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Synthesis and in vitro cytotoxic activity of novel pyrazolo[1,5-*a*]pyrimidines and related Schiff bases

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Abstract: The reaction of 5-amino-3-(4-methoxyphenylamino)-*N*-aryl-1*H*-pyrazole-4-carboxamides **1a**–**c** with ethyl acetoacetate **2** and 2-(4-fluorobenzylidene)malononitrile **6** yielded pyrazolo[1,5-*a*]pyrimidines **5a**–**c** and **9a**–**c**, respectively. On the other hand, Schiff bases **11a**–**f** were obtained upon treatment of carboxamides **1a**–**c** with some selected aldehydes **10a** and **b**. The newly synthesized compounds were characterized and confirmed by analytical and spectroscopic data (IR, MS, ^{1}H NMR, and ^{13}C NMR). Pyrazolo[1,5-*a*]pyrimidines **5a**–**c** and **9a**–**c** and Schiff bases **11b**–**f** were investigated for their cytotoxicity against four human cancer cell lines (colon HCT116, lung A549, breast MCF-7, and liver HepG2) according to SRB assay and the structure–activity relationship was discussed.

Key words: 5-Aminopyrazole, pyrazolopyrimidines, ferrocenyl-2-carboxaldehyde, Schiff bases, antitumor activity

1. Introduction

The main objective of organic and medicinal chemistry is the design, synthesis, and production of molecules having precious value as human therapeutic agents for the treatment of various human diseases, e.g., cancer, human immunodeficiency virus (HIV), and hepatitis C virus (HCV), which are the major scourges of humanity. A literature survey revealed that pyrazolo[1,5-*a*]pyrimidines are of considerable chemical and pharmacological importance as purine analogues. The class of pyrazolopyrimidines possesses a broad spectrum of biological effectiveness such as antimicrobial,[1] anti-inflammatory,[2] cytotoxicity,[3] and hepatitis C virus inhibitor[4] activities.

On the other hand, Schiff bases are an important class of compounds in the medicinal field, with biological applications including antimicrobial,[5] antioxidant,[6] anti-inflammatory,[7] antitumor,[8] and α-glucosidase enzyme inhibitor.[9] Furthermore, we have found that a Schiff base is a prominent group in the structures of some drugs, e.g., dantrolene (muscle relaxant), nifuroxazide (antibiotic), and thiacetazone (antituberculosis) (as shown in Figure 1).

In view of the above-mentioned biological importance of pyrazolo[1,5-*a*]pyrimidines and Schiff bases and as a continuation of our interest in the synthesis of novel compounds with expected biological activities,[10,11] we found that compounds 7-amino-6-cyano-2-(4-methoxyphenylamino)-5-(naphthalen-1-yl)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide (**I**) and 5-[(ferrocene-1-ylmethylidene)amino]-3-(phenylamino)-1*H*-pyrazole-4-carboxamide (**II**) as examples exerted promising anticancer activity against breast MCF7 and liver HepG2 cancer cell lines,

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respectively.[12] Furthermore, 2-(4-methoxyphenylamino)-5,7-dimethyl-*N*-phenylpyrazolo[1,5-*a*]pyrimidine-3-carboxamide (**III**) is an example of a pyrazolo[1,5-*a*]pyrimidine derivative that exhibited promising anticancer activity in Ehrlich ascites carcinoma assay (as shown in Figure 2).[13] We report herein the synthesis of a new series of pyrazolo[1,5-*a*]pyrimidine derivatives and Schiff bases based on 5-aminopyrazole derivatives, and also investigation of the cytotoxic activities of the synthesized compounds against four human tumor cell lines (HCT116 "colon", A549 "lung", HepG2 "liver", and MCF-7 "breast" cancers).

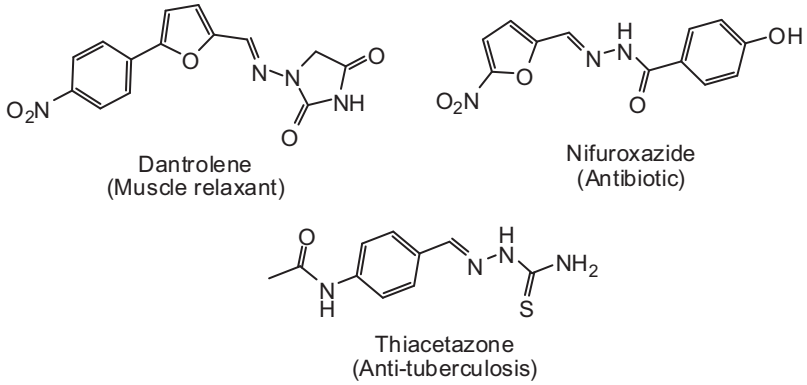


Figure 1. The structures of some drugs bearing Schiff base group.

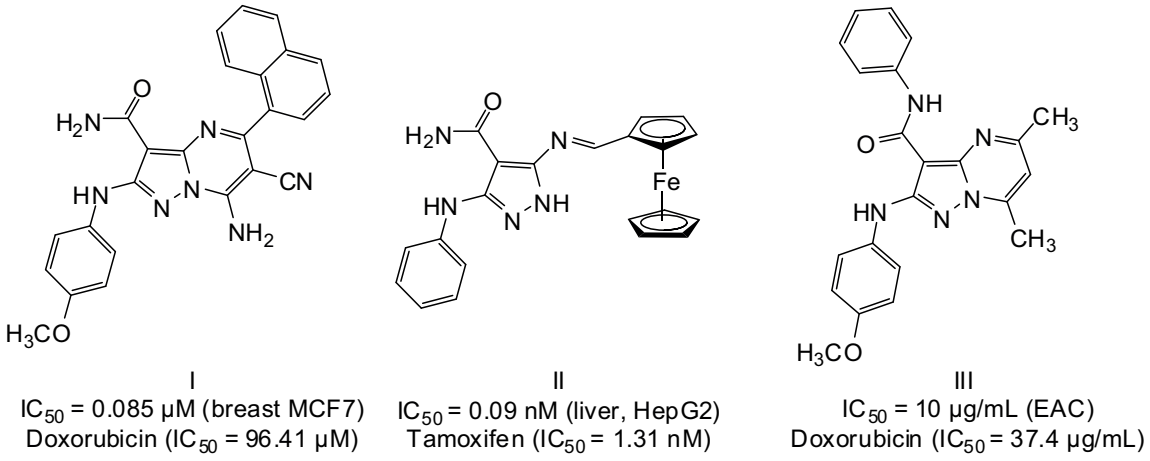


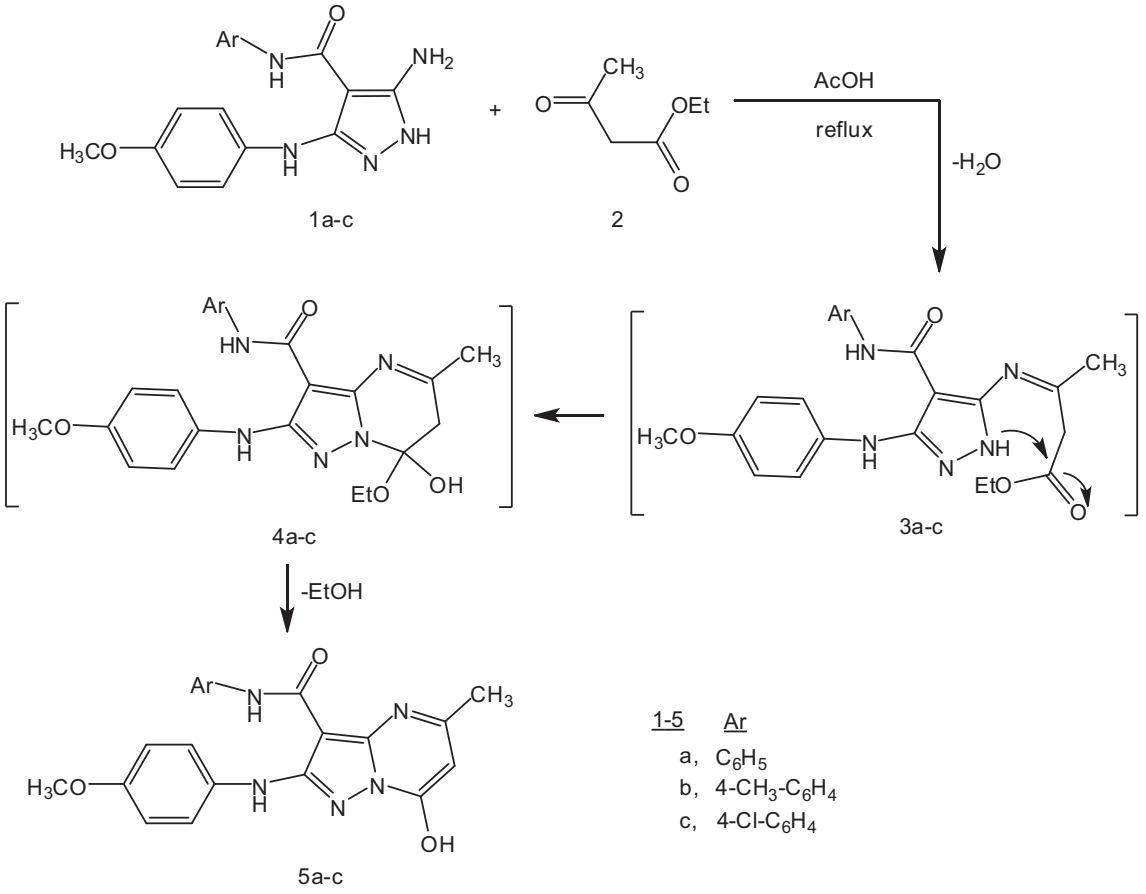
Figure 2. The structures of some anticancer agents.

2. Results and discussion

2.1. Chemistry

The starting compounds, 5-amino-3-(4-methoxyphenylamino)-*N*-aryl-1*H*-pyrazole-4-carboxamides **1a**–**c**,[13] were utilized for preparing the target compounds (Schemes 1–3). The reaction of compounds **1a**–**c** with ethyl acetoacetate **2** in glacial acetic acid under reflux temperature afforded the 7-hydroxy-5-methyl-*N*-(aryl)pyrazolo[1,5-*a*]pyrimidines **5a**–**c**. The formation of compounds **5a**–**c** was therefore assumed to proceed via the initial attack of the exocyclic amino group of **1a**–**c** on the keto group of ethyl acetoacetate **2**, followed by intramolecular cyclization via elimination of ethanol (Scheme 1). The structures of **5a**–**c** were confirmed on the basis of their analytical and spectral data. Compound **5c**, taken as a representative example, revealed the molecular formula

$C_{21}H_{18}ClN_5O_3$ (423.85) (m/z: 423 [M^+]) and its IR spectrum (KBr/cm^{-1}) showed strong absorption bands at 3299, 3101 corresponding to (OH, NH) and a band at 1658 due to 1667 (C=O) groups. Its ^{1}H NMR spectrum (300 MHz, δ ppm) showed two singlets at 2.34 and 3.71 due to CH_3 of the pyrimidine nucleus and –OCH_3 group protons, respectively, and a signal at 5.74 due to the H-6 proton of the pyrimidine nucleus. There were four doublets for the eight aromatic protons at 6.87 (2H, J_{HH} = 9.0 Hz), 7.40 (2H, J_{HH} = 9.0 Hz), 7.58 (2H, J_{HH} = 9.0 Hz), and 7.68 (2H, J_{HH} = 8.7 Hz). Finally, three singlets were present at 8.56, 9.78, and 11.94 assigned for the two –NH and –OH protons, which were D_2O exchangeable. Their ^{13}C NMR (75 MHz, δ ppm) spectrum was characterized by a signal at 21.0 assigned to a CH_3 (pyrazolopyrimidine) carbon, a signal at 161.5 corresponding to the carbonyl carbon, and a signal at 171.9 due to C_7 (C_7–OH) of the pyrazolopyrimidine nucleus.



Scheme 1. Synthesis of 7-hydroxy-5-methyl-*N*-(aryl)pyrazolo[1,5-*a*]pyrimidines (**5a**–**c**).

Fluorinated compounds have been of interest to medicinal chemists for many years because of their biological activities such as antiviral,[14] antitumor,[15] antitubercular,[16] anti-inflammatory, and antimicrobial.[17–19] In addition, we have found that some fluorinated compounds such as 5-fluorouracil, paroxetine, and ciprofloxacin are available as drugs (as shown in Figure 3). For these reasons, we were encouraged to synthesize a number of these derivatives via the reaction of 2-(4-fluorobenzylidene)malononitrile **6** with **1a**–**c** in ethanol and in the presence of a base under reflux conditions to give 7-amino-*N*-aryl-6-cyano-5-(4-fluorophenyl)-2-(4-methoxyphenylamino)pyrazolo[1,5-*a*]pyrimidine-3-carboxamides **9a**–**c**.

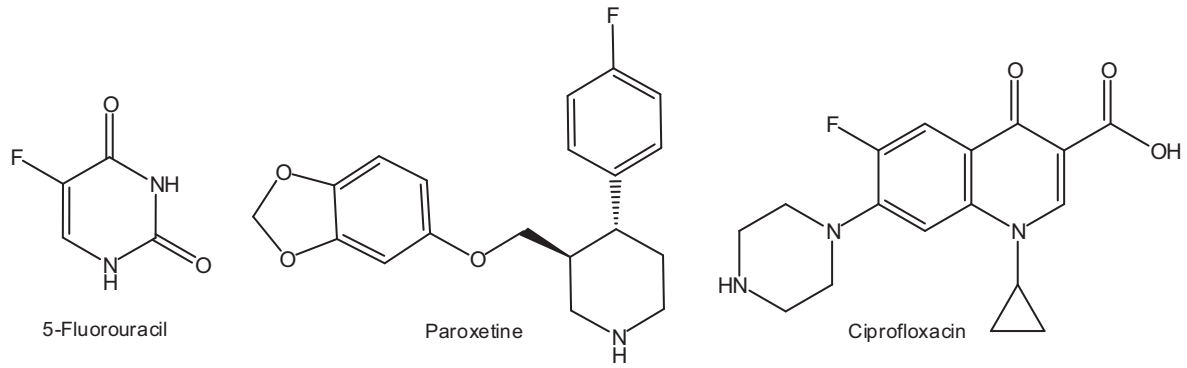
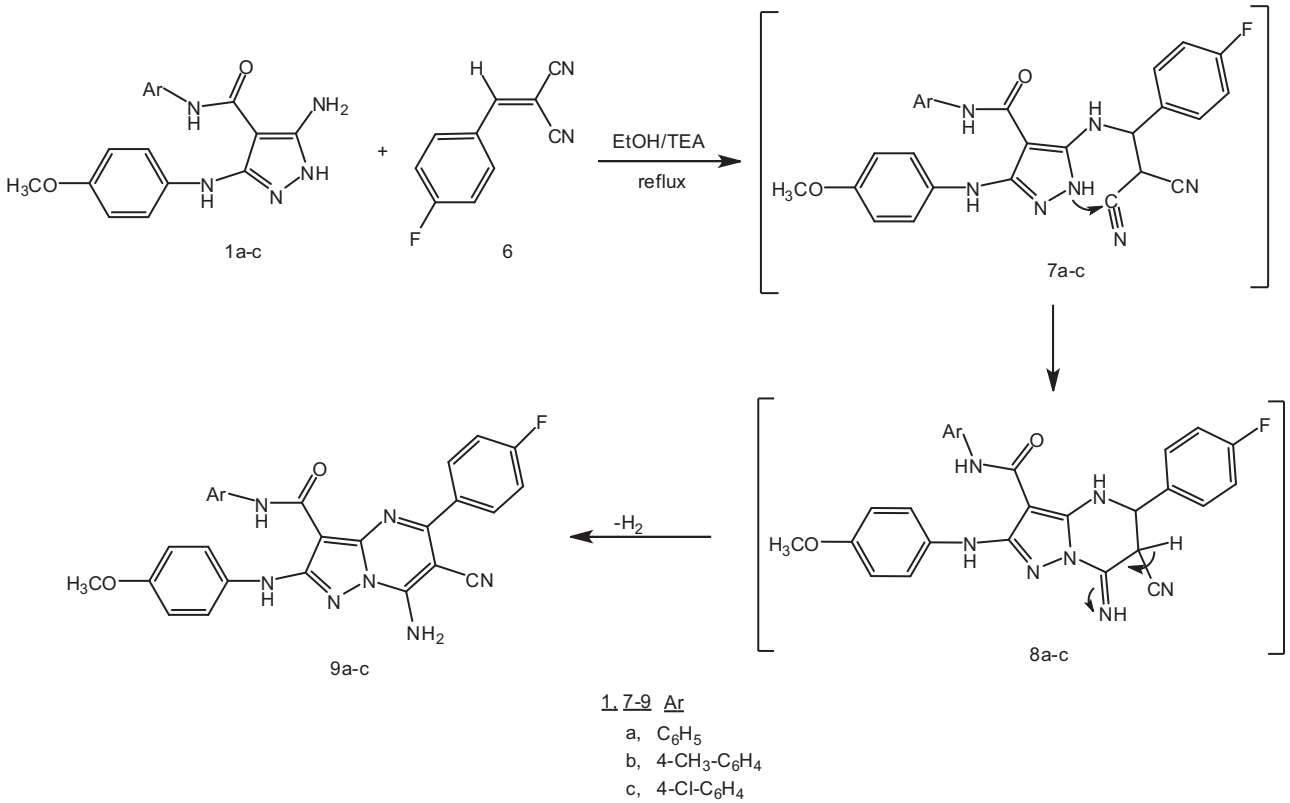


Figure 3. Some fluorinated drugs.

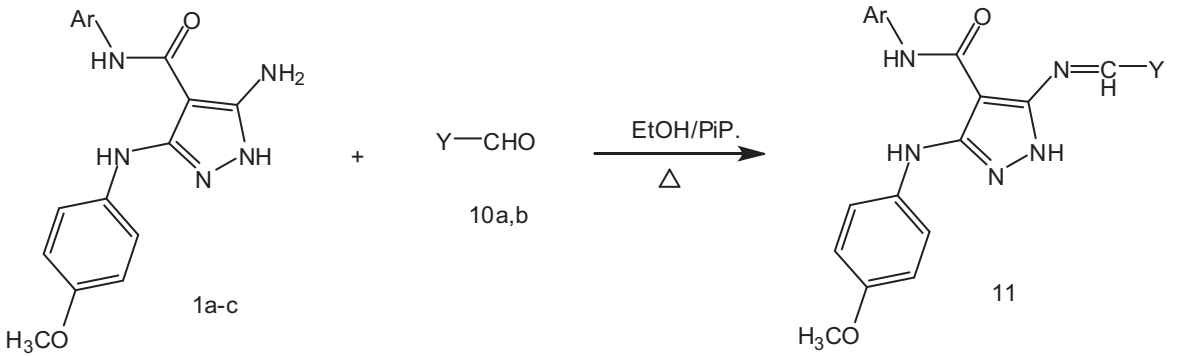
The formation of compounds **9a**–**c** was assumed to proceed via initial attack of the exocyclic amino function of the compounds **1a**–**c** on the α,β-unsaturated system in compound **6**, followed by intramolecular cyclization and spontaneous autooxidation through the loss of the H_2 molecule[20] (Scheme 2). The structures of **9a**–**c** were established based on their analytical and spectral data. Thus, as an example, the mass spectrum of compound **9a** [$C_{27}H_{20}FN_7O_2$ (493.49)] showed an ion peak at m/z 493 that corresponded to [M^+] and its IR spectrum (KBr/cm^{-1}) showed bands at 3445 and 3307 for (NH, NH_2), 2214 for C≡N, and 1668 for C=O groups. Its ^{1}H NMR spectrum (δ ppm) revealed the presence of a singlet at 3.75 corresponding to protons of



Scheme 2. Synthesis of 7-amino-*N*-aryl-6-cyano-5-(4-fluorophenyl)-2-(4-methoxyphenylamino)pyrazolo[1,5-*a*]pyrimidine-3-carboxamides (**9a**–**c**).

the –OCH_3; two triplets at 7.09 (1H) and 7.48 (2H) were assigned for three aromatic protons and five doublets at 6.91 (2H, J_{HH} = 9.0 Hz), 7.36 (2H, J_{HH} = 7.8 Hz), 7.60 (2H, J_{HH} = 8.7 Hz), 7.84 (2H, J_{HH} = 8.7 Hz), and 8.06 (2H, J_{HH} = 8.4 Hz) for ten aromatic protons. Moreover, the ^{1}H NMR spectrum (δ ppm) showed three singlets at 9.06, 9.24, and 10.04 due to –NH_2 and two –NH protons, which were D_2O exchangeable.

The importance of Schiff bases in the pharmaceutical field prompted us to synthesize some new Schiff bases **11a**–**f** by the condensation of 5-amino-*N*-aryl-1*H*-pyrazole-4-carboxamides **1a**–**c** with 5-methylfuran-2-carbaldehyde **10a** or ferrocene-2-carboxaldehyde **10b** in boiling ethanol using a catalytic amount of triethylamine (Scheme 3). The structures of **11a**–**f** were characterized and confirmed on the basis of analytical and spectral data (IR, MS, ^{1}H NMR, and ^{13}C NMR). Structure **11e** was taken as a representative example; the mass spectrum exhibited a molecular ion peak at m/z = 533 [M^+] $C_{29}H_{27}FeN_5O_2$, and its IR spectrum (KBr/cm^{-1}) showed strong absorption bands at 3274 and 1652 corresponding to NH and C=O groups respectively. Its ^{1}H NMR spectrum (δ ppm) showed two singlets at 2.25 and 3.69 due to –CH_3 and –OCH_3 protons, respectively, and 5H of the unsubstituted ferrocene ring appeared at 4.29 as a singlet, while 4H of the monosubstituted ferrocene ring appeared at 4.76 (2H) and 4.97 (2H) as singlets. In addition, there were two doublets at 6.86 (2H) and 7.54 (2H) for four aromatic protons (J_{HH} =8.4 Hz), two doublets at 7.16 (2H) and 7.38 (2H) for four aromatic protons (J_{HH} = 7.6 Hz), and a signal at 8.66 due to 1H of the –N=CH– group. Finally, three singlets at 8.84, 9.86, and 12.65 were assigned for three –NH protons, which were D_2O exchangeable. The ^{13}C NMR spectrum (δ ppm) was characterized by signals at 16.5, 70.1, 73.6, 78.9, and 148.6 assigned to CH_3, ferrocene ring, and –N=CH– carbon atoms.



1	Ar
a,	C_6H_5
b,	4-CH_3-C_6H_4
c,	4-Cl-C_6H_4

10	Y
a,	5-methylfuran-2-yl
b,	ferrocenyl

11	Ar	Y
a,	C_6H_5	5-methylfuran-2-yl
b,	4-CH_3-C_6H_4	5-methylfuran-2-yl
c,	4-Cl-C_6H_4	5-methylfuran-2-yl
d,	C_6H_5	ferrocenyl
e,	4-CH_3-C_6H_4	ferrocenyl
f,	4-Cl-C_6H_4	ferrocenyl

Scheme 3. Schiff bases (**11a**–**c**) and their ferrocenyl analogues (**11d**–**f**).

2.2. In vitro cytotoxic activity

The cytotoxic activity of the tested compounds was determined using the SRB assay[21] against four human cancer cell lines: colon HCT116, lung A549, liver HepG2, and breast MCF-7 (Table). The results are expressed as the IC_{50} (μg/mL), which is the concentration of a drug that causes a 50% reduction in the proliferation of

cancer cells when compared to the growth of the control cells. Doxorubicin was used as a reference drug. The tumor cells showed normal growth in the culture system and DMSO did not seem to have any noticeable effect on cellular growth.

Table. In vitro cytotoxicity ($IC_{50}\mu g/mL$, the concentration required for 50% inhibition of cell growth) of the tested compounds was determined by using the SRB assay on four human cancer cell lines.

The tested compound	Human cancer cell lines			
	Colon HCT116	Lung A549	Liver HepG2	Breast MCF-7
5a	N.A.	5.00 ± 0.50*	4.00 ± 0.44*	4.60 ± 0.55
5b	N.A.	5.60 ± 0.60	6.50 ± 0.75	5.90 ± 0.62
5c	N.A.	5.45 ± 0.62	6.10 ± 0.62	4.20 ± 0.60*
9a	N.A.	N.A.	N.A.	N.A.
9b	N.A.	N.A.	N.A.	N.A.
9c	N.A.	N.A.	4.50 ± 0.55	4.90 ± 0.50
11b	N.A.	N.A.	N.A.	32.00 ± 3.30
11c	N.A.	N.A.	19.20 ± 2.00	17.10 ± 1.80
11d	N.A.	N.A.	6.20 ± 0.70	7.00 ± 0.80
11e	N.A.	N.A.	N.A.	N.A.
11f	N.A.	N.A.	15.90 ± 1.70	24.70 ± 2.50
DMSO	N.A.	N.A.	N.A.	N.A.
Doxorubicin	6.30 ± 0.60	5.10 ± 0.50	4.20 ± 0.46	4.70 ± 0.55

IC_{50} ($\mu g/mL$) were expressed as mean ± SE {where mean ± SE = mean $\pm\frac{SD}{\sqrt{n}}$, n = 6 experiments}
N.A. is no activity
*The most potent compound

The results revealed that all the tested compounds {pyrazolo[1,5-*a*]pyrimidines **5a**–**c** and **9a**–**c**, and Schiff bases **11b**–**f**} did not exert any activity against human colon HCT116 cancer cell lines.

In the case of human lung A549 cancer cell lines, the tested compounds {pyrazolo[1,5-*a*]pyrimidines **9a**–**c** and Schiff bases **11b**–**f**} had no effect on the cancer cell lines, but compound **5a** (IC_{50} = 5.00 ± 0.50 $\mu g/mL$) was found to be more potent than the standard drug, doxorubicin (IC_{50} = 5.10 ± 0.50 $\mu g/mL$). Compounds **5b** (IC_{50} = 5.60 ± 0.60 $\mu g/mL$) and **5c** (IC_{50} = 5.45 ± 0.62 $\mu g/mL$) showed cytotoxicity close to that of the standard drug (IC_{50} = 5.10 ± 0.50 $\mu g/mL$).

For liver HepG2 cancer cell lines, while compounds **9a**, **9b**, **11b**, and **11e** had no effect on the cancer cell lines, compound **5a** (IC_{50} = 4.00 ± 0.44 $\mu g/mL$) was found to be more potent than the standard drug (IC_{50} = 4.20 ± 0.46 $\mu g/mL$). On the other hand, compound **8c** (IC_{50} = 4.50 ± 0.55 $\mu g/mL$) was nearly as potent as the reference drug (IC_{50} = 4.20 ± 0.46 $\mu g/mL$), but compounds **5b** (IC_{50} = 6.50 ± 0.75 $\mu g/mL$), **5c** (IC_{50} = 6.10 ± 0.62 $\mu g/mL$), and **11d** (IC_{50} = 6.20 ± 0.70 $\mu g/mL$) revealed slight activity in comparison with the standard drug (IC_{50} = 4.20 ± 0.46 $\mu g/mL$), while the rest of the tested compounds, **11c** (IC_{50} = 19.20 ± 2.00 $\mu g/mL$) and **11f** (IC_{50} = 15.90 ± 1.70 $\mu g/mL$), were less potent than the standard drug (IC_{50} = 4.20 ± 0.46 $\mu g/mL$).

From the estimation of the cytotoxic activity on the human breast MCF-7 cancer cell lines, compounds **9a**, **9b**, and **11e** had no effect on the cancer cells, but compounds **5a** (IC_{50} = 4.60 ± 0.55 $\mu g/mL$) and **5c** ($IC_{50=}$ 4.20 ± 0.60 $\mu g/mL$) showed cytotoxicity more potent than the standard drug (IC_{50} = 4.70 ± 0.55

μg/mL). Compound **9c** (IC_{50} = 4.90 ± 0.50 μg/mL) showed cytotoxic activity close to that of the standard drug (IC_{50} = 4.70 ± 0.55 μg/mL), but compounds **5b** (IC_{50} = 5.90 ± 0.62 μg/mL) and **11d** (IC_{50} = 7.00 ± 0.80 μg/mL) revealed slight activity in comparison with the standard drug (IC_{50} = 4.70 ± 0.55 μg/mL), while the rest of the tested compounds, **11b** (IC_{50} = 32.00 ± 3.30 μg/mL), **11c** (IC_{50} = 17.10 ± 1.80 μg/mL) and **11f** (IC_{50} = 24.70 ± 2.50 μg/mL), were less potent than the standard drug (IC_{50} = 4.70 ± 0.55 μg/mL).

Based on these results, it is evident that there is a structure–activity relationship (SAR). From the screening of the tested compounds against the lung A549, liver HepG2, and breast MCF-7 cell lines, some derivatives bearing the phenyl group were more active than those bearing the 4-chlorophenyl group and those bearing the 4-methylphenyl group. Thus, on lung A549 cell lines, **5a** (IC_{50} = 5.00 ± 0.50 μg/mL) > **5c** (IC_{50} = 5.45 ± 0.62 μg/mL) > **5b** (IC_{50} = 5.60 ± 0.60 μg/mL). Moreover, the screening of the tested compounds against the HepG2 (liver) cell lines showed that **5a** (IC_{50} = 4.00 ± 0.44 μg/mL) > **5c** (IC_{50} = 6.10 ± 0.62 μg/mL) > **5b** (IC_{50} = 6.50 ± 0.75 μg/mL) and **11d** (IC_{50} = 6.20 ± 0.70 μg/mL) > **11f** (IC_{50} = 15.90 ± 1.70 μg/mL) > **11e** (N.A.). Furthermore, on breast MCF-7 cell lines, **11d** (IC_{50} = 7.00 ± 0.80 μg/mL) > **11f** (IC_{50} = 24.70 ± 2.50 μg/mL) > **11e** (N.A.).

Finally, compound **5a** showed cytotoxic activity and was more potent against the lung A549 and liver HepG2 cell lines, with IC_{50} = 5.00 ± 0.50 μg/mL and IC_{50} = 4.00 ± 0.44 μg/mL, respectively, and compound **5c** showed cytotoxic activity and was more potent against the breast MCF-7 cell lines, with IC_{50} = 4.20 ± 0.60 μg/mL.

3. Conclusion

In the present work, we report the synthesis, characterization, and in vitro cytotoxic activity of novel pyrazolo[1,5-*a*]pyrimidines **5a–c** and **9a–c** and Schiff bases **11a–f**. The cytotoxicity results of the above-mentioned compounds against four human cancer cell lines (colon HCT116, lung A549, liver HepG2, and breast MCF-7) indicated that two compounds, **5a** and **5c**, showed cytotoxicity and growth inhibitor activity on lung A549, liver HepG2, and breast MCF-7 cancer cell lines at low concentrations in comparison with the reference drug considered (doxorubicin).

4. Experimental

All melting points were measured on a Gallenkamp melting point apparatus and are uncorrected. The IR spectra were recorded (KBr disk) on a PerkinElmer 1650 FT-IR instrument. ^{1}H NMR (300 or 500 MHz) and ^{13}C NMR (75 or 125 MHz) spectra were recorded on a Varian spectrometer using DMSO-d_6 as a solvent and TMS as an internal standard. Chemical shifts are recorded in ppm. Mass spectra were recorded on a Varian MAT 112 spectrometer at 70 eV. Elemental analyses were obtained from the Micro Analytical Center at Cairo University, Egypt.

Progress of the reactions was monitored by thin-layer chromatography (TLC) using aluminum sheets coated with silica gel F_{254} (Merck); viewing under a short-wavelength UV lamp effected detection. All evaporations were carried out under reduced pressure at 40 °C.

4.1. Chemistry

4.1.1. Synthesis of 5-amino-3-(4-methoxyphenylamino)-*N*-aryl-1*H*-pyrazole-4-carboxamides (1a–c)

Compounds of this series (**1a–c**) were prepared according to the literature procedure.[13]

4.1.2. Synthesis of 7-hydroxy-2-(4-methoxyphenylamino)-5-methyl-*N*-(aryl)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide (5a–c)

A mixture of compounds **1a**–**c** (0.01 mol) with ethyl acetoacetate **2** (0.01 mol) in glacial acetic acid (20 mL) was refluxed for 6 h, then poured onto crushed ice, and the separated solid was filtered off, dried well, and recrystallized from ethanol to afford compounds **5a**–**c**.

4.1.3. 7-Hydroxy-2-(4-methoxyphenylamino)-5-methyl-*N*-phenylpyrazolo[1,5-*a*]pyrimidine-3-carboxamide (5a)

Pale yellow crystals, mp > 300 °C, yield (84%). IR (KBr) ν_{max}/cm^{-1} 3294, 3057 (OH, NH), 1662 (C=O). ^{1}H NMR (300 MHz, δ ppm) 2.34 (s, 3H, CH_3 pyrimidine), 3.71 (s, 3H, OCH_3), 5.75 (s, 1H, pyrimidine H-6), 6.90 (d, 2H, Ar-H, J_{HH} = 9.0 Hz), 7.10 (t, 1H, Ar-H, J_{HH} = 7.8 Hz), 7.36 (t, 2H, Ar-H, J_{HH} = 7.8 Hz), 7.60 (d, 2H, Ar-H, J_{HH} = 9.3 Hz), 7.65 (d, 2H, Ar-H, J_{HH} = 7.5 Hz), 8.59 (s, 1H, NH, D_2O exchangeable), 9.66 (s, 1H, NH, D_2O exchangeable), 11.86 (s, 1H, OH, D_2O exchangeable). MS m/z: 389 [M$^+$]. Anal. Calcd. (%) for $C_{21}H_{19}N_5O_3$ (389.41): C, 64.77; H, 4.92; N, 17.98. Found: C, 64.70; H, 4.96; N, 18.03%.

4.1.4. 7-Hydroxy-2-(4-methoxyphenylamino)-5-methyl-*N*-(4-methylphenyl)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide (5b)

Pale yellow crystals, mp 260–261 °C, yield (79%). IR (KBr) ν_{max}/cm^{-1} 3339, 3055 (OH, NH), 1667 (C=O). ^{1}H NMR (300 MHz, δ ppm) 2.29 (s, 3H, CH_3), 2.34 (s, 3H, CH_3 pyrimidine), 3.72 (s, 3H, OCH_3), 5.74 (s, 1H, pyrimidine H-6), 6.89 (d, 2H, Ar-H, J_{HH} = 9.0 Hz), 7.16 (d, 2H, Ar-H, J_{HH} = 8.4 Hz), 7.53 (d, 2H, Ar-H, J_{HH} = 8.4 Hz), 7.58 (d, 2H, Ar-H, J_{HH} = 8.7 Hz), 8.64 (s, 1H, NH, D_2O exchangeable), 9.56 (s, 1H, NH, D_2O exchangeable), 11.84 (s, 1H, OH, D_2O exchangeable). MS m/z: 403 [M$^+$]. Anal. Calcd. (%) for $C_{22}H_{21}N_5O_3$ (403.43): C, 65.50; H, 5.25; N, 17.36. Found: C, 65.55; H, 5.28; N, 17.40%.

4.1.5. *N*-(4-Chlorophenyl)-7-hydroxy-2-(4-methoxyphenylamino)-5-methylpyrazolo[1,5-*a*]pyrimidine-3-carboxamide (5c)

Pale yellow crystals, mp > 300 °C, yield (75%). IR (KBr) ν_{max}/cm^{-1} 3299, 3101 (OH, NH), 1667 (C=O). ^{1}H NMR (300 MHz, δ ppm) 2.34 (s, 3H, CH_3 pyrimidine), 3.71 (s, 3H, OCH_3), 5.74 (s, 1H, pyrimidine H-6), 6.87 (d, 2H, Ar-H, J_{HH} = 9.0 Hz), 7.40 (d, 2H, Ar-H, J_{HH} = 9.0 Hz), 7.58 (d, 2H, Ar-H, J_{HH} = 9.0 Hz), 7.68 (d, 2H, Ar-H, J_{HH} = 8.7 Hz), 8.56 (s, 1H, NH, D_2O exchangeable), 9.78 (s, 1H, NH, D_2O exchangeable), 11.94 (s, 1H, OH, D_2O exchangeable). ^{13}C NMR (75 MHz, DMSO-d_6,δ ppm) 21.0 (-CH_3, pyrazolopyrimidine), 55.2 (–OCH_3), 87.4 (C_3, pyrazolopyrimidine), 98.3 (C_6, pyrazolopyrimidine), 114.0, 118.8, 122.2 (6C, Ar), 127.1 (C_{3a}, pyrazolopyrimidine), 128.3, 134.4, 137.5, 153.2 (6C, Ar), 153.6 (C_2, pyrazolopyrimidine), 154.8 (C_5, pyrazolopyrimidine), 161.5 (C=O), 171.9 (C_7, pyrazolopyrimidine). MS m/z: 423 [M$^+$]. Anal. Calcd. (%) for $C_{21}H_{18}ClN_5O_3$ (423.85): C, 59.51; H, 4.28; N, 16.52. Found: C, 59.60; H, 4.25; N, 16.55%.

4.1.6. Synthesis of 7-amino-6-cyano-5-(4-fluorophenyl)-2-(4-methoxyphenylamino)-*N*-(aryl)-pyrazolo[1,5-*a*]pyrimidine-3-carboxamide (9a–c)

A mixture of compounds **1a**–**c** (0.01 mol) with 2-(4-fluorobenzylidene)malononitrile **5** (0.01 mol) and a catalytic amount of triethylamine (four drops) in absolute ethanol (30 mL) was refluxed for 6 h. The solvent was concentrated under reduced pressure and the solid obtained was collected and recrystallized from ethanol to give **9a**–**c**.

4.1.7. 7-Amino-6-cyano-5-(4-fluorophenyl)-2-(4-methoxyphenylamino)-*N*-phenylpyrazolo[1,5-*a*] pyrimidine-3-carboxamide (9a)

Orange crystals, mp > 300 °C, yield (72%). IR (KBr) ν_{max}/cm^{-1} 3445, 3307 (NH, NH_2), 2214 (C≡N), 1668 (C=O). ^{1}H NMR (300 MHz, δ ppm) 3.75 (s, 3H, OCH_3), 6.91 (d, 2H, Ar-H, J_{HH} = 9.0 Hz), 7.09 (t, 1H, Ar-H, J_{HH} = 7.2 Hz), 7.36 (d, 2H, Ar-H, J_{HH} = 7.8 Hz), 7.48 (t, 2H, Ar-H, J_{HH} = 8.7 Hz), 7.60 (d, 2H, Ar-H, J_{HH} = 8.7 Hz), 7.84 (d, 2H, Ar-H, J_{HH} = 8.7 Hz), 8.06 (d, 2H, Ar-H, J_{HH} = 8.4 Hz), 9.06 (s, 2H, NH_2, D_2O exchangeable), 9.24 (s, 1H, NH, D_2O exchangeable), 10.04 (s, 1H, NH, D_2O exchangeable). MS m/z: 493 [M$^+$]. Anal. Calcd. (%) for $C_{27}H_{20}FN_7O_2$ (493.49): C, 65.71; H, 4.08; N, 19.87. Found: C, 65.75; H, 4.05; N, 19.90%.

4.1.8. 7-Amino-6-cyano-5-(4-fluorophenyl)-2-(4-methoxyphenylamino)-*N*-(4-methylphenyl)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide (9b)

Yellow crystals, mp > 300 °C, yield (78%). IR (KBr) ν_{max}/cm^{-1} 3414, 3299 (NH, NH_2), 2210 (C≡N), 1650 (C=O). ^{1}H NMR (500 MHz, δ ppm) 2.20 (s, 3H, CH_3), 3.67 (s, 3H, OCH_3), 6.75 (d, 2H, Ar-H, J_{HH} = 6.8 Hz), 7.36 (d, 2H, Ar-H, J_{HH} = 8.3 Hz), 7.45 (d, 2H, Ar-H, J_{HH} = 7.4 Hz), 7.53 (d, 2H, Ar-H, J_{HH} = 7.9 Hz), 7.81 (d, 2H, Ar-H, J_{HH} = 6.9 Hz), 8.09 (d, 2H, Ar-H, J_{HH} = 6.2 Hz), 8.91 (s, 2H, NH_2, D_2O exchangeable), 9.14 (s, 1H, NH, D_2O exchangeable), 9.93 (s, 1H, NH, D_2O exchangeable). MS m/z: 507 [M$^+$]. Anal. Calcd. (%) for $C_{28}H_{22}FN_7O_2$ (507.52): C, 66.26; H, 4.37; N, 19.32. Found: C, 66.35; H, 4.34; N, 19.40%.

4.1.9. 7-Amino-*N*-(4-chlorophenyl)-6-cyano-5-(4-fluorophenyl)-2-(4-methoxyphenylamino)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide (9c)

Orange crystals, mp > 300 °C, yield (82%). IR (KBr) ν_{max}/cm^{-1} 3463, 3310 (NH, NH_2), 2216 (C≡N), 1667 (C=O). ^{1}H NMR (300 MHz, δ ppm) 3.74 (s, 3H, OCH_3), 6.88 (d, 2H, Ar-H, J_{HH} = 9.0 Hz), 7.38 (d, 2H, Ar-H, J_{HH} = 8.7 Hz), 7.47 (d, 2H, Ar-H, J_{HH} = 8.7 Hz), 7.58 (d, 2H, Ar-H, J_{HH} = 9.0 Hz), 7.78 (d, 2H, Ar-H, J_{HH} = 8.7 Hz), 8.05 (d, 2H, Ar-H, J_{HH} = 8.7 Hz), 9.04 (s, 2H, NH_2, D_2O exchangeable), 9.15 (s, 1H, NH, D_2O exchangeable), 10.04 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (75 MHz, δ ppm) 54.0 (–OCH_3), 88.9 (C_6, pyrazolopyrimidine), 97.8 (C_3, pyrazolopyrimidine), 114.0 (2C, Ar), 115.4 (–C≡N), 115.7, 118.9, 120.4, 128.8, 131.0, 131.2 (11C, Ar), 132.8 (C_{3a}, pyrazolopyrimidine), 133.0, 133.1, 138.2, 153.8 (4C, Ar), 156.1 (C_2, pyrazolopyrimidine), 158.5 (C, Ar), 159.9 (C_5, pyrazolopyrimidine), 161.5 (C=O), 164.2 (C_7, pyrazolopyrimidine). MS m/z: 527 [M$^+$]. Anal. Calcd. (%) for $C_{27}H_{19}ClFN_7O_2$ (527.94): C, 61.43; H, 3.63; N, 18.57. Found: C, 61.50; H, 3.60; N, 18.60%.

4.2. Synthesis of Schiff bases (11a–c) and their ferrocenyl analogues (11d–f)

A mixture of compounds **1a**–**c** (0.01 mol) with 5-methylfuran-2-carbaldehyde **10a** or ferrocene-2-carboxaldehyde **10b** (0.01 mol) in absolute ethanol (30 mL) and a catalytic amount of triethylamine (four drops) was refluxed for 6 h. The solvent was concentrated under reduced pressure and the solid obtained was collected and recrystallized from ethanol to give **11a**–**f**.

4.2.1. 3-(4-Methoxyphenylamino)-5-((5-methylfuran-2-yl)methyleneamino)-*N*-phenyl-1*H*-pyrazole-4-carboxamide (11a)

Yellow crystals, mp 200–202 °C, yield (74%). IR (KBr) ν_{max}/cm^{-1} 3228 (NH), 1649 (C=O). ^{1}H NMR (500 MHz, δ ppm) 2.30 (s, 3H, CH_3), 3.69 (s, 3H, OCH_3), 6.45 (d, 1H, furan H-4, J =3.0 Hz), 6.85 (d, 2H, Ar-H, J_{HH} = 8.4 Hz), 6.87 (d, 1H, furan H-3, J =3.0 Hz), 7.03 (t, 1H, Ar-H, J_{HH} =6.9 Hz), 7.15 (d, 2H, Ar-H, J_{HH} = 7.65 Hz), 7.35 (d, 2H, Ar-H, J_{HH} = 7.65 Hz), 7.69 (d, 2H, Ar-H, J_{HH} = 7.65 Hz), 8.61 (s, 1H, –N=CH–), 8.64 (s, 1H, NH, D_2O exchangeable), 10.58 (s, 1H, NH, D_2O exchangeable), 12.82 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (125 MHz, δ ppm) 14.6 (–CH_3), 55.6 (–OCH_3), 94.9 (C_4, pyrazole), 104.0 (C_4, furan), 110.9 (C_3, furan), 114.9, 118.8, 123.5, 129.6, 134.5, 139.5 (11C, Ar), 146.5 (–N=CH–), 147.9 (C_2, furan), 150.2 (C, Ar & C_5, pyrazole), 154.4 (C_3, pyrazole), 159.2 (C_5, furan), 163.3 (C=O, amide). Anal. Calcd. (%) for $C_{23}H_{21}N_5O_3$ (415.44): C, 66.49; H, 5. 09; N, 16.86. Found: C, 66.35; H, 5.20; N, 17.00%.

4.2.2. 3-(4-Methoxyphenylamino)-5-((5-methylfuran-2-yl)methyleneamino)-*N*-(4-methylphenyl)-1*H*-pyrazole-4-carboxamide (11b)

Yellow crystals, mp 202–204 °C, yield (80%). IR (KBr) ν_{max}/cm^{-1} 3236 (NH), 1646 (C=O). ^{1}H NMR (500 MHz, δ ppm) 2.25 (s, 3H, CH_3), 3.29 (s, 3H, CH_3), 3.68 (s, 3H, OCH_3), 6.44 (d, 1H, furan H-4), 6.84 (d, 2H, Ar-H, J_{HH} =7.65 Hz), 6.90 (d, 1H, furan H-3), 7.15 (d, 2H, Ar-H, J_{HH} = 6.1 Hz), 7.50 (d, 2H, Ar-H, J_{HH} = 7.65 Hz), 7.58 (d, 2H, Ar-H, J_{HH} = 8.4 Hz), 8.60 (s, 1H, –N=CH–), 8.63 (s, 1H, NH, D_2O exchangeable), 10.46 (s, 1H, NH, D_2O exchangeable), 12.78 (s, 1H, NH, D_2O exchangeable). Anal. Calcd. (%) for $C_{24}H_{23}N_5O_3$ (429.47): C, 67.12; H, 5.40; N, 16.31. Found: C, 67.00; H, 5.50; N, 16.20%.

4.2.3. 3-(4-Methoxyphenylamino)-5-((5-methylfuran-2-yl)methyleneamino)-*N*-(4-chlorophenyl)-1*H*-pyrazole-4-carboxamide (11c)

Yellow crystals, mp 202–204 °C, yield (80%). IR (KBr) ν_{max}/cm^{-1} 3224 (NH), 1657 (C=O). ^{1}H NMR (500 MHz, δ ppm) 3.29 (s, 3H, CH_3), 3.69 (s, 3H, OCH_3), 6.49 (d, 1H, furan H-4), 6.86 (d, 2H, Ar-H, J_{HH} = 7.65 Hz), 7.13 (d, 1H, furan H-3), 7.32 (d, 2H, Ar-H, J_{HH} = 7.65 Hz), 7.40 (d, 2H, Ar-H, J_{HH} = 8.4 Hz), 7.70 (d, 2H, Ar-H, J_{HH} = 8.4 Hz), 8.55 (s, 1H, –N=CH–), 8.63 (s, 1H, NH, D_2O exchangeable), 10.64 (s, 1H, NH, D_2O exchangeable), 12.84 (s, 1H, NH, D_2O exchangeable). Anal. Calcd. (%) for $C_{23}H_{20}ClN_5O_3$ (449.89): C, 61.40; H, 4.48; N, 15.57. Found: C, 61.60; H, 4.30; N, 15.80%.

4.2.4. 3-(4-Methoxyphenylamino)-5-(ferrocen-2-ylmethyleneamino)-*N*-phenyl-1*H*-pyrazole-4-carboxamide (11d)

Reddish-brown crystals, mp 108–110 °C, yield (70%). IR (KBr) ν_{max}/cm^{-1} 3270 (NH), 1650 (C=O). ^{1}H NMR (500 MHz, δ ppm) 3.68 (s, 3H, OCH_3), 4.29 (s, 5H, C_5H_5, ferrocene ring), 4.77, 4.98 (2s, 4H, C_5H_4,

ferrocene ring), 6.86–7.65 (m, 9H, Ar-H), 8.67 (s, 1H, –N=CH–), 8.87 (s, 1H, NH, D_2O exchangeable), 10.01 (s, 1H, NH, D_2O exchangeable), 12.59 (s, 1H, NH, D_2O exchangeable). Anal. Calcd. (%) for $C_{28}H_{25}FeN_5O_2$ (519.14): C, 64.75; H, 4.85; N, 13.48. Found: C, 64.50; H, 5.00; N, 13.60%.

4.2.5. 3-(4-Methoxyphenylamino)-5-(ferrocen-2-ylmethyleneamino)-*N*-(4-methylphenyl)-1*H*-pyrazole-4-carboxamide (11e)

Reddish-brown crystals, mp 132–134 °C, yield (81%). IR (KBr) ν_{max}/cm^{-1} 3274 (NH), 1652 (C=O). ^1H NMR (500 MHz, δ ppm) 2.25 (s, 3H, CH_3), 3.69 (s, 3H, OCH_3), 4.29 (s, 5H, C_5H_5, ferrocene ring), 4.76, 4.97 (2s, 4H, C_5H_4, ferrocene ring), 6.86 (d, 2H, Ar-H, J_{HH} = 8.4 Hz), 7.16 (d, 2H, Ar-H, J_{HH} = 7.6 Hz), 7.38 (d, 2H, Ar-H, J_{HH} = 7.6 Hz), 7.54 (d, 2H, Ar-H, J_{HH} = 8.4 Hz), 8.66 (s, 1H, –N=CH–), 8.84 (s, 1H, NH, D_2O exchangeable), 9.86 (s, 1H, NH, D_2O exchangeable), 12.65 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (125 MHz, δ ppm) 16.5 (–CH_3), 55.6 (–OCH_3), 70.1, 73.6, 78.9 (10C, ferrocene ring), 92.9 (C_4, pyrazole), 114.5, 119.2, 130.0, 132.6, 134.9, 136.6 (11C, Ar), 148.6 (–N=CH–), 149.9 (C, Ar), 152.3 (C_5, pyrazole), 154.1 (C_3, pyrazole), 163.4 (C=O, amide). MS m/z (%): 533 (1.24%) [M^+]. Anal. Calcd. (%) for $C_{29}H_{27}FeN_5O_2$ (533.40): C, 65.30; H, 5.10; N, 13.13. Found: C, 65.15; H, 5.35; N, 13.00%.

4.2.6. 3-(4-Methoxyphenylamino)-5-(ferrocen-2-ylmethyleneamino)-*N*-(4-chlorophenyl)-1*H*-pyrazole-4-carboxamide (11f)

Reddish-brown crystals, mp 110–112 °C, yield (69%). IR (KBr) ν_{max}/cm^{-1} 3268 (NH), 1652 (C=O). ^1H NMR (500 MHz, δ ppm) 3.68 (s, 3H, OCH_3), 4.30 (s, 5H, C_5H_5, ferrocene ring), 4.78, 4.98 (2s, 4H, C_5H_4, ferrocene ring), 6.85–7.68 (m, 8H, Ar-H), 8.60 (s, 1H, –N=CH–), 8.81 (s, 1H, NH, D_2O exchangeable), 9.96 (s, 1H, NH, D_2O exchangeable), 12.75 (s, 1H, NH, D_2O exchangeable). Anal. Calcd. (%) for $C_{28}H_{24}ClFeN_5O_2$ (553.82): C, 60.72; H, 4.37; N, 12.65. Found: C, 65.50; H, 4.50; N, 12.50%.

4.3. Evaluation of cytotoxic activity in vitro

The cytotoxic activity was measured in vitro using the Sulforhodamine-B stain (SRB) assay according to the previously reported standard procedure.[21] Cells were inoculated in a 96-well microtiter plate (10^4 cells/well) for 24 h before treatment with the tested compounds to allow attachment of cells to the wall of the plate. The tested compounds were dissolved in DMSO and diluted with saline to the appropriate volume. Different concentrations of the tested compounds under testing (0–100 μg/mL) were added to the cells. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h at 37 °C and in an atmosphere of 5% CO_2. After 48 h, cells were fixed, washed, and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. The unbound dye was removed by four washes with 1% acetic acid and attached stain was recovered with *tris*-EDTA buffer. Color intensity was measured in an ELISA reader at wavelength 540 nm. The relation between the surviving fraction and drug concentration was plotted to obtain the survival curve for each cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and the results are given in the Table.

4.4. Statistical analysis

The results are reported as mean ± standard error (S.E.) {where mean ± SE = mean $\pm\frac{SD}{\sqrt{n}}$; n = 6 experiments}.

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