicle control. The percentage growth was evaluated spectrophotometrically at 570 nm versus controls not treated with test agents.

Using the seven absorbance measurements [time zero, (T_z), control growth in the absence of drug (C), and test growth in the presence of drug at 100 µM concentration (T_i)], the growth percentage for each compound was calculated as

$$\text{Growth percentage} = [(T_i - T_z)/(C - T_z)] \times 100$$

Five dose assay

Potential cytotoxicity of compounds was tested by the method of Skehan *et al.*, (1990) as follows Cells were plated in 96-multiwell plate (5×10^3 cells) for 24 h before treatment with the compounds **5e**, **5f**, **5g**, **5h**, **5j**, and **9d** as well as compounds **5a**, **5d,** and **6** to allow attachment of cells to the wall of the plate. Different concentrations of the compounds (0, 5, 12.5, 25, and 50 µg/ml) were added to

the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds under test for 48 h at 37 °C and in atmosphere of 5 % CO_2. After 48 h, cells were fixed with trichloroacetic acid, stained for 30 min with 0.4 % (wt/vol) SRB stain, and dissolved in 1 % acetic acid. Unbound dye was removed by four washes with 1 % acetic acid, and protein-bound dye was extracted with 10 mM buffered Tris EDTA [tris (ethylene diamine tetra acetic acid)] for determination of color intensity in an ELISA reader. For comparison purposes, the cytotoxicity of Doxorubicin, a standard antitumor drug, was evaluated under the same conditions. The relation between surviving fraction and drug concentration is plotted to get the survival curve of the specified compound against each tumor cell line.

Molecular modeling

Energy minimization and conformational search procedure

The compounds **5a**, **5h**, and **9d** were drawn on ChemDraw Ultra 8 (Cambridge) and saved as mol file. The latter were subjected to energy minimization using Force Field MMFF94x and systemic conformational search by molecular operating environment (MOE) software (MOE Chemical Computing Group Inc; Montreal).

Electrostatic potential map (EPS)

The relevant energy minimized compounds were opened by MOE software, their partial charges are calculated and their EPS were obtained on Gaussian surface.

Source of target protein

The crystal structure of human NAT1 with the covalently bound 2-bromoacetanilide inhibitor (PDB ID code: 2PQT) was downloaded from the Protein Data Bank and opened by MOE software. MOE was also used to calculate the best score between the ligands and the enzymes' binding site. Previous studies of bacterial NATs revealed a cysteine protease-like catalytic core. The Cys-His-Asp triad (C^{68}, H^{101} and D^{122} in human NATs) is conserved among prokaryotic and eukaryotic NATs, including Mycobacterium smegmatis, Pseudomonas aeruginosa, and in human NATs for which the structures have been determined (Sinclair *et al.*, 2000).

Docking procedure

The modeling studies presented in this report were conducted as follows. The α-substituted acetanilide moiety was removed from Cys^{68} of the deposited structure of human NAT1 (2PQT) before docking being performed. The NAT1 structure was used after the restoration of the terminal thiol motif of Cys^{68}. Hydrogen atoms were added to NAT1 and subsequently minimized by the force MMFF94x field. The synthesized target compounds were opened by the MOE software. The docking was done by the default MOE-DOCK, where the option Rotate Bonds was selected to give flexible ligand-rigid receptor docking. Thirty conformers of the ligand were retained with highest and best score by default. The top score ligand–receptor docking was then demonstrated by 2D and 3D ligand–receptor interactions.

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Conflict of interest None.

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