Full Paper

Synthesis and Biological Evaluation of Some Novel Fused Pyrazolopyrimidines as Potential Anticancer and Antimicrobial Agents*

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Synthesis and evaluation of anticancer and antimicrobial activity of some novel pyrazolopyrimidines and fused pyrazolopyrimidines are reported. Twelve analogs were selected to be evaluated for their *in vitro* anticancer potential against a panel of three human tumor cell lines: hepatocellular carcinoma HepG2, cervical carcinoma HelaS3 and colon carcinoma CaCo. The obtained data revealed that eight compounds namely; **6b, 6d**, **7c**, **8c**, **10b**, **12b**, **13a** and **13b** were able to exhibit variable degrees of anticancer activities against the three used cell lines, of which compound **6d** proved to be the most active. On the other hand, all the newly synthesized compounds were subjected to *in vitro* antibacterial and antifungal screening. Almost all the tested compounds were found to possess variable degrees of antimicrobial activities. Collectively, compounds **7c**, **8c**, **12b**, **13a** and **13b** could be considered as possible agents with dual anticancer and antimicrobial activities.

Keywords: Antibacterial / Anticancer Activity / Antifungal / Pyrazolopyrimidines / Synthesis

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Introduction

Intervention with cell cycle is an attractive strategy for combating microbes as well as diseases associated with abnormal cellular proliferation like cancer [1]. DNA is one of the promising targets in this field. A planar or semi-planar pharmacophore with a polyaromatic ring, capable of intercalation into DNA, is the common characteristic feature of DNA-intercalating anticancer drugs. Many of these intercalators, allocated in literature have tricyclic ring system [2–7]. In addition, protein kinases are involved in regulation of all cell functions. Uncontrolled activation of many of these kinases has been shown to result in uncontrolled cell growth. Based on literature reports, fused tricyclic core systems have been used as scaffold for kinase inhibitors [8–11].

The chemistry of pyrazolopyrimidines has drawn great attention due to their pharmacological importance and structural resemblance to purines. Pyrazolopyrimidine core has been used as scaffold for the design of antitumor agents [12–14], antimicrobial agents [15, 16] and inhibitors of kinases [17–20]. In addition, several pyrazolopyrimidines have proved to elicit inhibitory activity on the growth of several human tumor cell lines besides being active against cyclin dependent kinases (CDKs) [21–23], Moreover, purine derivatives such as olomoucine and roscovitine; structurally related to pyrazolopyrimidines, were found to exhibit moderate activity but good selectivity toward several CDKs [24]. Furthermore, some pyrazolopyrimidines proved to possess potent inhibitory activity against other enzymes that contribute to the cell cycle such as glycogen synthase kinase-3 [25, 26] and B-Raf kinase [27, 28].

Patients with neoplastic disorders that are subjected to chemotherapeutic treatment are highly susceptible to

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microbial infections due to subsequent lack of immunity. Co-administration of multiple drugs for treating patients suffering from cancer disease accompanied with microbial infections might inflect some added health problems especially in patients with impaired liver and/or kidney functions. Therefore, the concept of monotherapy by a single drug which possesses dual utility might be advantageous from both therapeutic and cost-effective stand points.

Encouraged by the afore-mentioned findings and in a continuation of an ongoing program aiming at finding new structural leads with potential chemotherapeutic activities [29–32], it was rationalized to synthesize some pyrazolopyrimidines and their annulated tricyclic analogs that would produce dual anticancer and antimicrobial activities. The proposed candidates were supported with a variety of pharmacophoric groups which would impart various electronic and lipophilic properties. The fact that some substituted hydrazines were found to possess antineoplastic activity [33] prompted the synthesis of compounds bearing hydrazine, acetohydrazide or amino functionality hoping to enhance their anticipated biological activities. Synthesis of compounds with an amide group was rationalized on the fact that many antitumor antibiotics such as bleomycin, and pyrazofurin incorporate in their structures amidic group [34]. Moreover, various chemotherapeutic activities associated with many hydrazones [35–37] motivated synthesis of new ones and investigation of their antitumor as well as antimicrobial activities before cyclization into their tricyclic analogs. Furthermore, owing to the contribution of various triazoles to the potential antineoplastic activities [38–41], it became of interest to incorporate triazole ring into a series of pyrazolopyrimidines with the hope of improving their biological impact. In addition, the fact that various imidazoles [42–44] and pyrimidine ring systems [45–47] were found to exhibit various chemotherapeutic activities spurred to establish tricyclics having imidazole or pyrimidine ring system engaged to corner stone-pyrazolopyrimidine nucleus aiming to shed light on the activity of such type of compounds.

Results and discussions

Synthesis of the intermediate and target compounds was accomplished according to the steps illustrated in schemes 1 and 2. Compounds **1a,b**, 5-amino-1-aryl-*1H*-pyrazole-4-carbonitriles [21] were reacted with triethyl orthoformate/acetic anhydride to give the corresponding ethoxymethyleneamino derivatives; **2a**,**b**. The latter compounds were either treated with hydrazine hydrate in ethanol under reflux to afford the iminoamines **3a,b** [48] or stirred with hydrazine hydrate in benzene at room temperature to give hydrazine derivatives **4a,b** [21, 49]. The 3-methyltriazolo derivatives **5a,b** were obtained by boiling **4a,b** in acetic anhydride. Furthermore, 3-arylamino derivatives **6a-d** were obtained when **4a,b** were treated with aryl isothiocyanates in refluxing dioxane/ethanol. The reaction proceeded via thiosemicarbazide intermediate with concomitant dehydrosulfurization. Condensing **4a,b** with aromatic aldehydes furnished the hydrazones **7a–d**. The latter compounds could be cyclized into **8a–d** using bromine and sodium acetate in glacial acetic acid (Scheme 1).

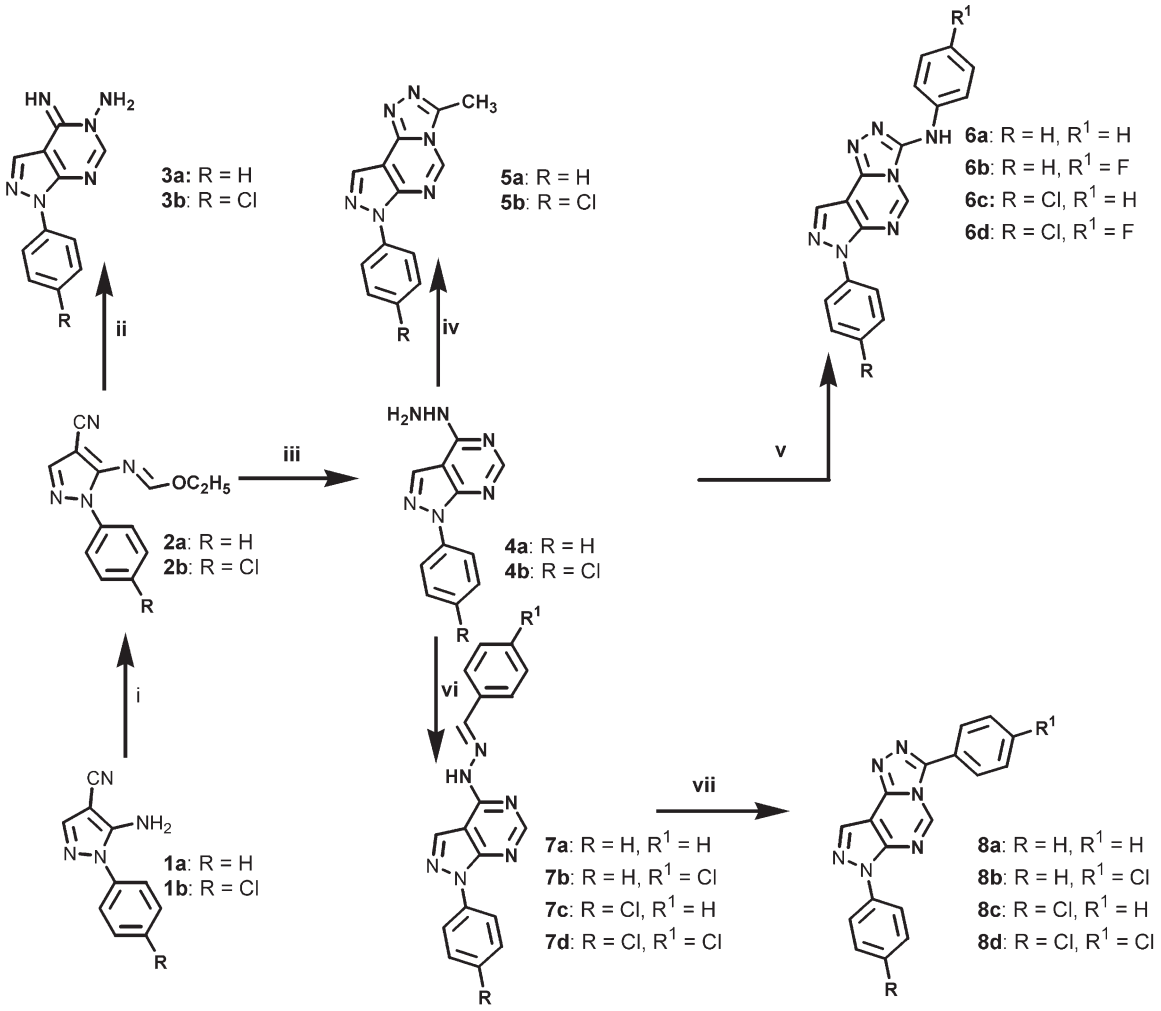
In Scheme 2, the amine derivatives **9a,b** [21] were obtained upon refluxing **1a,b** in formamide. Cyclizing **9a** with different phenacyl bromides afforded imidazopyrazolopyrimidine derivatives **10a–c**. Fusion of **9a,b** with diethyl ethoxymethylenemalonate at 120–130°C afforded the diesters **11a,b** which were cyclized upon heating in diphenyl ether affording the ketoesters **12a,b**, which in their turn were refluxed with hydrazine hydrate in ethanol to yield the aminoesters **13a,b** instead of the expected acid hydrazides. Investigation of ^{1}H-NMR spectrum of **13b** revealed two deuterium exchangeable signals due to NH and NH_2 moieties in addition to a triplet and a quartet corresponding to ethyl ester fragment. Other protons were located at their expected chemical shifts. In addition, its ^{13}C-NMR, HMQC, HMBC and mass spectral data were found to agree with the postulated rather than the hydrazide structure. Direct heating of **12a,b** with benzylamine funished the amides **14a,b**. Mass spectrum of compound **14a** was in favor of the unexpected structure as it showed its molecular ion peak at 386. ^{1}H-NMR spectrum of **14a** revealed three deuterium exchangeable signals due to two NH and NH_2. On the other hand, heating of the amine derivatives **9a,b** with ethyl ethoxymethylenecyanoacetate at 120–130°C afforded the corresponding cyanoesters **15a,b.** Trails to cyclize the latter compounds by refluxing in glacial acetic acid went in vain and instead, iminoesters **16a,b** were obtained. ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC spectral data of **16b** agreed with the postulated rather than the expected pyrazolopyrimidopyrimidine structure. In addition, mass spectra of compounds **16a,b** were in favor of the unexpected structure as they showed their molecular ion peaks at 324 (**16a**) and 360 and 358 (**16b**).

Preliminary *in-vitro* Anticancer Screening

In-vitro MTT cytotoxicity assay

Out of the newly synthesized compounds, twelve analogs namely; **3b**, **4b**, **5b**, **6b**, **6d**, **7c**, **8c**, **10b**, **12b**, **13a**, **13b** and **16b** were selected to be evaluated for their *in vitro* anticancer effect via the standard MTT method [50-52], against a panel of three human tumor cell lines namely; hepatocellular carcinoma HepG2, cervical carcinoma HelaS3 and colon carcinoma CaCo.

MTT assay is a standard colorimetric assay for measuring cell growth. It is used to determine cytotoxicity of potential

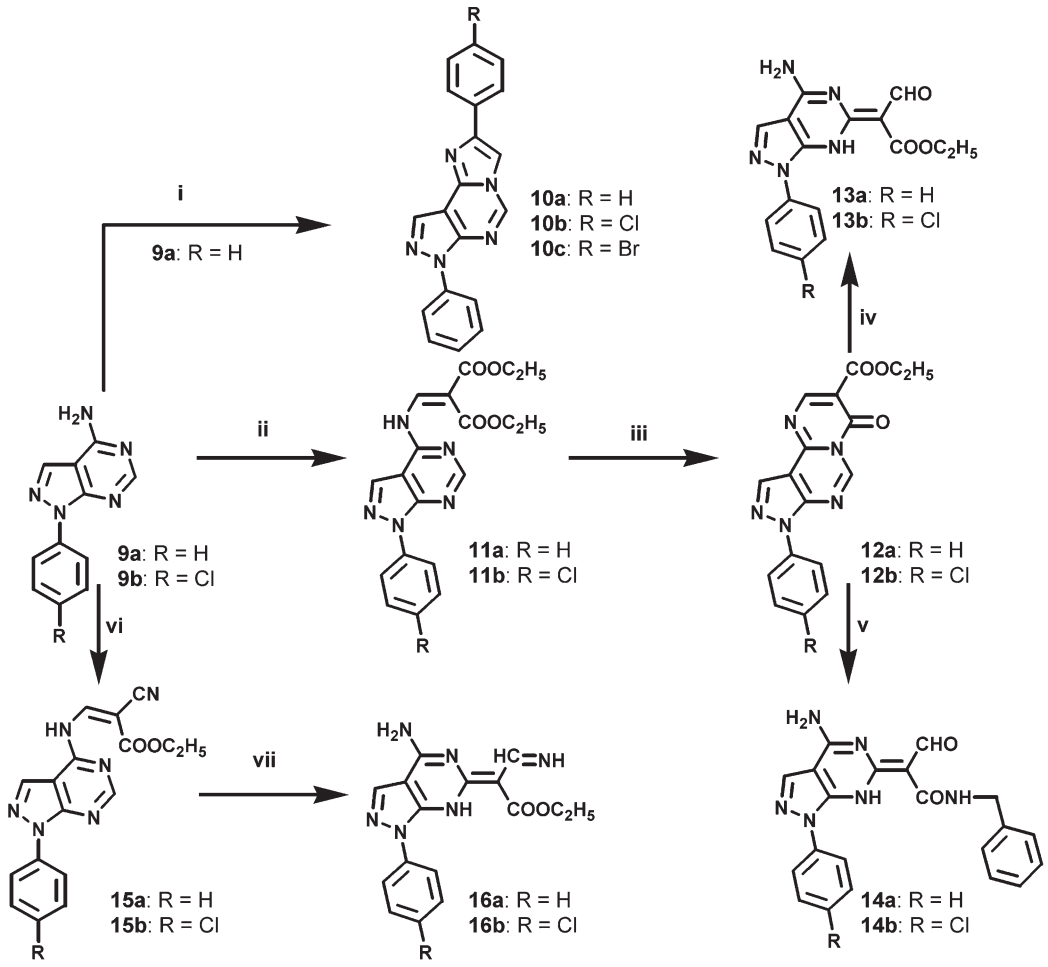


Reaction conditions: i: Ac_2O / $HC(OC_2H_5)_3$, reflux, **ii**: $NH_2NH_2.H_2O$ / EtOH, reflux, **iii**: $NH_2NH_2.H_2O$ / C_6H_6, R.T., **iv:** Ac_2O, reflux, **v:** $R^1C_6H_4NCS$ / dioxane / EtOH, reflux, **vi**: $R^1C_6H_4CHO$ / EtOH, reflux, **vii**: Br_2 / HOAc / NaOAc, R.T.

Scheme 1. Synthesis of intermediate and target compounds.

medicinal agents and other toxic materials. In brief, yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan by mitochondrial dehydrogenases of living cells. A suitable solvent is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength. When the amount of purple formazan produced by cells treated with an agent is compared with that produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve.

The obtained results revealed that eight of the tested compounds namely; **6b**, **6d**, **7c**, **8c**, **10b**, **12b**, **13a** and **13b** exhibited variable degrees of inhibitory activity towards the three tested human tumor cell lines. As for activity against hepatocellular carcinoma HepG2, the highest cytotoxic activity was displayed by compounds **6d** and **13b** which showed almost similar activity (% inhibition = 64.5 and 55.3, respectively in 48 h and % inhibition = 80.6 and 86.2 in 72 h, respectively). Remarkable inhibitory activity was also demonstrated by compounds **8c**, **10b** and **12b** in 72 h. The cervical carcinoma HelaS3 cell line showed highest sensitivity towards the tested compounds, as its growth was found to be inhibited by seven compounds. The best activity was demonstrated by compounds **6b**, **10b** and **13b** which were nearly equipotent (about 85% inhibition) in 72 h. The remaining compounds exhibited less inhibitory activity with %



Reaction conditions: i: 4-$RC_6H_4COCH_2Br$ / EtOH, reflux, **ii:** $C_5H_5OCH{=}C(COOC_2H_5)_2$, **iii:** diphenyl ether, reflux, **iv**: $NH_2NH_2.H_2O$ / EtOH, reflux, **v**: $C_6H_5CH_2NH_2$, **vi**: $C_2H_5OCH{=}C(CN)COOC_2H_5$, **vii**: HOAc, reflux.

Scheme 2. Synthesis of intermediate and target compounds.

Table 1. Percentage growth inhibitory effects (GI%) of the active compounds on some human tumor cell lines using the MTT assay.

Compound	Human hepatocellular carcinoma HepG2			Cervical carcinoma HelaS3			Colon carcinoma CaCo		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
6b	–[a]	–	–	45[b]	58.1	85.1	9.2	34.7	51.7
6d	26.4	64.5	80.6	30.2	41.2	65.3	30.3	52.4	90.97
7c	–	–	–	30.4	35.91	60	–	–	–
8c	18.2	37.2	74.9	50.4	53.2	58.2	–	–	–
10b	8.5	66.82	76.3	19.6	46.3	84.7	–	–	–
12b	0.5	31.5	69.6	11	28.3	54.8	–	–	–
13a	6.1	46.4	59.3	–	–	–	–	–	–
13b	30	55.3	86.2	35.8	42.1	85.4	25.4	53	54.5

[a] Not active
[b] Growth inhibitory activity (%)

inhibition range of 54.8–65.3. On the other hand, colon carcinoma CaCo was proved to be the least sensitive among the cell lines tested as it was affected by only three test compounds. However, an outstanding growth inhibition potential was shown by compound **6d** with % inhibition = 52.4 and 90.97 in 48 h and 72 h, respectively. The remaining two active compounds, namely **6b**, and **13b**, showed mild activity against the same cell line with % inhibition = 51.7 and 54.5 in 72 h, respectively.

Further interpretation of the results revealed that compounds **6d** and **13b** showed a considerable broad spectrum of anticancer activity against the three tested human tumor cell lines. In particular, compound **6d** proved to be the most active member in this study with special effectiveness against human colon carcinoma CaCo and hepatocellular carcinoma HepG2 cell lines. Whereas, compound **13b** was found to possess high activity against hepatocellular carcinoma HepG2 and cervical carcinoma HelaS3 cell lines.

In-vitro antibacterial and antifungal activities

All the newly synthesized compounds were evaluated for their *in-vitro* antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* as Gram-positive bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* as Gram-negative bacteria. They were also evaluated for their *in-vitro* antifungal potential against *Candida albicans.* Their inhibition zones using the cup-diffusion technique [53] were measured and further evaluation was carried out to determine their minimum inhibitory concentration (MIC) using the twofold serial dilution method [54]. Ampicillin was used as standard antibacterial while clotrimazole was used as antifungal reference. Dimethylsulfoxide (DMSO) was used as blank and showed no antimicrobial activity.

Regarding the antibacterial activity, results revealed that 32 out of the tested 33 compounds displayed variable inhibitory effects on the growth of the tested Gram positive and Gram negative bacterial strains. Among the Gram positive bacteria tested, *B. subtilis* showed relative higher sensitivity towards the tested compounds than *S. aureus*. Compounds **3b**, **5a**, **6a**, **8b**, **11a**, **13b** and **15a** (MIC 25 μg/mL) showed 20% of the activity of ampicillin against *S. aureus*. With regard to the activity against *B. subtilis*, the best activity was displayed by compounds **3a**, **7a** and **8a** (MIC 12.5 μg/mL), i.e. equipotent to ampicillin. In addition, compounds **3b**, **4b**, **5a**, **8d**, **10b**, **12a**, **14b** and **15a** (MIC 25 μg/mL) showed 50% of the activity of ampicillin against *B. subtilis*. Whereas, compounds **5b**, **6a**, **7b**, **8b**, **13b**, **14a** and **16b** (MIC 50 μg/mL) showed 25% of the activity of ampicillin against the same organism. On the other hand, investigation of the antibacterial activity against Gram negative strains revealed that compound **7b** showed four times the activity of ampicillin against *P. aeruginosa* whereas, compounds **3a**, **7a**, **7c**, **10a**, **10c**, **12b** and **15a** exhibited two times the potency of the reference standard against the same organism. Meanwhile, compounds **5b**, **6c**, **7d**, **8c**, **11b**, **13a**, **13b** and **14b** (MIC 50 μg/mL) were found to be equipotent to ampicillin against the same organism. On the other hand, compounds **3a**, **7b** and **14a** (MIC 12.5 μg/mL) were found to be nearly equipotent to ampicillin (MIC 10 μg/mL) against *E. coli*. Whereas, compounds **4b**, **5a**, **6c**, **7c** and **14b** demonstrated moderate inhibitory activity (MIC 25 μg/mL) against the same organism. Concerning the antifungal activity of the tested compounds against *C. albicans*, compounds **4a**, **13a** and **15a** (MIC 12.5 μg/mL) showed 40% the activity of clotrimazole (MIC 5 μg/mL). Most of the remaining compounds revealed moderate inhibitory activity (MIC 25–50 μg/mL) against *C. albicans*.

A close examination of the structures of the active compounds presented in Table 2 revealed that the iminoamine **3a** displayed a broad spectrum of antimicrobial activity against Gram positive and Gram negative bacteria, it was found to be equipotent to ampicillin against *B. subtilis*, and nearly equipotent to the standard against *E. coli*. Meanwhile, it showed two times the activity of the reference against *P. aeruginosa*. On the other hand, its chlorophenyl analog **3b** was found to be less potent except against *S. aureus*. The hydrazine analogs **4a,b** exerted less antibacterial activities but more antifungal activity. Cyclization of the former into methylpyrazolotriazolopyrimidines **5a,b** generally was found to enhance the antibacterial activity and decrease the antifungal potency. Aminopyrazolotriazolopyrimidines **6a–d** were found to possess moderate antimicrobial potential. Generally, a broad spectrum of antimicrobial activity was demonstrated by hydrazones **7a–d** in which compounds **7a,b** were found to be the most potent. Compound **7a** was found to be as potent as ampicillin against *B. subtilis.* Meanwhile, it exerted two times the activity of the reference against *P. aeruginosa*. On the other hand, compound **7b** demonstrated four times the potency of the reference against *P. aeruginosa* whereas, it was found to be nearly as potent as ampicillin against *E. coli*. It could be recognized that generally a decreased antibacterial activity was encountered with compounds **8a-d** than their precursor hydrazones **7a–d**. Among which, **8a** was found to be as potent as the standard against *B. subtilis*, **8b** demonstrated 20% the activity of the reference against *S. aureus*, whereas, **8c** exhibited equal potency to ampicillin against *P. aeruginosa*.

Concerning the antimicrobial activity of the imidazopyrazolopyrimidine series (Scheme 2), the phenyl derivative **10a** and its 4-bromophenyl analog **10c** were found to possess almost similar antimicrobial potency and spectrum. They were found to be twice as potent as ampicillin against *P. aeruginosa*, whereas, compound **10a** (MIC 50 μg/mL) exerted higher antifungal activity against *C. albicans* than **10c**. On the other hand, compound **10b** (R = 4–Cl) demonstrated decreased activity

Table 2. Minimum inhibitory concentrations (MIC) of the tested compounds in μg/mL.

Compound	*S. aureus*	*B. subtilis*	*P. aeruginosa*	*E. coli*	*C. albicans*
3a	50	12.5	25	12.5	100
3b	25	25	100	100	25
4a	100	100	100	100	12.5
4b	100	25	100	25	25
5a	25	25	100	25	100
5b	100	50	50	100	50
6a	25	50	100	50	50
6b	50	100	100	50	25
6c	100	100	50	25	100
6d	50	100	100	100	100
7a	50	12.5	25	50	25
7b	100	50	12.5	12.5	50
7c	100	100	25	25	25
7d	100	100	50	100	50
8a	100	12.5	100	50	50
8b	25	50	100	100	100
8c	100	100	50	50	100
8d	100	25	100	50	50
10a	50	100	25	50	50
10b	100	25	100	100	25
10c	50	100	25	50	100
11a	25	100	100	100	50
11b	50	100	50	100	25
12a	50	25	100	50	50
12b	50	100	25	50	100
13a	100	100	50	50	12.5
13b	25	50	50	50	50
14a	100	50	100	12.5	100
14b	100	25	50	25	100
15a	25	25	25	50	12.5
15b	100	100	100	100	50
16a	100	100	100	100	100
16b	100	50	100	100	50
A[a]	5	12.5	50	10	–
C[b]	–[c]	–	–	–	5

[a] A: Ampicillin trihydrate (standard broad spectrum antibiotic).
[b] C: Clotrimazole (standard broad spectrum antifungal agent).
[c] (–): Totally inactive (MIC ≥ 200 μg/mL).

against *S. aureus, P. aeruginosa* and *E. coli*. Whereas, its activity against *B. subtilis* was enhanced four times (MIC 25 μg/mL). Meanwhile, its antifungal activity was also enhanced.

Regarding the activity of the diesters **11a,b**, moderate antimicrobial activity was displayed by compound **11a** (MIC 25–100 μg/mL), whereas, **11b** was found to be equipotent to the standard against *P. aeruginosa.*

The ketoester **12a** was found to possess 50% the potency of ampicillin against *B. subtilis* whereas, its chlorophenyl analog **12b** was found to be two times as potent as ampicillin against *P. aeruginosa*. Although, the ester **13a** was found to possess weak antimicrobial activity (MIC 100 μg/mL) against the tested Gram-positive bacteria, its chlorophenyl analog **13b** was found to elicit moderate inhibitory activity against Gram-positive bacteria (MIC 25–50 μg/mL). Meanwhile, both **13a,b** were found to be equipotent to ampicillin against *P. aeruginosa*. The amides **14a,b** varies in their antimicrobial potential, of which **14b** was found to be equipotent to ampicillin against *P. aeruginosa* whereas compound **14a** (MIC 12.5 μg/mL) was found to be nearly equipotent to the standard (MIC 10 μg/mL) against *E. coli*. The cyanoester **15a** was found to possess antimicrobial activity against Gram-positive and Gram-negative strains (MIC 25–50 μg/mL), besides displaying appreciable antifungal activity (MIC 12.5 μg/mL). Introduction of a chlorine atom in **15b** (R = 4–Cl) resulted in dramatic reduction in the antibacterial activity. On the other hand, replacing the aldehydic group in **13a,b** by an imino moiety (compounds **16a,b**) resulted in a noticeable decrease in their antimicrobial activity.

Collectively, compounds **3a, 7a, 13b** and **15a** are considered to be the most active antimicrobial members identified in this study with a broad spectrum of antibacterial activity against both Gram positive and Gram negative bacteria.

Conclusion

The objective of the present study was to synthesize and investigate the anticancer and antimicrobial activities of some pyrazolopyrimidines as well as their annulated tricyclic analogs with the hope of discovering new structure leads serving as dual anticancer-antimicrobial agents. The results of the anticancer screening revealed that eight compounds were found to exhibit variable degrees of anticancer activities against the three used cell lines. Compounds **6d** and **13b** showed a considerable broad spectrum of anticancer activity against the three tested human tumor cell lines. In particular, compound **6d** proved to be the most active member in this study with special effectiveness against the human colon carcinoma CaCo and hepatocellular carcinoma HepG2 cell lines. Compound **13b** was found to possess high activity against hepatocellular carcinoma HepG2 and cervical carcinoma HelaS3 cell lines.

On the other hand, it has been found that 32 out of the 33 tested compounds displayed variable *in vitro* antibacterial and antifungal inhibitory effects. Compounds **3a, 7a, 13b** and **15a** could be considered as the most active broad spectrum antimicrobial members identified in this study. Collectively, the anticancer and antimicrobial results would suggest that compounds **7c, 8c, 12b, 13a** and **13b** could be considered as possible dual antimicrobial-anticancer agents.

Chemistry

Melting points were determined in open glass capillaries on a Stuart melting point apparatus and were uncorrected. The infrared (IR) spectra were recorded on Perkin-Elmer 1430 infrared spectrophotometer using the KBr plate technique. ^{1}H-NMR, ^{13}C-NMR, HMQC and HMBC spectra were determined on Jeol spectrometer (500 MHz) at the Microanalytical unit, Faculty of Science, Alexandria University and on a Varian spectrometer (300 MHz), Faculty of Science, Cairo University 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; t: triplet; m: multiplet. Mass spectra were carried out using a Schimadzu GCMS-QP-1000EX mass spectrometer at 70 eV, Faculty of Science, Cairo University. Microanalyses were performed at the Microanalytical Unit, Faculty of Science, Cairo University and at the Central lab, Faculty of Pharmacy, Alexandria University, Egypt. The found values were within ±0.4% of the theoretical values. Follow up of the reactions and checking the homogeneity of the compounds were made by TLC on silica gel-protected glass plates and the spots were detected by exposure to UV-lamp at λ 254.

1-Aryl-4-imino-1,4-dihydropyrazolo[3,4-d]pyrimidin-5-ylamines ***3a,b***

To a solution of **2a,b** (0.001 mol) in EtOH (4 mL), hydrazine hydrate (0.5 g, 0.49 mL, 0.01 mol) was added. The reaction mixture was heated under reflux for 1 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH, dried and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 3342-3337, 3206-3201 (NH); 1660-1657 (C=N). ^{1}H-NMR (δ ppm) for **3a**: 4.76 (s, 1H, NH, D_2O exchangeable); 4.93 (s, 2H, NH_2, D_2O exchangeable); 7.33 (t, J = 7.5 Hz, 1H, phenyl-C_4-H); 7.52 (t, J = 7.5 Hz, 2H, phenyl-$C_{3,5}$-H); 8.21 (d, J = 7.5 Hz, 2H, phenyl-$C_{2,6}$-H); 8.58 (s, 1H, pyrazolopyrimidine-C_3-H); 9.29 (s, 1H, pyrazolopyrimidine-C_6-H). ^{1}H-NMR (δ ppm) for **3b**: 4.70 (s, 1H, NH, D_2O exchangeable); 4.92 (s, 2H, NH_2, D_2O exchangeable); 7.52, 7.93 (two d, J = 8.4 Hz, each 2H, chlorophenyl-$C_{2,6}$-H and $C_{3,5}$-H); 8.55 (s, 1H, pyrazolopyrimidine-C_3-H); 9.30 (s, 1H, pyrazolopyrimidine-C_6-H).

[1-Aryl-1H-pyrazolo[3,4-d]pyrimidin-4-yl]hydrazines ***4a,b***

To a solution of **2a,b** (0.001 mol) in benzene (3 mL), a solution of hydrazine hydrate (0.3 g, 0.29 mL, 0.006 mol) in H_2O (2 mL) was added. The reaction mixture was stirred at R.T. for 1 h. The obtained precipitate was filtered, washed with H_2O, dried and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 3336–3302, 3156–3150 (NH); 1656–1652 (C=N). ^{1}H-NMR (δ ppm) for **4a**: 5.60 (s, 2H, NH_2, D_2O exchangeable); 7.33 (t, J = 8.4 Hz, 1H, phenyl-C_4-H); 7.49 (t, J = 8.4 Hz, 2H, phenyl-$C_{3,5}$-H); 7.97 (d, J = 8.4 Hz, 2H, phenyl-$C_{2,6}$-H); 8.12 (s, 1H, pyrazolopyrimidine-C_3-H); 8.24 (s, 1H, pyrazolopyrimidine-C_6-H). ^{1}H-NMR (δ ppm) for **4b**: 5.60 (s, 2H, NH_2, D_2O exchangeable); 7.52, 7.93 (two d, J = 8.4 Hz, each 2H, chlorophenyl-$C_{2,6}$-H and $C_{3,5}$-H); 8.07 (s, 1H, pyrazolopyrimidine-C_3-H); 8.21 (s, 1H, pyrazolopyrimidine-C_6-H).

7-Aryl-3-methyl-7H-pyrazolo[4,3-e][1,2,4]triazolo-[4,3-c]pyrimidines ***5a,b***

A suspension of **4a,b** (0.001 mol) in HOAc or Ac_2O (2 mL) was heated under reflux for 1 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH, dried and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 1658-1652 (C=N). ^{1}H-NMR (δ ppm) for **5a**: 2.52 (s, 3H, CH_3); 7.43 (t, J = 8.4 Hz,

Table 3. Physicochemical and analytical data of compounds **3–16**

Compd.	R	R^1	Yield (%)	M.p. (°C) cryst. solvent*	Mol. Formula (mol. wt.)**
3a	H	–	44	234–235[a] E	$C_{11}H_{10}N_6$ (226.24)
3b	Cl	–	72	257–259 E	$C_{11}H_9ClN_6$ (260.68)
4a	H	–	66	192–194[b,c] E	$C_{11}H_{10}N_6$ (226.24)
4b	Cl	–	73	221–223 E	$C_{11}H_9ClN_6$ (260.68)
5a	H	–	92	198–200 E	$C_{13}H_{10}N_6$ (250.26)
5b	Cl	–	93	240–242 E	$C_{13}H_9ClN_6$ (284.70)
6a	H	H	65	295–297 D/E	$C_{18}H_{13}N_7$ (327.34)
6b	H	F	63	296–298 D/E	$C_{18}H_{12}FN_7$ (345.33)
6c	Cl	H	70	286–288 D/E	$C_{18}H_{12}ClN_7$ (361.79)
6d	Cl	F	76	>300 D/E	$C_{18}H_{11}ClFN_7$ (379.78)
7a	H	H	49	284–286 D/E	$C_{18}H_{14}N_6$ (314.34)
7b	H	Cl	55	280–282 D/E	$C_{18}H_{13}ClN_6$ (348.79)
7c	Cl	H	70	299–301 D/E	$C_{18}H_{13}ClN_6$ (348.79)
7d	Cl	Cl	74	>300 D/E	$C_{18}H_{12}Cl_2N_6$ (383.23)
8a	H	H	92	222–224 D/E	$C_{18}H_{12}N_6$ (312.33)
8b	H	Cl	97	279–281 D/E	$C_{18}H_{11}ClN_6$ (346.77)
8c	Cl	H	93	262–264 D/E	$C_{18}H_{11}ClN_6$ (346.77)
8d	Cl	Cl	87	>300 D/E	$C_{18}H_{10}Cl_2N_6$ (381.22)
10a	H	–	44	238–240 D/E	$C_{19}H_{13}N_5$ (311.34)
10b	Cl	–	46	252–254 DMF/E	$C_{19}H_{12}ClN_5$ (345.78)
10c	Br	–	58	268–270 D	$C_{19}H_{12}BrN_5$ (390.24)
11a	H	–	49	110–112 E	$C_{19}H_{19}N_5O_4$ (381.39)
11b	Cl	–	70	162–164 D	$C_{19}H_{18}ClN_5O_4$ (415.83)
12a	H	–	90	225–226 D/E	$C_{17}H_{13}N_5O_3$ (335.32)
12b	Cl	–	93	290–292 D/E	$C_{17}H_{12}ClN_5O_3$ (369.76)
13a	H	–	72	288–290 D	$C_{16}H_{15}N_5O_3$ (325.32)
13b	Cl	–	87	>300 DMF/E	$C_{16}H_{14}ClN_5O_3$ (359.77)
14a	H	–	70	>300 DMF/E	$C_{21}H_{18}N_6O_2$ (386.41)
14b	Cl	–	79	>300 DMF/E	$C_{21}H_{17}ClN_6O_2$ (420.85)
15a	H	–	34	211–213 E	$C_{17}H_{14}N_6O_2$ (334.33)

continued

Table 3. (*continued*)

Compd.	R	R^1	Yield (%)	M.p. (°C) cryst. solvent*	Mol. Formula (mol. wt.)**
15b	Cl	–	72	217–219 D/E	$C_{17}H_{13}ClN_6O_2$ (368.78)
16a	H	–	81	208–210 E	$C_{16}H_{16}N_6O_2$ (324.34)
16b	Cl	–	97	255–257 E	$C_{16}H_{15}ClN_6O_2$ (358.78)

*Crystallization solvent (s): DMF (*N*,*N*-dimethylformamide), E: ethanol, D: 1,4-dioxane.
**Found values are within ±0.4% of the calculated values.
[a] Reported m.p. 235 [48]
[b] Reported m.p. 184–186 [21]
[c] Reported m.p. 192–192.5 [49]

1H, phenyl-C_4-H); 7.59 (t, J = 8.4 Hz, 2H, phenyl-$C_{3,5}$-H); 8.08 (d, J = 8.4 Hz, 2H, phenyl-$C_{2,6}$-H); 8.69 (s, 1H, pyrazolotriazolopyrimidine-C_9-H); 9.58 (s, 1H, pyrazolotriazolopyrimidine-C_5-H). ^{1}H-NMR (δ ppm) for **5b**: 2.53 (s, 3H, CH_3); 7.67, 8.16 (two d, J = 8.4 Hz, each 2H, chlorophenyl-$C_{2,6}$-H and $C_{3,5}$-H); 8.71 (s, 1H, pyrazolotriazolopyrimidine-C_9-H); 9.61 (s, 1H, pyrazolotriazolopyrimidine-C_5-H).

Aryl-(7-aryl-7H-pyrazolo[4,3-e][1,2,4]triazolo-[4,3-c]pyrimidin-3-yl)amines ***6a–d***

To a suspension of **4a,b** (0.001 mol) in dioxane/EtOH, 3/1 (4 mL), the appropriate isothiocyanate (0.001 mol) was added. The reaction mixture was heated under reflux for 12 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH, dried and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 3287–3282 (NH); 1658–1654 (C=N). ^{1}H-NMR (δ ppm) for **6a**: 6.92 (t, J = 8.4 Hz, 1H, NH-phenyl-C_4-H); 7.30 (t, J = 8.4 Hz, 2H, NH-phenyl-$C_{3,5}$-H); 7.41 (t, J = 8.4 Hz, 1H, phenyl-C_4-H); 7.58 (t, J = 8.4 Hz, 2H, phenyl-$C_{3,5}$-H); 7.70 (d, J = 8.4 Hz, 2H, NH-phenyl-$C_{2,6}$-H); 8.11 (d, J = 8.4 Hz, 2H, phenyl-$C_{2,6}$-H); 8.64 (s, 1H, pyrazolotriazolopyrimidine-C_9-H); 9.46 (s, 1H, pyrazolotriazolopyrimidine-C_5-H); 9.96 (s, 1H, NH, D_2O exchangeable). ^{1}H-NMR (δ ppm) for **6d**: 7.16 (d, J = 8.4 Hz, 2H, fluorophenyl-$C_{2,6}$-H); 7.64–7.71 (m, 4H, Ar-H); 8.19 (d, J = 8.4 Hz, 2H, chlorophenyl-$C_{3,5}$-H); 8.68 (s, 1H, pyrazolotriazolopyrimidine-C_9-H); 9.48 (s, 1H, pyrazolotriazolopyrimidine-C_5-H); 10.00 (s, 1H, NH, D_2O exchangeable).

N-(4-Substituted benzylidene)-N′-(1-aryl-1H-pyrazolo-[3,4-d]pyrimidin-4-yl)hydrazines ***7a–d***

To a suspension of **4a,b** (0.001 mol) in EtOH (3 mL), the appropriate aromatic aldehyde (0.001 mol) was added. The reaction mixture was heated under reflux for 12 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH, dried and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 3203–3183 (NH); 1599–1596 (C=N). ^{1}H-NMR (δ ppm) for **7b**: 7.58–7.66 (m, 3H, phenyl-$C_{3,4,5}$-H); 7.68, 8.12 (two d, J = 9.15 Hz, each 2H, chlorophenyl-$C_{2,6}$-H and $C_{3,5}$-H); 7.96 (d, J = 6.5 Hz, 2H, phenyl-$C_{2,6}$-H); 8.72 (s, 1H, =CH); 8.78 (s, 1H, pyrazolopyrimidine-C_3-H); 9.30 (s, 1H, pyrazolopyrimidine-C_6-H); 9.96 (s, 1H, NH, D_2O exchangeable). ^{1}H-NMR (δ ppm) for **7c**: 7.44–7.58 (m, 3H, phenyl-$C_{3,4,5}$-H); 7.64, 8.29 (two d, J = 9.15 Hz, each 2H, chlorophenyl-$C_{2,6}$-H and $C_{3,5}$-H); 7.83 (d, J = 6.5 Hz, 2H, phenyl-$C_{2,6}$-H); 8.32 (s, 1H, =CH); 8.51 (s, 1H, pyrazolopyrimidine-C_3-H); 8.66 (s, 1H, pyrazolopyrimidine-C_6-H); 12.27 (s, 1H, NH, D_2O exchangeable).

3,7-Diaryl-7H-pyrazolo[4,3-e][1,2,4]triazolo-[4,3-c]pyrimidines ***8a–d***

To a mixture of **7a–d** (0.001 mol) and anhydrous sodium acetate (0.25 g, 0.003 mol) in glacial HOAc (3 mL), Br_2 (0.1 mL, 0.002 mol) was added. The reaction mixture was stirred at r.t. over night then poured onto ice-cold H_2O. The obtained precipitate was filtered, washed with H_2O, dried and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 1649–1646 (C=N). ^{1}H-NMR (δ ppm) for **8c**: 7.58-7.66 (m, 3H, phenyl-$C_{3,4,5}$-H); 7.68, 8.12 (two d, J = 9.15 Hz, each 2H, chlorophenyl-$C_{2,6}$-H and $C_{3,5}$-H); 7.96 (d, J = 6.5 Hz, 2H, phenyl-$C_{2,6}$-H); 8.78 (s, 1H, pyrazolotriazolopyrimidine-C_9-H); 9.30 (s, 1H, pyrazolotriazolopyrimidine-C_5-H).

2-Aryl-7-phenyl-7H-imidazo[1,2-c]pyrazolo-[4,3-e]pyrimidines ***10a–c***

To a suspension of **9a** (0.21 g, 0.001 mol) in EtOH (3 mL), the appropriate phenacyl bromide (0.001 mol) was added. The reaction mixture was heated under reflux for 6 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH, dried and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 1639 (C=N). ^{1}H-NMR (δ ppm) for **10c**: 7.40 (t, J = 7.65 Hz, 1H, phenyl-C_4-H); 7.58 (t, J = 7.65 Hz, 2H, phenyl-$C_{3,5}$-H); 7.63 (d, J = 7.65 Hz, 2H, phenyl-$C_{2,6}$-H); 7.94, 8.09 (two d, J = 7.6 Hz, each 2H, bromophenyl-$C_{2,6}$-H and $C_{3,5}$-H); 8.55 (s, 1H, imidazopyrazolopyrimidine-C_3-H); 8.63 (s, 1H, imidazopyrazolopyrimidine-C_9-H); 9.31 (s, 1H, imidazopyrazolopyrimidine-C_5-H). MS *m/z* (relative abundance%) for **10c**: 392 [M^+ + 3] (18.6), 391

$[M^+ + 2]$ (73.7), 390 $[M^+ + 1]$ (100), 389 $[M^+]$ (78.5), 388 (95.9), 387 (17.5), 364 (8.7), 363 (9.7), 362 (10.3), 361 (7.5), 310 (6.7), 309 (6.8), 260 (6.4), 258 (5.2), 195 (5.1), 194 (7.7), 182 (6.2), 181 (10.8), 180 (14.9), 179 (16.2), 155 (10.9), 154 (15.6), 153 (10.0), 152 (10.9), 142 (6.9), 141 (10.0), 140 (8.3), 128 (8.1), 127 (15.1), 126 (17.3), 115 (10.6), 114 (8.1), 113 (7.4), 103 (7.2), 102 (14.2), 101 (16.7), 100 (13.4), 89 (17.8), 88 (23.5), 77 (76.8), 76 (86.3), 75 (28.1), 74 (18.1), 62 (14.2), 52 (8.2), 51 (52.8), 50 (61.8).

Diethyl 2-{[1-aryl-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino]methylene}malonates ***11a,b***

A mixture of **9a,b** (0.001 mol) and diethyl ethoxymethylenemalonate (0.22 g, 0.2 mL, 0.001 mol) was heated at 120–130 for 1 h. The reaction mixture was allowed to cool then triturated with ether. The obtained precipitate was filtered, washed with ether, dried and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 3285–3269, 3221–3211 (NH); 1728–1727 (C=O); 1662–1656, 1623–1617 (C=N); 1250, 1069, 1032 (C–O–C). ^{1}H-NMR (δ ppm) for **11a**: 1.22, 1.27 (two t, $J = 6.85$ Hz, each 3H, 2 × CH_2CH_3); 4.15, 4.25 (two q, $J = 6.85$ Hz, each 2H, 2 × CH_2CH_3); 7.35 (t, $J = 8.4$ Hz, 1H, phenyl-C_4-H); 7.53 (t, $J = 8.4$ Hz, 2H, phenyl-$C_{3,5}$-H); 8.12 (d, $J = 8.4$ Hz, 2H, phenyl-$C_{2,6}$-H); 8.60 (s, 1H, pyrazolopyrimidine-C_3-H); 8.72 (s, 1H, =CH); 8.96 (s, 1H, pyrazolopyrimidine-C_6-H); 11.04 (s, 1H, NH, D_2O exchangeable). MS *m/z* (relative abundance%) for **11b**: 417 $[M^+ + 2]$ (6.2), 416 $[M^+ + 1]$ (3.6), 415 $[M^+]$ (23.8), 370 (9.8), 369 (10.7), 345 (12.4), 344 (37.6), 343 (34.3), 342 (100), 341 (18.1), 326 (11.0), 324 (15.5), 316 (8.6), 315 (9.7), 314 (23.04), 299 (6.8), 298 (9.5), 297 (15.4), 272 (9.1), 271 (9.9), 270 (9.3), 269 (27.1), 268 (6.5), 256 (6.14), 255 (7.6), 241 (8.3), 231 (5.9), 230 (9.5), 229 (37.3), 228 (22.7), 201 (5.84), 194 (7.1), 176 (8.1), 175 (5.8), 162 (5.0), 128 (5.2), 111 (11.3).

Ethyl 8-aryl-4-oxopyrazolo[4,3-e]pyrimido-[1,2-c]pyrimidine-3-carboxylates ***12a,b***

A mixture of **11a,b** (0.002 mol) and diphenyl ether (3 mL) was heated under reflux for 1 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH, dried and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 3340, 3114 (NH); 1742–1741, 1692–1688 (C=O); 1625–1621 (C=N); 1032 (C–O–C). ^{1}H-NMR (δ ppm) for **12a**: 1.28 (t, $J = 6.85$ Hz, 3H, CH_2CH_3); 4.26 (q, $J = 6.85$ Hz, 2H, CH_2CH_3); 7.48 (t, $J = 7.65$ Hz, 1H, phenyl-C_4-H); 7.62 (t, $J = 7.65$ Hz, 2H, phenyl-$C_{3,5}$-H); 8.04 (d, $J = 7.65$ Hz, 2H, phenyl-$C_{2,6}$-H); 8.84 (s, 1H, pyrazolopyrimidopyrimidine-C_2-H); 8.87 (s, 1H, pyrazolopyrimidopyrimidine-C_{10}-H); 9.64 (s, 1H, pyrazolopyrimidopyrimidine-C_6-H). MS *m/z* (relative abundance%) for **12b**: 372 $[M^+ + 3]$ (8.9), 371 $[M^+ + 2]$ (45.3), 370 $[M^+ + 1]$ (15.4), 369 $[M^+]$ (100), 341 (12.7), 327 (5.8), 326 (20.2), 325 (24.5), 324 (92.6), 323 (13.9), 299 (34.5), 298 (25.4), 297 (91.6), 296 (11.6), 271 (21.6), 270 (6.8), 269 (40.6), 255 (16.8), 242 (5.0), 241 (5.3), 231 (18.2), 230 (18.7), 229 (46.6), 228 (27.2), 213 (5.1), 207 (5.2), 203 (11.0), 202 (27.7), 201 (19.5), 194 (24.7), 193 (6.5), 176 (6.4), 175 (15.5), 166 (5.6), 165 (6.2).

Ethyl 2-(4-amino-1-aryl-1,7-dihydropyrazolo-[3,4-d]pyrimidin-6-ylidene)-3-oxopropionoates ***13a,b***

To a suspension of **12a,b** (0.001 mol) in EtOH (3 mL), hydrazine hydrate (0.2 g, 0.19 mL, 0.004 mol) was added. The reaction mixture was heated under reflux for 1 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH, dried and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 3284–3276, 3105–3101 (NH); 1693–1688 (C=O); 1614-1612 (C=N); 1032 (C–O–C). ^{1}H-NMR (δ ppm) for **13b**: 1.22 (t, $J = 6.85$ Hz, 3H, CH_2CH_3); 4.17 (q, $J = 6.85$ Hz, 2H, CH_2CH_3); 7.27 (s, 2H, NH_2, D_2O exchangeable); 7.52–7.60 (m, 4H, chlorophenyl-H); 8.37 (s, 1H, pyrazolopyrimidine-C_3-H); 8.52 (s, 1H, CHO); 12.54 (s, 1H, NH, D_2O exchangeable). ^{13}C-NMR (δ ppm) for **13b**: 14.77 (CH_2CH_3), 60.44 (CH_2CH_3), 95.45 (C-3a), 110.79 (C=C), 126.20 (chlorophenyl-C-2,6), 130.03 (chlorophenyl-C-3,5), 132.62 (chlorophenyl-C-4), 136.93 (chlorophenyl-C-1), 140.01 (C-3), 150.35 (C-7a), 157.60 (C-4), 159.10 (C-6), 160.40 (C=O aldehyde), 164.15 (C=O ester). MS *m/z* (relative abundance%) for **13a**: 326 $[M^+ + 1]$ (20.6), 325 $[M^+]$ (100), 279 (99.9), 278 (37.8), 252 (9.2), 251 (40.1), 238 (14.5), 223 (7.2), 184 (10.9), 183 (16.0), 139 (8.4), 93 (7.1), 92 (19.9), 91 (6.0) 77 (28.1), 69 (11.6), 53 (8.5), 52 (6.3), 51 (7.1). MS *m/z* (relative abundance%) for **13b**: 361 $[M^+ + 2]$ (37.8), 360 $[M^+ + 1]$ (28.2), 359 $[M^+]$ (100), 358 (9.8), 315 (31.7), 314 (31.7), 313 (85.0), 312 (28.0), 287 (15.3), 286 (9.3), 285 (30.2), 272 (9.5), 271 (5.2), 257 (5.1), 218 (7.7), 217 (8.3), 156 (5.3), 127 (6.0), 126 (11.9), 125 (5.0), 113 (5.3), 111 (16.4), 75 (11.0), 69 (17.9), 68 (6.7), 67 (5.2), 53 (12.9), 52 (8.9).

2-(4-Amino-1-aryl-1,7-dihydropyrazolo[3,4-d]pyrimidin-6-ylidene)-N-benzyl-3-oxopropionamides ***14a,b***

A suspension of **12a,b** (0.001 mol) in benzylamine (0.43 g, 0.44 mL, 0.004 mol) was heated at 160–170 for 30 min. The reaction mixture was allowed to cool then triturated with 2 portions of ether (2 × 15 mL). The obtained precipitate was filtered, washed with ether, dried and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 3354–3353, 3300–3296, 3115–3108 (NH); 1681–1673 (C=O); 1614–1613 (C=N); 1032 (C–O–C). ^{1}H-NMR (δ ppm) for **14a**: 4.49 (d, $J = 6.15$ Hz, 2H, $CH_2C_6H_5$); 7.21–7.55 (m, 12H, Ar-H and NH_2); 8.38 (s, 1H, pyrazolopyrimidine-C_3-H); 8.67 (s, 1H, CHO); 9.41, 12.87 (two s, each 1H, 2NH, D_2O exchangeable). MS *m/z* (relative abundance%) for **14a**: 387 $[M^+ + 1]$ (2.1), 386 $[M^+]$ (6.0), 325 (6.8), 281 (5.3), 280 (13.4), 279 (9.0), 278 (5.2), 225 (6.0), 185 (11.0), 184 (7.9), 183 (9.2), 131

(5.8), 119 (6.2), 106 (100), 93 (7.3), 92 (11.6), 91 (25.4), 90 (7.5), 79 (10.3), 78 (5.9), 77 (28.8), 69 (8.1), 65 (6.4), 53 (5.6), 51 (5.0).

*Ethyl 3-(1-aryl-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino)-2-cyanoacrylates **15a,b***

A mixture of **9a,b** (0.001 mol) and ethyl ethoxymethylenecyanoacetate (0.17 g, 0.001 mol) was heated at 120–130 for 5 min. The reaction mixture was allowed to cool then triturated with ether. The obtained precipitate was filtered, washed with ether, dried and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 3278–3276, 3197–3196 (NH); 2229–2227 (C≡N); 1734–1732 (C=O); 1646–1642 (C=N); 1032 (C–O–C). ^{1}H-NMR (δ ppm) for **15a**: 1.25 (t, J = 6.9 Hz, 3H, CH_2CH_3); 4.23 (q, J = 6.9 Hz, 2H, CH_2CH_3); 7.38 (t, J = 7.65 Hz, 1H, phenyl-C_4-H); 7.56 (t, J = 7.65 Hz, 2H, phenyl-$C_{3,5}$-H); 8.14 (d, J = 7.65 Hz, 2H, phenyl-$C_{2,6}$-H); 8.80 (s, 1H, CH=); 8.86 (s, 1H, pyrazolopyrimidine-C_3-H); 9.24 (s, 1H, pyrazolopyrimidine-C_6-H); 12.21 (s, 1H, NH, D_2O exchangeable). ^{1}H-NMR (δ ppm) for **15b**: 1.31 (t, J = 6.9 Hz, 3H, CH_2CH_3); 4.29 (q, J = 6.9 Hz, 2H, CH_2CH_3); 7.57, 7.60 (two d, J = 9.15 Hz, each 2H, chlorophenyl-$C_{2,6}$-H and $C_{3,5}$-H); 8.82 (s, 1H, CH=); 8.88 (s, 1H, pyrazolopyrimidine-C_3-H); 9.25 (s, 1H, pyrazolopyrimidine-C_6-H); 12.15 (s, 1H, NH, D_2O exchangeable). MS m/z (relative abundance%) for **15b**: 370 [M^+ + 2] (16.8), 369 [M^+ + 1] (11.3), 368 [M^+] (39.5), 349 (5.2), 324 (9.5), 323 (10.9), 322 (17.0), 307 (15.7), 298 (7.9), 297 (30.9), 296 (32.3), 295 (100), 294 (6.5), 270 (8.8), 229 (16.7), 202 (5.3), 176 (6.5), 150 (5.7), 149 (33.9).

*Ethyl 2-(4-amino-1-aryl-1,7-dihydropyrazolo-[3,4-d]pyrimidin-6-ylidene)-3-iminopropionoates **16a,b***

A mixture of **15a,b** (0.002 mol) and glacial acetic acid (5 mL) was heated under reflux for 10 h. The reaction mixture was then cooled and poured onto cold water. The obtained precipitate was filtered, washed with water, dried and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 3281–3271 (NH); 1691–1688 (C=O); 1623–1622 (C=N); 1032 (C–O–C). ^{1}H-NMR (δ ppm) for **16a**: 1.27 (t, J = 6.9 Hz, 3H, CH_2CH_3); 4.25 (q, J = 6.9 Hz, 2H, CH_2CH_3); 6.94 (s, 2H, NH_2, D_2O exchangeable); 7.37 (t, J = 7.65 Hz, 1H, phenyl-C_4-H); 7.48–7.62 (m, 5H, phenyl-$C_{2,3,5,6}$-H and NH); 7.92 (s, 1H, pyrazolopyrimidine-C_3-H); 8.03 (s, 1H, NH, D_2O exchangeable); 8.68 (s, 1H, CH=). ^{1}H-NMR (δ ppm) for **16b**: 1.27 (t, J = 6.9 Hz, 3H, CH_2CH_3); 4.25 (q, J = 6.9 Hz, 2H, CH_2CH_3); 6.98 (s, 2H, NH_2, D_2O exchangeable); 7.52–7.62 (m, 5H, chlorophenyl-H and NH); 7.93 (s, 1H, pyrazolopyrimidine-C_3-H); 8.01 (s, 1H, NH, D_2O exchangeable); 8.68 (s, 1H, CH=). ^{13}C-NMR (δ ppm) for **16b**: 14.72 (CH_2CH_3), 60.82 (CH_2CH_3), 99.72 (C=C), 102.22 (C-3a), 125.72 (chlorophenyl-C-2,6), 129.89 (chlorophenyl-C-3,5), 131.91 (chlorophenyl-C-4), 137.74 (chlorophenyl-C-1), 141.22 (C-3), 149.21 (C-7a), 159.50 (CH=NH), 162.26 (C-4), 163.95 (C-6), 166.18 (C=O). MS m/z (relative abundance%) for **16a**: 325 [M^+ + 1] (24.8), 324 [M^+] (100), 323 (22.6), 296 (17.7), 295 (10.5), 279 (6.1), 278 (5.8), 93 (11.7), 92 (10.3), 77 (20.1), 67 (5.6), 52 (6.9), 51 (6.4). MS m/z (relative abundance%) for **16b**: 360 [M^+ + 2] (36.3), 359 [M^+ + 1] (26.8), 358 [M^+] (100), 357 (11.9), 332 (7.1), 330 (20.9), 313 (7.5), 312 (6.1), 186 (8.6), 127 (9.8), 126 (9.9), 75 (6.1), 67 (6.2), 52 (6.9).

Biology

Anticancer screening

Estimation of the concentration of a test chemical producing a 50% inhibition (IC_{50}) by MTT assay

Isolation of lymphocytes

Isolation of lymphocytes from whole human blood using Ficoll-Pague™ Plus, ready to use density gradient medium for purifying lymphocytes in high yield and purity from human peripheral blood. 5 mL of human blood in a heparinized syring was mixed gently with one part of Hank's Balance Salt Solution (HBSS). Layer carefully the diluted blood sample onto 10 mL Ficoll. Three layers will be obtained of the centrifugation at 2000 rpm for 25 min, using Pasteur pipette withdraw the middle lymphocyte layer. Lymphocytes were suspended in 1 mL of HBSS and were centrifuged at 2000 rpm for 10 min. Then, final pellet was resuspended in 1 mL RPMI. Under the microscope count the cells in the central 25 squares using hemocytometer and Trypan blue dye.

Measurement cytotoxicity

5×10^4 lymphocyte cells were seeded per well in 96 well plates and the plates were incubated in RPMI media containing a test chemical (**3b**, **4b**, **5b**, **6b**, **6d**, **7c**, **8c**, **10b**, **12b**, **13a**, **13b** and **16b**) with different concentrations (1.5, 7.5, 15, 22.5 and 30 μg/mL) for 24 h in 5% CO_2 incubator. Next, the media was removed, wells were washed with HBSS, and the fraction of viable lymphocyte cells was measured by the MTT assay.

Effect of IC_{50} of a test chemical on tumor cell viability

To measure tumor cell viability tumor (HelaS3, HepG2 and CaCo), 2×10^4 cells were seeded per well in 96 well plates and plates were incubated in Ham's F-l2, RPMI and DMEM, respectively for 24 h in 5% CO_2 incubator for cell attachment. Next, the media was changed to media containing 1% of IC_{50} of test chemicals (0.44, 0.2, 0.18, 0.5, 0.4, 0.45, 0.49, 0.2, 0.21, 0.43, 1.05, and 0.45 μg/mL, respectively) and cells were incubated in that media for 24, 48, and 72 h. Media without test chemical was used as the negative control and media containing solvent (DMSO) as solvent control. At each time point, the media was removed, wells were washed with phosphate buffer saline (PBS) and fractions of viable cells were measured by the MTT assay.

MTT assay

Yellow MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan in the mitochondria of viable cells. 100 μL of the MTT working solution (0.5 mg/mL) was added to each well and incubated at 37°C for 4 h in 5% CO_2 incubator. Next, media was removed, wells were washed with PBS, and 100 μL DMSO were added to solubilize the formazan crystalline product. The absorbance was measured with a plate reader at 590 nm [50–52].

Antimicrobial screening

Inhibition-zone measurements

All the synthesized compounds were evaluated by the agar cup diffusion technique [53] using a 1 mg/mL solution in DMSO. The test organisms were *Staphylococcus aureus* (DSM 1104) and *Bacillus subtilis* (ATCC 6633) as Gram-positive bacteria; *Escherichia coli* (ATCC 11775) and *Pseudomonas aeruginosa* (ATCC 10145) as Gram-negative bacteria. *Candida albicans* (DSM 70014) was also used as a representative for fungi. Each 100 mL of sterile molten agar (at 45°C) received 1 mL of 6-h broth culture and then the seeded agar was poured into sterile Petri dishes. Cups (8 mm in diameter) were cut in the agar. Each cup received 0.1 mL of the 1 mg/mL solution of the test compounds. The plates were then incubated at 37°C for 24 h or, in case of *C. albicans*, for 48 h. A control using DMSO without the test compound was included for each organism. Ampicillin was used as standard antibacterial, while clotrimazole was used as antifungal reference.

Minimal inhibitory concentration (MIC) measurement

The minimal inhibitory concentrations (MIC) of the most active compounds were measured using the twofold serial broth dilution method [54]. The test organisms were grown in their suitable broth: 24 h for bacteria and 48 h for fungi at 37°C. Twofold serial dilutions of solutions of the test compounds were prepared using 200, 100, 50, 25, and 12.5 μg/mL. The tubes were then inoculated with the test organisms; each 5 mL received 0.1 mL of the above inoculum and were incubated at 37°C for 48 h. Then, the tubes were observed for the presence or absence of microbial growth. The MIC values of the prepared compounds are listed in Table 2.

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