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Original article

Synthesis of new pyrrolo[2,3-*d*]pyrimidine derivatives and evaluation of their activities against human colon cancer cell lines

Saritha Jyostna Tangeda, Achaiah Garlapati*

University College of Pharmaceutical Sciences, Kakatiya University Warangal 506 009, Andhra Pradesh, India

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ABSTRACT

New pyrrolo[2,3-*d*]Pyrimidines with heteroaryl substitution at 5th position through sulfur linker were synthesized incorporating putative pharmacophoric moieties like benzimidazole and benzothiazole as heteroaryl groups. Cytotoxic effect of all the compounds was carried out on HCT116 colon cancer cell lines. Compounds **6c** and **6h** with nitrobenzimidazole and pyrimidyl heterocycles attached at 5th position via sulfur were the most potent of all with IC50 values ≈17.6 μM. Among the four compounds tested for apoptosis induction activity, **6c** induced apoptosis in a dose dependent manner.

1. Introduction

TS (Thymidylate Synthase) plays an important role in the biosynthesis of nucleic acid precursors [1,2]. TS which catalyses the conversion of deoxyuridylate (dump) to deoxy thymidylate (dTMP), has been recognized as an attractive target for the development of anti-tumor agents [3]. 5-Fluorouracil, the first known TS inhibitor, has been used clinically as an anti-tumor agent. The emergence of resistance as well as the insensitivity of certain tumor types to 5-FU has prompted the design of novel folate analogues as potential TS inhibitors and anti-tumor agents [4]. Fig. 1 illustrates important examples of clinically used TS inhibitors such as Raltitrexed [5] and Pemetrexed [6].

Classical antifolates containing L-glutamic acid moiety in the molecule have short-comings such as drug resistance due to decreased folyl poly-γ-glutamate synthetase (FPGS) and impairment of reduced folate carrier (RFC) system required to enter into the cell [7]. The problems associated with classical antifolates are overcome by non-classical lipophilic analogues by deleting (or) modifying L-glutamic acid component from the folate analogues, which would not be dependent on FPGS for their potency and could passively diffuse into cells [8,9]. Nolatrexed is the first non-classical TS inhibitor to reach clinical trials. Gangjee et al. reported the synthesis of 2-amino-4-oxo-5-thiopyridyl-6-methyl pyrrolo(2,3-*d*)pyrimidine (**1**) as a potent TS inhibitor (IC_{50} = 0.34 μM) [10,11].

Over the past years benzimidazole derivatives are one of the most extensively studied classes of heterocyclic compounds. Molecules with benzimidazole nucleus have been reported to posses anticancer, anti-infective, antibacterial and antifungal properties due to their structural similarity with naturally occurring purines as they can easily interact with biomolecules of the living systems [12,13]. FB642 is a member of the benzimidazole carbamate exhibited in vitro anti-tumor activity against both the murine B16 melanoma (IC_{50} = 8.5 μM) and human HT-29 colon carcinoma (IC_{50} = 9.5 μM) cell lines [14,15]. Similarly benzothiazole constitutes an important Scaffold of drugs possessing several pharmacological functions, rendering this molecule and its derivatives powerful anti-tumor agents [16,17]. DF203 is identified as anticancer agent with remarkable differential activity in vitro and in vivo [18,19].

In spite of the above and although many new pyrrolo[2,3-*d*]pyrimidine derivatives have been synthesized as potential anti-tumor agents, there is scarce literature data on pyrrolo(2,3-*d*)pyrimidines possessing benzimidazole and benzothiazole as substituent. Therefore it is thought worthwhile to synthesize pyrrolo[2,3-*d*]pyrimidine derivatives bearing various heterocyclic nuclei such as benzothiazole, benzimidazole etc and evaluate them for their anti-tumor activity. The newly proposed synthesized compounds, characterized by spectral and analytical properties, are evaluated for anti-tumor against human colon cancer cell lines (HCT 116) (Fig. 2).

2. Chemistry

2-Amino-6-methyl-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-*d*]pyrimidine (**5**) the key intermediate for the synthesis of title compounds

* Corresponding author. Tel.: +91 9440601054; fax: +91 870 2453508.
E-mail address: achaiah_g@yahoo.co.in (A. Garlapati).

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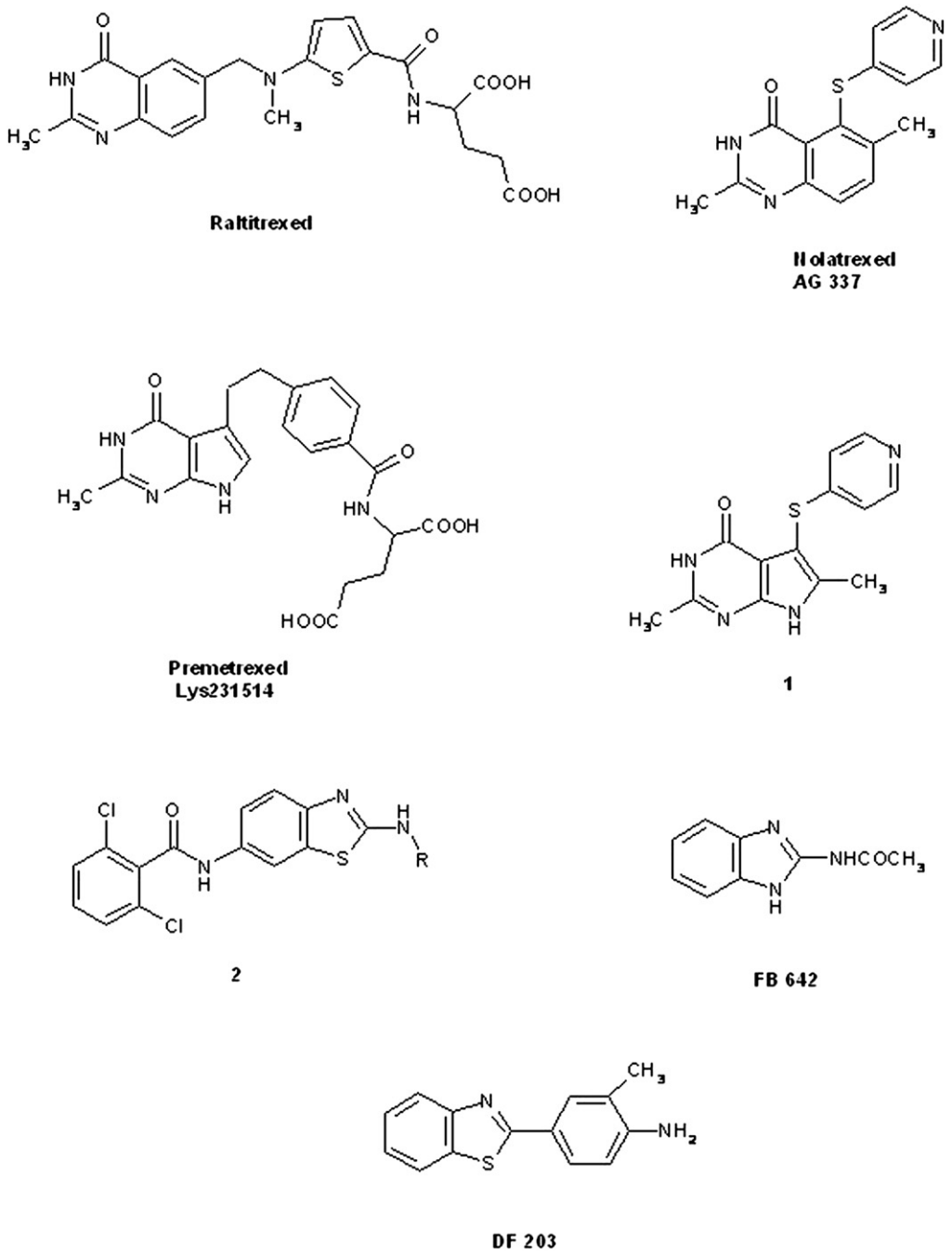


Fig. 1. Examples of some potential TS inhibitors.

6a–j (Scheme 1) was prepared by the addition of chloroacetone (**4**) to a solution of 2,6-diamino-4-hydroxy pyrimidine (**3**) in sodium acetate and water as reported earlier [11]. Various heteroaryl moieties like benzimidazole, benzothiazole, pyridyl, substituted oxadiazoles etc. were linked via sulfur to the 5th position of pyrrolopyrimidines in a one step oxidative addition of corresponding mercapto heteroaryl compounds in presence of iodine [20]. Completion of the reaction was monitored by TLC. The title compounds **6a–j** were obtained in good yields (32–67%) [21].

3. Biological studies

All the synthesized compounds (**6a–j**) were evaluated for cytotoxic activity against the colon cancer HCT 116 cells using trypan blue exclusion method [21]. The inhibitory potencies (IC_{50}) are listed in Table 1. Compound **6c** and **6h** were equipotent and the most active of series with IC_{50} close to 17.60 μM. The compound with 5-nitrobenzimidazole (**6c**) exhibited more than 2 times potency when compared to the one without 5-nitro substitution (**6b**; IC_{50} = 38.04 μM). However, introduction of electron withdrawing chloro substituent at 5th position of benzimidazole nucleus did not result in any significant increase in potency (**6d**; IC_{50} = 36.23 μM). On contrary 5-bromo substitution decreased the potency (**6c**; IC_{50} = 45.27 μM). However substitution with electron releasing methyl group at 5th position resulted in a little increase in potency (**6f**; IC_{50} = 34.56 μM). Compound **6a** and **6i** showed lower activity which indicates phenyl substituted oxadiazole and pyridyl moieties did not contribute much to the activity. However,

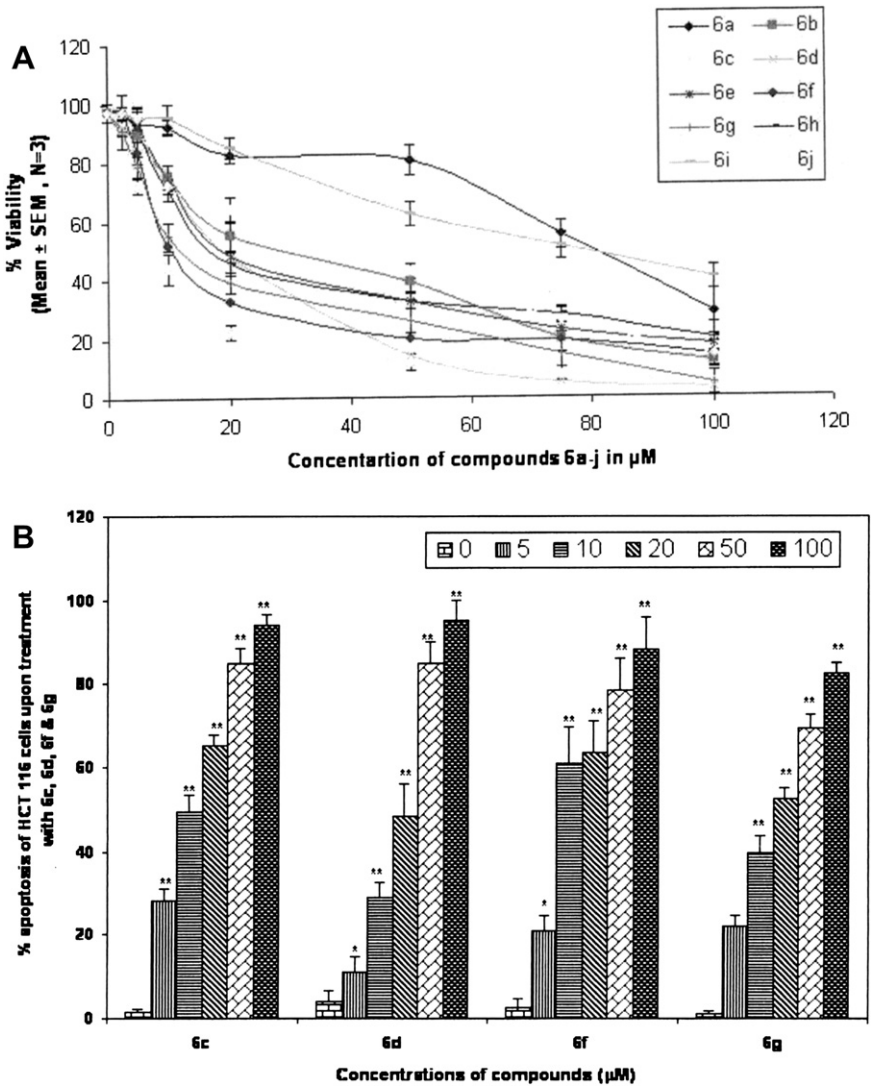


Fig. 2. (A) A cytotoxic effect of compounds **6a–j**. (B) Apoptosis of HCT 116 cells upon treatment with **6c**, **6d**, **6f** and **6g**. Mean of three independent experiments; Significance levels; $^{*}p < 0.005$; $^{**}p < 0.0005$.

introduction of another nitrogen into the pyridine ring (**6i**) to result 2-pyrimidinyl compound (**6h**) greatly enhanced the potency ($IC_{50} = 17.60$ μM).

Compound **6g** with benzothiazole linked to pyrrolopyrimidine exhibited greater potency ($IC_{50} = 26.27$ μM) when compared to its benzimidazole containing compound (**6b**). Compound **6j** with *N*-methyl imidazole substitution exhibited lower potency ($IC_{50} = 48.90$ μM) indicating the importance of benzo fusion to imidazole for activity. Among all the compounds synthesized, four have been selected for their effect on apoptosis by EtBr/AO assay [22]. **6c**, **6d**, **6f** and **6g** induced apoptosis in HCT116 cells in a dose dependent manner (6c, 28.33% in 5 μmol/L, 49.33% in 10 μmol/L, 65% in 20 μmol/L, 85% in 50 μmol/L and 94% in a 100 μmol/L) versus untreated cells.

4. Conclusions

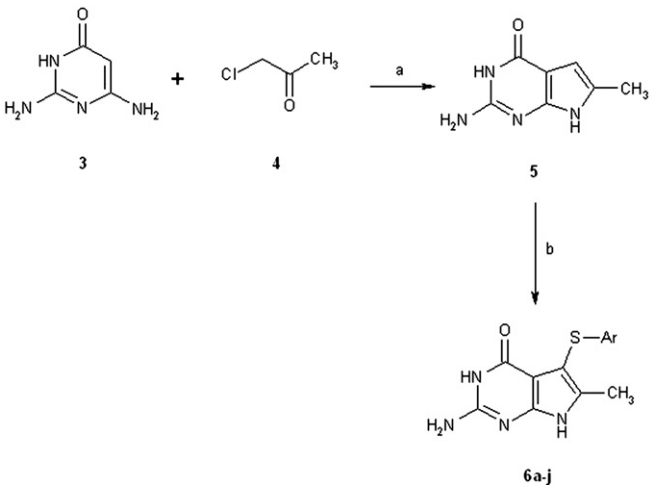
The synthetic methodology adopted resulted in sulfanyl heteroaryl linked pyrrolopyrimidines in overall high yields. The compounds thus obtained were evaluated for cytotoxic activity against colon cancer cell lines to obtain the IC_{50} values.

Among all the compounds, **6c** with nitrobenzimidazole heterocycle exhibited maximum activity. Other electron withdrawing groups like chloro on benzimidazole did not result in such increase in potency. Benzothiazole containing compound, **6g** exhibited much greater potency when compared to the benzimidazole counter part (**6b**) which further suggests that nitro substitution on benzothiazole might result in further improvement in potency. The results suggested that compounds **6a–j** suppressed cell proliferation and a few of them induced apoptosis in human colon cancer cell lines. It has been well established that excessive proliferation and lack of apoptosis leads to tumor growth and above study clearly suggests that compound **6c** can be a potential candidate for further studies as an anti-tumor agent.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on Shital scientific melting point apparatus and are uncorrected. ^{1}H NMR spectra were



Scheme 1. Reagents and conditions (a) NaOAc/H_2O, 80 °C, 4–5 h (b) ArSH, I_2, EtOH/H_2O (3:2), 90 °C, upto 3 h.

determined on a Bruker AVANCE-300 spectrometer. All NMR spectra were measured in DMSO-d_6 solutions using tetra methyl silane as an internal standard and ^{1}H chemical shifts are reported as ppm. Mass spectra were recorded by using electrospray ionization technique (ESI) on the VG170708H mass spectrometer. Solvents were dried by conventional methods. Silicagel 60–120 mesh (Merck) was used as an adsorbent for column chromatography. TLC was performed on 5–10 cm aluminum plates coated with silicagel 60F-254 (Merck) in an appropriate solvent.

*5.1.1. 2-Amino-6-methyl-3,4-dihydro-4-oxo-7H-pyrrolo [2,3-d]-pyrimidine **5***

A suspension of 2,6-diamino-4-hydroxy pyrimidine (1.26 g, 10 mmol) in 25 mL of water containing sodiumacetate (0.82 g, 10 mmol) was heated to 80 °C until it formed a clear solution. Chloroacetone (0.79 mL, 10 mmol) was added to this solution in one lot, following which a precipitate began to form within 30 min. The reaction mixture was heated with stirring at 80 °C for an additional 4 h, cooled and filtered. The crude residue thus obtained was recrystallised with water to get **5** as a off white solid, yield 68%, mp 250 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.14 (3H, s, CH_3), 5.84 (1H, s, 5-CH), 5.96 (2H, s, NH_2), 10.13 (1H, s, NH), 10.79 (1H, s, NH); Anal. $C_7H_8N_4O$ (C, H, N).

*5.1.2. 2-Amino-6-methyl-5-[2′-(5′-phenyl-1′,3′,4′-oxadizolyl)sulfanyl]-3,4-dihydro-4-oxo-7H-pyrrolo [2,3-d] pyrimidine **6a***

To a solution of **5** (0.164 g, mmol) in a mixture of ethanol, water (3:2; 40 ml) was added 2-mercapto-5-phenyl-1,3,4-oxadiazole (0.356 g, 2 mmol) and the reaction miture was heated to 90 °C for 30 min. Then I_2 (0.500 g, 2 mmol) was added and the heating continued with stirring until starting material disappeared as monitored by TLC.. To this mixture was added excess sodium thiosulfate and filtered. The residue was washed well with water and air dried. The crude solid obtained was purified through column chromatography on silicagel using 1–8% methanol in dichloromethane. Fractions containing the desired spot were pooled and evaporated to dryness to give **6a** as a pale yellow solid (32%); mp 200–204 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.26 (3H, s, CH_3), 6.17 (2H, s, NH_2), 7.54 (3H, m, H ar), 7.83 (2H, d, H ar), 10.32 (1H, s, NH), 11.57 (1H, s, NH); Anal. $C_{15}H_{12}N_6O_2S$ (C, H, N).

*5.1.3. 2-Amino-6-methyl-5-[2′-(benzimidazolyl)sulfanyl] 3,4-dihydro-4-oxo-7H-pyrrolo [2,3-d] pyrimidine **6b***

Compound **6b** (synthesized as described for **6a**); yield 53%; pale white solid; mp 280–284 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.22 (3H, s, CH_3), 6.15 (2H, s, NH_2), 7.05–7.41 (4H, m, H ar), 10.33

Table 1
Cytotoxicity IC50 values in μM for the tested compounds against HCT116 Cancer cell lines.

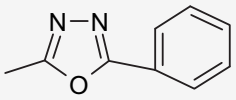
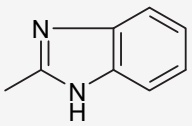
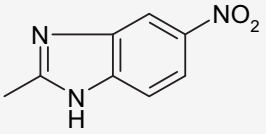
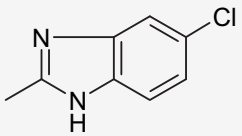
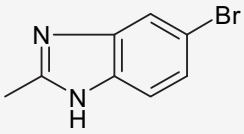
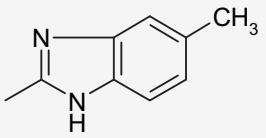
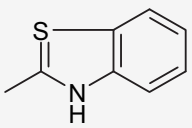
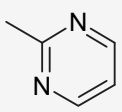
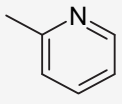
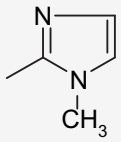
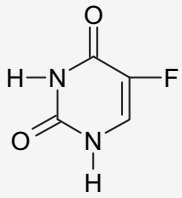
Compound	Ar	HCT 116 (colon)
6a		88.00
6b		38.04
6c		17.61
6d		36.23
6e		45.27
6f		34.56
6g		26.27
6h		17.60
6i		50.00

Table 1 (*continued*)

Compound	Ar	HCT 116 (colon)
6j		48.90
5-Fluorouracil		3.03

(1H, s, NH), 11.50 (1H, s, NH), 11.77 (1H, s, NH); MS (ESI) *m*/*z* 313 $[M + 1]^+$; Anal. $C_{14}H_{12}N_6OS$ (C, H, N).

5.1.4. 2-Amino-6-methyl-5-[2′-(5′-nitrobenzimidazolyl)sulfanyl]-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d] pyrimidine ***6c***

Compound **6c** (synthesized as described for **6a**); yield 52%; yellow solid; mp 280–284 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.23 (3H, s, CH_3), 6.18 (2H, s, NH_2), 7.50 (1H, d, H ar), 8.03 (1H, m, H ar), 8.20 (1H, d, H ar), 10.50 (1H, s, NH), 11.60 (1H, s, NH), 11.61 (1H, s, NH); Anal. $C_{14}H_{11}N_7OS$ (C, H, N).

5.1.5. 2-Amino-6-methyl-5-[2′-(5′-chlorobenzimidazolyl)sulfanyl]-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d] pyrimidine ***6d***

Compound **6d** (synthesized as described for **6a**); yield 56%; off white solid; mp 260–264 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.22 (3H, s, CH_3), 6.16 (2H, s, NH_2), 7.05–7.45 (3H, m, H ar), 10.31 (1H, s, NH), 11.50 (1H, s, NH), 11.80 (1H, s, NH); Anal. $C_{14}H_{11}N_6OSCl$ (C, H, N).

5.1.6. 2-Amino-6-methyl-5-[2′-(5′-bromobenzimidazolyl)sulfanyl]-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d] pyrimidine ***6e***

Compound **6e** (synthesized as described for **6a**); yield 57%; light brown solid; mp 270–274 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.22 (3H, s, CH_3), 6.16 (2H, s, NH_2), 7.19–7.59 (3H, m, H ar), 10.32 (1H, s, NH), 11.54 (1H, s, NH), 11.86 (1H, s, NH); Anal. $C_{14}H_{11}N_6OSBr$ (C, H, N).

5.1.7. 2-amino-6-methyl-5-[2′-(5′-methylbenzimidazolyl)sulfanyl]-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidine ***6f***

Compound **6f** (synthesized as described for **6a**); yield 57%; off white solid; mp 260–264 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.22 (3H, s, CH_3), 2.33 (3H, s, CH_3), 6.20 (2H, s, NH_2), 6.84–7.31 (3H, m, H ar), 10.34 (1H, s, NH), 11.50 (1H, s, NH), 11.70 (1H, s, NH); Anal. $C_{15}H_{14}N_6OS$ (C, H, N).

5.1.8. 2-Amino-6-methyl-5-[2′-(benzothiazolyl)sulfanyl]-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d] pyrimidine ***6g***

Compound **6g** (synthesized as described for **6a**); yield 67%; off white solid; mp 240–246 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.25 (3H, s, CH_3), 6.19 (2H, s, NH_2), 7.25 (1H, t, H ar), 7.39 (1H, t, H ar), 7.76 (1H, d, H ar) 7.82 (1H, d, H ar), 10.36 (1H, s, NH), 11.70 (1H, s, NH); MS (ESI) *m*/*z* 330.2 $(M + 1)^+$; Anal. $C_{14}H_{11}N_5OS_2$ (C, H, N).

5.1.9. 2-Amino-6-methyl-5-[(2′-pyrimidinyl)sulfanyl]-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d] pyrimidine ***6h***

Compound **6h** (synthesized as described for **6a**); yield 37%; paleyellow solid; mp 240–246 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.13 (3H, s, CH_3), 6.08 (2H, s, NH_2), 7.11 (1H, t, H ar), 8.50 (2H, d, H ar), 10.20 (1H, bs, NH), 11.30 (1H, s, NH); MS (ESI) *m*/*z* 275.2 $[M + 1]^+$; Anal. $C_{11}H_{10}N_6OS$ (C, H, N).

5.1.10. 2-Amino-6-methyl-5-[2′-(pyridinyl)sulfanyl]-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-i] pyrimidine ***6i***

Compound **6i** (synthesized as described for **6a**); yield 48%; yellow solid; mp 250–256 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.16 (3H, s, CH_3), 6.12 (2H, s, NH_2), 6.67–8.30 (4H, m, H ar), 10.24 (1H, s, NH), 11.45 (1H, s, NH); Anal. $C_{12}H_{11}N_5OS$ (C, H, N).

5.1.11. 2-Amino-6-methyl-5-[2′-(1′-methylimidazolyl)sulfanyl]-3,4-dihydro-4-oxo-7H-pyrrolo-[2,3-d] pyrimidine ***6j***

Compound **6j** (synthesized as described for **6a**); yield 51%; white solid; mp 220–226 °C dec. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.23 (3H, s, CH_3), 3.73 (3H, s, CH_3) 6.07 (2H, s, NH_2), 6.80 (1H, s, H ar), 7.12 (1H, s, H ar), 10.16 (1H, s, NH), 11.23 (1H, s, NH); Anal. $C_{11}H_{12}N_6OS$ (C, H, N).

5.2. Evaluation of cell cytotoxicity

5.2.1. Cell culture

Human colon cancer HCT116 cells, purchased from American Type Culture Collection (Manassas, VA) were used and maintained in McCoy's 5A medium containing L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/mL streptomycin at 37 °C and 5% CO_2 atmosphere and sub-cultured after trypsinization (0.5% trypsin/2.6 mM EDTA). For all experiments, cells were seeded at 1×10^6 cells in culture dishes (100 mm) and grown to 60–70% confluence. To study the growth arrest and apoptosis, various doses of test compounds ranging from 2.5 μM to 100 μM were used.

5.2.2. Cytotoxicity assay

Trypan blue exclusion assay has the ability to stain cells with damaged plasma membrane blue. Hence blue stained cells are considered as died and unstained cells are viable [23]. HCT116 cells (10,000 cells) are layered in a 6 well plate for 24 h, treated with various concentrations (2.5–100 μM) of test compounds (**6a**–**j**) and then incubated at 37 °C. Viability assay is done for HCT116 cells after 24 h of treatment with test compounds. The floating and adherent cells were collected, suspended in 25 μL of phosphate buffered saline (PBS) and mixed with 0.4% trypan blue stain. After a few minutes, stained and unstained cells were counted on a haemocytometer by light microscopy. Values are expressed as % of viable cells.

5.2.3. Detection of apoptosis

Apoptosis in cells induced by test compounds is detected by their ability to get stained with fluorescent dyes, acridine orange and ethidium bromide. Apoptotic cells that have lost their membrane integrity appear orange due to costain with ethidium bromide showing condensed chromatin. Alive cells stain uniformly green. HCT 116 cells were exposed to **6c**, **6d**, **6f** and **6g** (2.5–100 μM) for 48 h 25 μL of the cell suspension ($\sim 5 \times 10^6$ per mL) was added with 1 μL of acridine orange/ethidium bromide (one part each of 100 μg/mL acridine orange and 100 μg/mL ethidium bromide in PBS) just prior to microscopy. A 10 μL aliquot of the gently mixed suspension was placed on microscope slides, covered with glass slips and examined under an Olympus AX71 microscope (Tokyo, Japan) connected to a digital imaging system with SPOT RT software version 3.0. Cells were scored into following categories: C1, cells with large, green, noncondensed nuclei as non-apoptotic, viable cells; C2, cells with red/orange nuclei that showed signs of nuclear bead formation as apoptotic cells and C3, cells with large red nuclei that did not show signs of nuclear condensation or bead formation

as necrotic cells. At least 200 cells/sample were counted and scored. The apoptotic index (percent) was calculated by dividing the sum of apoptotic cells C2 × 100 by the total number of cells scored.

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