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Synthesis and in vitro cytotoxic activity of novel pyrazolo[3,4-*d*]pyrimidines and related pyrazole hydrazones toward breast adenocarcinoma MCF-7 cell line

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ABSTRACT

New series of pyrazolo[3,4-*d*]pyrimidines (**7a–e** and **13a–d**) and pyrazole hydrazones **17a–d** were synthesized and evaluated for their antiproliferative activity against human breast adenocarcinoma MCF-7 cell line. Most of the tested compounds exploited potent to moderate growth inhibitory activity, in particular compound **7e** exhibited superior potency to the reference drug cisplatin (IC_{50} = 7.60 and 13.29 μM, respectively). The antitumor activity of the new compounds was accompanied by significant increase in the activity of superoxide dismutase with concomitant decrease in the activities of catalase and glutathione peroxidase and reduced glutathione level. Accordingly, the overproduction of hydrogen peroxide, nitric oxide and other free radicals allowed reactive oxygen species (ROS)-mediated tumor cells death, as monitored by reduction in the synthesis of protein and nucleic acids.

1. Introduction

Although there have been great advances in the detection and treatment of cancer, it remains one of the greatest medical challenges, with the incidence of some malignancies continuing to increase.[1] For many tumor types, established treatments such as cytotoxic chemotherapy and radiotherapy provide only transient therapeutic benefits despite severe side effects.[2] Therefore, the need for better treatments has stimulated research to develop new efficient chemotherapeutic agents for management of cancer.

In the past few years, several studies have been devoted to the antiproliferative activity of aroylhydrazone derivatives. Where, a plethora of hydrazone compounds has been reported to have inhibitory effect on the growth of a number of tumor cells including leukemia, colon, ovarian, renal, lung and breast cancer cell lines.[3–5] For example, a series of 2-arylamino-6-trifluoromethyl-3-(hydrazonocarbonyl)pyridines **1** was found to have excellent growth inhibitory activity against the full panel of human tumor cell lines.[6] In addition, aroylhydrazones of 2-phenylindole-3-carbaldehydes were able to cause cell cycle arrest accompanied by apoptosis in MCF-7 human breast adenocarcinoma cell line.[7] Moreover, a number of guanylhydrazone derivatives were able to induce apoptosis and to cause oxidative stress in HT-29 colon cancer and HL-60 leukemia cell lines.[8,9]

On the other hand, extensive studies have been conducted on pyrazolo[3,4-*d*]pyrimidines as anticancer agents. Several members of this family were found to induce apoptosis and/or reduce cell proliferation in different solid tumor and leukemia cell lines.[10–15] Different mechanisms account for the cytotoxic activity of this class of compounds, where they have been reported to act as epidermal growth factor (EGFR) inhibitors,[11,16] mammalian target of rapamycin (mTOR) inhibitors,[10] Src or dual Src/Abl inhibitors,[17–19] cyclin dependent kinase (CDK) inhibitors,[20] glycogen synthase kinase-3β (GSK-3β) inhibitors,[21,22] xanthine oxidase inhibitors[23] or through modulating oxygen stress in cancer cells.[24]

Stimulated by the successful applications of pyrazolo[3,4-*d*]pyrimidines and aroylhydrazones as antitumor agents, our objective was to synthesize a new class of pyrazolo[3,4-*d*]pyrimidines endowed with the structural elements of hydrazones, hoping that the hybrid of these pharmacophoric features would produce enhanced antitumor activity. During a previous study,[25] 6-methyl-1-phenyl-5-(quinolin-3-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **2** was found to exploit potent cytotoxic activity against MCF-7 cell line. Since the antitumor activity associated with hydrazones has been attributed to the presence of the CONH–N=CH pharmacophore with active azomethine proton,[3] therefore a new series of N^1-(4-chlorophenyl) pyrazolo[3,4-*d*]

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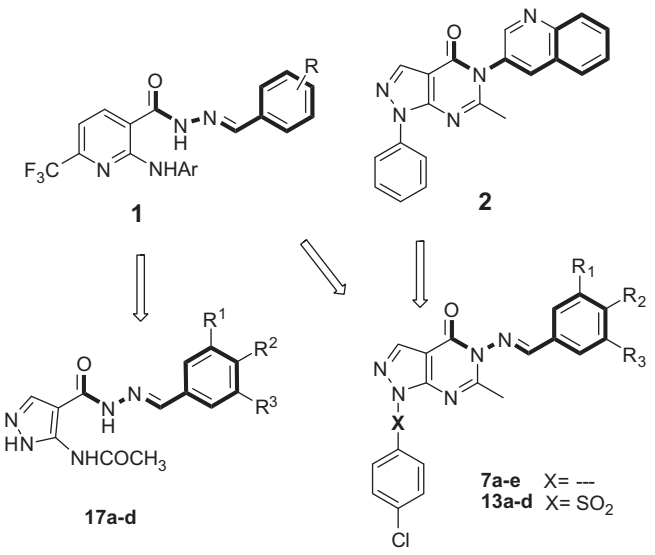


Figure 1. Strategies for the design of the newly synthesized compounds.

pyrimidine hydrazones **7a–e** was designed in which the 5-quinoline ring in compound **2** was replaced with 5-arylideneamino moiety (Fig. 1). Furthermore, arylsulfonylimidazolidinone[26,27] and arylsufonylpyrazole[28] derivatives were claimed to exhibit excellent activity against a broad spectrum of human tumor cell lines. Accordingly, a second series of N^1-(4-chlorophenylsulfonyl)pyrazolo[3,4-*d*]pyrimidine hydrazones **13a–d** was synthesized to explore the influence of incorporating sulfonyl group on the antitumor activity. In an attempt to prepare the N^1-phenylsulfonyl and N^1-4-methylphenylsulfonyl analogues of **13a–d**, only the 4-(arylidene)hydrazinecarbonylpyrazole congeners **17a–d** were obtained. The cytotoxic activity of the produced pyrazoles **17a–d** was evaluated due to structural similarity with the lead compound **1** (Fig. 1).

Oxygen is the essential molecule for all aerobic organisms, and plays predominant role in ATP generation, namely, oxidative phosphorylation. During this process, reactive oxygen species (ROS), such as superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are produced as by-products,[29] under physiological conditions; cells have a series of defense systems to counteract these reactive insults. Such defense systems include intracellular superoxide dismutase (SOD) that converts $O_2^{\cdot-}$ to H_2O_2, catalase (CAT) and glutathione peroxidase (GSH-Px) that eliminate H_2O_2, in addition to free radical scavenging compounds like glutathione (GSH).[29,30] The balance of ROS formation and antioxidative defense level is crucial to cell survival and growth.[29] On the contrary, disturbance of this balance can produce oxidative stress. This state of oxidative stress can result in injury to all the important cellular components like proteins, DNA and membrane lipids which can cause cell death.[31–33]

Tumor cells have higher levels of ROS and are frequently more deficient in most crucial antioxidative enzymes than normal cells, therefore they are more vulnerable to additional oxidative stress.[34,35] Accordingly, a unique antitumor strategy named 'oxidative therapy' was developed by delivering excess oxidative stress or disrupting antioxidative defense system in cancer cells.[36,37] Many conventional anticancer drugs like camptothecin, doxorubicin, cisplatin and anthracyclines exhibit antitumor activity by generating ROS.[38] In addition, several pyrazolo[3,4-*d*]pyrimidines and hydrazones are known to induce apoptotic death in cancer cells via generation of reactive oxygen species.[7,8,24] Hence, the effect of the prepared compounds on the activities of the free-radical-metabolizing enzymes as well as the levels of the oxidative stress parameters in MCF-7 cells were estimated.

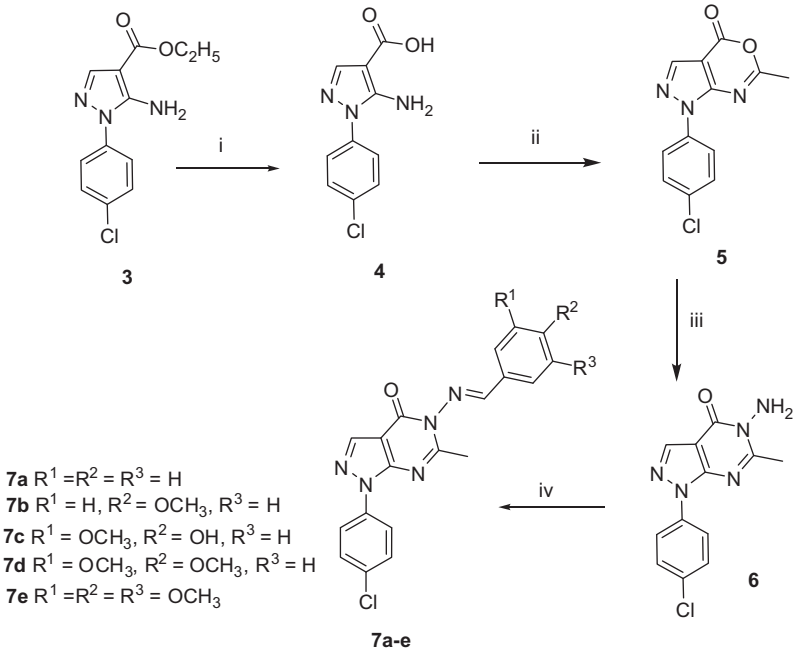
2. Results and discussion

2.1. Chemistry

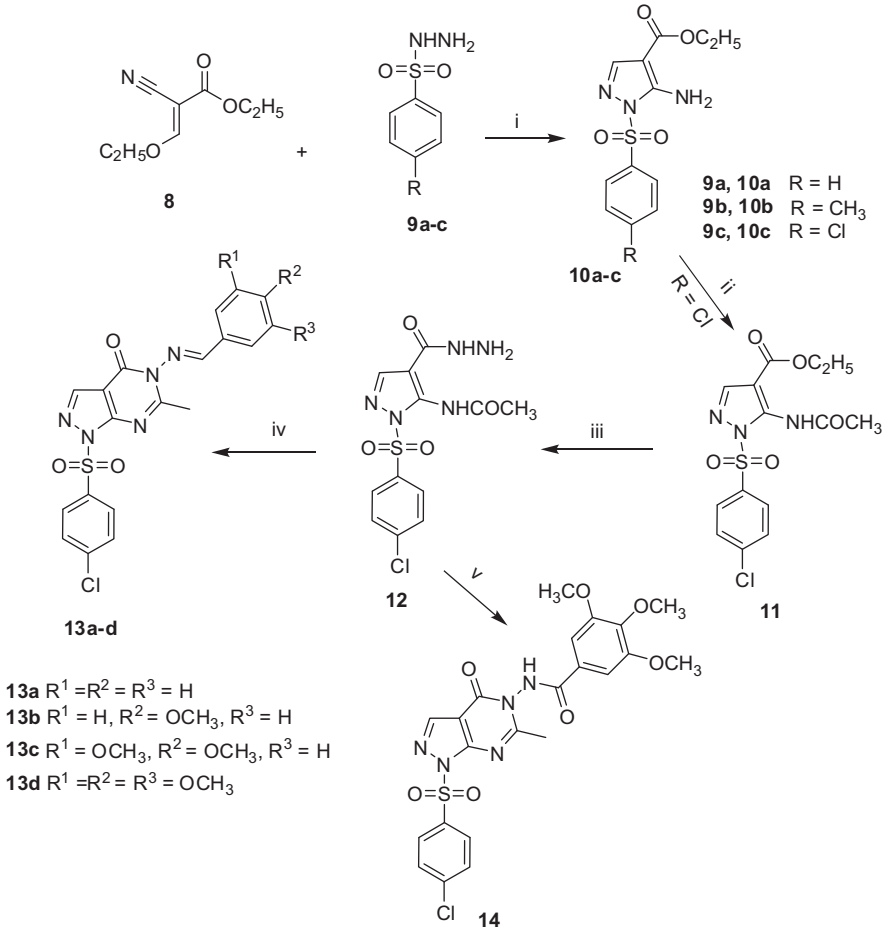
The synthetic approaches adopted to obtain the target compounds are outlined in Schemes 1–3. Hydrolysis of ethyl 5-amino-1-(4-chlorophenyl)-1*H*-pyrazole-4-carboxylate **3**[39] with alcoholic sodium hydroxide followed by neutralization afforded the corresponding carboxylic acid **4**,[40] which was then refluxed with acetic anhydride to give 1-(4-chlorophenyl)-6-methylpyrazolo[3,4-*d*][1,3]oxazin-4(1*H*)-one **5**. Condensation of the pyrazoloxazine **5** with hydrazine hydrate (99%) afforded the 5-amino pyrazolo[3,4-*d*]pyrimidine derivative **6**. The latter compound was reacted with certain substituted aromatic aldehydes to produce the corresponding Schiff's bases **7a–e** (Scheme 1). IR spectra of compounds **7a–e** showed disappearance of absorption bands for (NH_2). Also, ^{1}H NMR spectra revealed singlet signal resonating at δ 8.62–10.04 ppm for (N=CH) proton. According to literature,[41] the presence of singlet downfield (CH=N) signal accounts for formation of *E*-isomers exclusively.

One of the principal objectives of the present work was the synthesis of N^1-arylsulfonylpyrazolo[3,4-*d*]pyrimidine derivatives (Scheme 2). The ethyl carboxylate intermediates **10a–c** were prepared by the cyclocondensation of ethyl ethoxymethylenecyanoacetate **8** with 4-(un) substituted phenylsulfonyl hydrazines **9a–c** in refluxing acetonitrile. Reacting **10a–c** with acetic anhydride yielded the desired acetylated derivative **11** in case of 4-chlorophenylsulfonyl derivative only. Hydrazinolysis of **11** furnished the respective acid hydrazide **12**. Coupling of **12** with different aromatic aldehydes, followed by in situ intramolecular cyclization yielded the pyrazolo[3,4-*d*]pyrimidine derivatives **13a–d**. While, the reaction of acid hydrazide **12** with 3,4,5-trimethoxybenzoyl chloride afforded compound **14**. The structures of **13a–d** and **14** were established on the basis of their elemental analyses and spectral data. IR spectra of **13a–d** were characterized by the absence of (NH and NH_2) bands and the presence of two sharp peaks around 1303.88–1323.17 and 1126.43–1186.86 cm^{-1} for (SO_2). Their ^{1}H NMR spectra showed singlet downfield (CH=N) signals and two doublets at δ 7.14–7.65 and 7.65–7.93 ppm for 4-chlorophenyl protons. It is worthy to mention that the lack of (NH) bands in the IR spectra and exchangeable (NH) signals in the ^{1}H NMR spectra asserted the production of the cyclic pyrazolo[3,4-*d*] pyrimidines **13a–d**. In contrast, the IR spectral data for compound **14** exhibited characteristic absorption bands for (NH) at 3317.56 cm^{-1} and (SO_2) at 1327.03 and 1157.29 cm^{-1}. Its ^{1}H NMR spectrum showed a typical AB system of 4-chlorophenyl along with single exchangeable signal at δ 10.48 ppm corresponding to the amide NH, which affirmed cyclization.

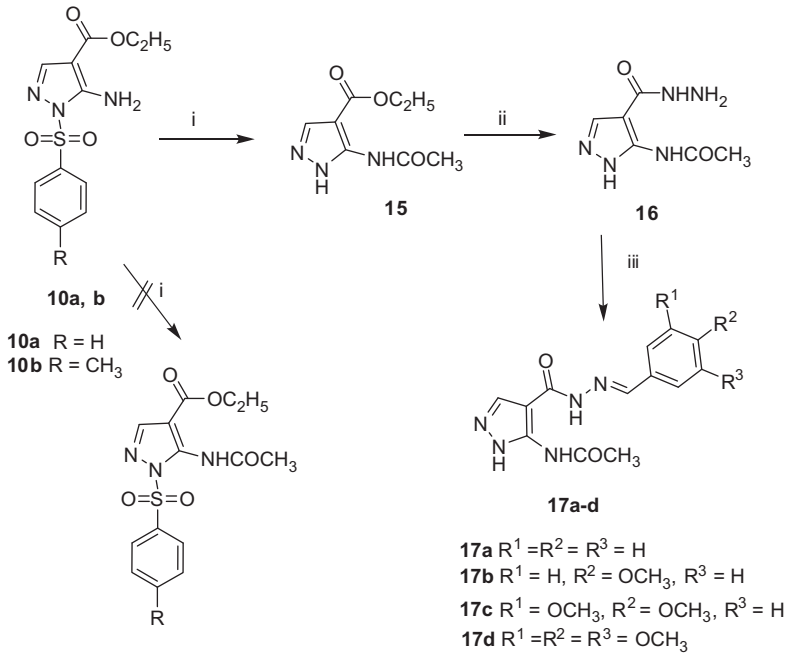
On the other hand, the acetylation of 5-amino-N^1-phenylsulfonyl and 5-amino-N^1-(4-methylphenylsulfonyl)pyrazoles **10a**,**b** produced ethyl 5-acetamido-1*H*-pyrazole-4-carboxylate **15**[42] as a result of hydrolytic cleavage of the SO_2–N bond. Treatment of the ester **15** with hydrazine hydrate furnished the acid hydrazide **16**. Subsequent condensation of **16** with various aromatic aldehydes in refluxing glacial acetic acid for 24 h in an attempt to obtain the cyclized pyrazolo[3,4-*d*]pyrimidine derivatives was not successful. Instead, the 4-(arylidene)hydrazinecarbonylpyrazoles **17a–d** were isolated (Scheme 3). The structure of compounds **17a–d** was supported by analytical and spectral data. IR spectra of compounds **17a–d** displayed no absorption bands of (SO_2) group, while those derived from (NH) and (C=O) were observed. The ^{1}H NMR spectra of compounds **17b–d** in DMSO-d_6 displayed



Scheme 1. Reagents and conditions: (i) NaOH, CH_3OH, reflux 10 h; (ii) acetic anhydride, reflux, 4 h; (iii) $NH_2NH_2{\cdot}H_2O$, *n*-butanol, reflux 24 h; (iv) Ar-CHO, glacial acetic acid, reflux 16 h.



Scheme 2. Reagents and conditions: (i) CH_3CN, reflux 6 h; (ii) acetic anhydride, reflux,4 h; (iii) $NH_2NH_2{\cdot}H_2O$, *n*-butanol, reflux 24 h; (iv) Ar-CHO, glacial acetic acid, reflux 24 h; (v) $(OCH_3)_3C_6H_2COCl$, dry benzene, stir room temperature 3 h.



Scheme 3. Reagents and conditions: (i) acetic anhydride, reflux, 4 h; (ii) $NH_2NH_2{\cdot}H_2O$, *n*-butanol, reflux 24 h; (iii) Ar-CHO, glacial acetic acid, reflux 24 h.

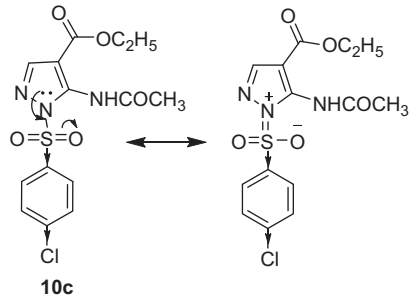


Figure 2. Effect of electron withdrawing chlorine.

a mixture of tautomers, since the proton of cyclic NH is mobile between the two annular nitrogen atoms.[42] Moreover, the ^{1}H NMR spectra confirmed the production of the uncyclized pyrazoles where three pairs of exchangeable singlet signals derived from amide (NH) at δ 10.26–10.64, hydrazide (NH) at δ 11.10–11.60 and pyrazole (NH) at δ 12.84–13.15 were detected.

A point of interest in this investigation was the different behavior of various 5-amino-N^1-arylsulfonylpyrazoles **10a–c** during acetylation with acetic anhydride. The stability of N^1-(4-chlorophenylsulfonyl)pyrazole derivative **10c** to acid hydrolysis could be attributed to the presence 4-chloro substituent. Presumably, the presence of an electron withdrawing chlorine atom on the phenyl ring draws electrons away from the sulfonyl group. Therefore the neighboring nitrogen can feed its lone pair of electrons into the sulfonyl to form a dipolar resonance structure (Fig. 2). The latter is resistant to nucleophilic attack as reflected by their resistance to hydrolysis. Apparently, this resonance stabilization is not favored in case of phenyl and 4-methylphenyl analogues **10a**,**b**.

2.2. In vitro cytotoxic activity

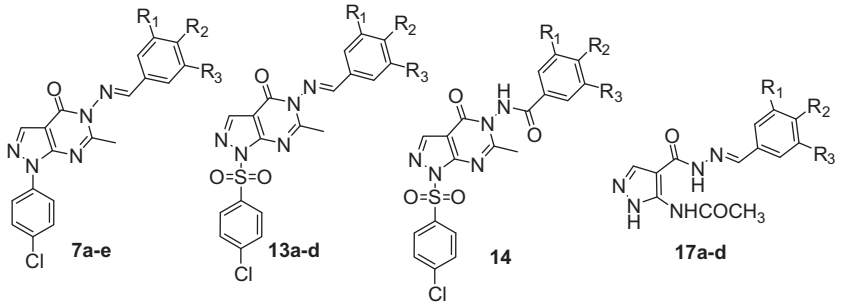
The cytotoxic activity of the newly synthesized compounds was evaluated against MCF-7 cancer cell line using Sulforhodamine B (SRB) colorimetric assay, in comparison with cisplatin as a reference drug.[43]

The cytotoxic activities are expressed by median growth inhibitory concentration (IC_{50}) and provided in Table 1. From the results, it is evident that all the tested compounds displayed potent to moderate growth inhibitory activity, in particular compound **7e** (IC_{50} = 7.60 μM) was found to be more potent and efficacious than cisplatin (IC_{50} values 13.29 μM).

Although the number of tested compounds in this study is limited, some structural features that are important for explanation of their cytotoxic effects can be referred. In general, the pyrazolo[3,4-*d*]pyrimidine containing compounds **7a–e** and **13a–d** were more potent than those possessing pyrazole ring **17a–d**. Moreover, it was envisioned that the incorporation of sulfonyl functionality between pyrazolo[3,4-*d*]pyrimidine scaffold and 4-chlorophenyl moiety in **13a–d** may increase the anticancer activity of compounds **7a–e**. Results in Table 1 illustrated that, adding sulfonyl group had inconsistent effect on activity. While, compound **13a** almost showed the same activity as **7a**, compounds **13b–d** exhibited decline in potency compared with the congeneric compounds **7b–e**, probably due to lower stability of the pyrazole-sulfone linkage. In addition, to verify the hypothesis that integrating an active azomethine proton to the pyrazolo[3,4-*d*]pyrimidine motif would be profitable to the cytotoxic activity, compound **14** having an amide linkage was prepared. As expected, the replacement of azomethine linker in **13d** by an amide linker in **14** led to significant decrease in activity (IC_{50} = 27.80 and 35.77 μM, respectively).

From the other point of view, it has been reported that the presence of electron donating substituent as methoxy or ethoxy group seems to be a contributing factor for high antitumor activity.[44,45] Therefore, the effect of introducing single or multiple methoxy substituents to the arylidene moiety on the activity of the synthesized compounds has been explored. The results indicated that the position and number of methoxy groups contributed to the growth inhibitory activity. Increasing the number of methoxy groups on the arylidene motif was accompanied by enhancement of antiproliferative activity and reached the highest activity with the most potent trimethoxybenzylidene derivative **7e** (IC_{50} = 7.60 μM). However, compounds bearing unsubstituted benzylidene **7a**, **13a**, **17a** demonstrated higher activity compared to those having a single *p*-methoxy group **7b**, **13b**, **17b**. This suggested that *m*-methoxy sub-

Table 1
In vitro cytotoxic activities of the synthesized compounds against MCF-7 cancer cell line



Compound	R^1	R^2	R^3	IC_{50} (μM)
Cisplatin	—	—	—	13.29 ± 1.08
2[25]	—	—	—	1.53 ± 1.12
7a	H	H	H	23.09 ± 1.50
7b	H	OCH_3	H	38.59 ± 3.02
7c	OCH_3	OH	H	19.76 ± 1.57
7d	OCH_3	OCH_3	H	25.01 ± 1.43
7e	OCH_3	OCH_3	OCH_3	7.60 ± 0.71
13a	H	H	H	22.44 ± 1.48
13b	H	OCH_3	H	41.93 ± 2.53
13c	OCH_3	OCH_3	H	33.61 ± 1.86
13d	OCH_3	OCH_3	OCH_3	27.80 ± 2.05
14	OCH_3	OCH_3	OCH_3	35.77 ± 2.19
17a	H	H	H	42.04 ± 3.35
17b	H	OCH_3	H	61.73 ± 3.90
17c	OCH_3	OCH_3	H	37.12 ± 2.69
17d	OCH_3	OCH_3	OCH_3	32.10 ± 2.35

Data are expressed as means ± SE of three separate experiments.

stituent seems to be more favorable than *p*-methoxy substituent for antitumor activity. The cytotoxic ability of this class of compounds appears to depend on the position of methoxy group rather than its electronic effect. This assumption could be supported by the fact that replacing the *p*-methoxy in **7d** by *p*-hydroxy in **7c**—that has the same electronic characters—was accompanied by increase in potency (IC_{50} = 25.01 and 19.76 μM, respectively).

2.3. Biochemical assays

To elucidate the mechanism by which the new compounds exert their antitumor activities, six compounds of various levels of antiproliferative activity were selected to estimate their ability to induce oxidative stress in cancer cells. We estimated the activities of the free-radical-metabolizing enzymes including SOD, CAT and GSH-Px as well as the levels of the oxidative stress parameters including H_2O_2, nitric oxide (NO) and reduced GSH in MCF-7 cells treated with the prepared compounds. Additionally, the effect of these compounds on the levels of total protein and nucleic acids was determined.

As shown in Table 2, in general treatment of the cells with different compounds or cisplatin (at the 1/10 of IC_{50} values)[24] resulted in a significant increase in the activity of SOD and the level of H_2O_2 higher than those of control, accompanied with a significant decrease in the activity of CAT and GSH-Px, and depletion in GSH level. This means that the antitumor activity of these compounds was accompanied with high activity of SOD with subsequent increase in H_2O_2 production. The produced H_2O_2 should be rapidly removed through the activation of CAT and GSH-Px. The present results showed that activities of CAT and GSH-Px and the level of reduced GSH were lowered in groups treated with the prepared compounds compared to control cells. Consequently, the excess H_2O_2 produced in tumor cells with the compounds can not be removed. In other words, the accumulation of H_2O_2 and other free radicals in tumor cells should be partly the cause of tumor cell killing.

It should be mentioned that, the changes in the activities of the free-radical-metabolizing enzymes and oxidative stress parameters were in the order of **7e > 7c > 7a > 13d > 17d > 7b** which is in accordance with the order of cytotoxic activity of the tested compounds. The highest activity was found for the most potent antitumor compound **7e**, which resulted in the highest SOD activity and H_2O_2 and low activities of CAT and GSH-Px as well as GSH level than the other tested compounds. The consistency between cytotoxic activity and biochemical assay results indicated that the antitumor effect of the present compounds may be exerted at least partly by production of ROS.

Moreover, results in Table 3 illustrated that, treatment with these compounds led to significant increase in the level of NO. There is a growing body of evidence indicating that NO is able to induce apoptosis by helping to dissipate the membrane potential of mitochondria making it more permeable.[46] In addition; the elevated level of NO was accompanied with depletion in the levels of total protein and nucleic acids compared to control. This can be explained by several cytotoxic effects that include reaction of NO with proteins and nucleic acids. The main targets of NO in proteins are the thiol group[47] and iron of active sites.[48] In the nucleus, NO has been shown to cause gene mutation,[49] to inhibit DNA repair enzymes,[50] and to mediate DNA strand breaks.[51] Moreover, Bienvenu et al.,[52] reported that most chemotherapeutic agents cause cells to over-generate ROS and thus, are capable of inducing apoptosis, and causing oxidative damage to DNA and proteins. The cascade of signals mediating apoptosis often involves a ROS intermediate messenger, and ROS can short circuit the pathway, bypassing the need for upstream signals for cell suicide. Later, Huang et al.[37] reported that regulation of free radical-producing agents may also have important clinical applications. This mechanism for the effects of ROS generating anticancer agents has been understood recently, as previously the mechanism of most anticancer agents was believed to be mainly due to direct interaction with DNA, interference with DNA regulatory machinery (e.g., topoi-

Table 2
Effect of treatment with the prepared compounds on the activities of SOD, CAT, GSH-Px, as well as the levels of reduced GSH and H_2O_2 in MCF-7 treated cells

Treatment (μg/ml)	SOD U/mg protein	CAT U/mg protein	GSH-Px U/mg protein	GSH nmol/mg protein	H_2O_2 nmol/mg protein
Control (DMSO)	40.30 ± 4.75	7.60 ± 0.70	9.30 ± 1.00	40.00 ± 5.00	15.70 ± 1.60
Cisplatin	130.80 ± 15.65[a]	2.96 ± 0.22[a]	4.40 ± 0.40[a]	21.60 ± 2.40[a]	47.50 ± 5.70[a]
7a	95.25 ± 11.00[a]	3.82 ± 0.42[a]	6.00 ± 0.60[a]	30.40 ± 3.20[b]	30.00 ± 2.80[a,b]
7b	50.00 ± 5.20[b]	7.40 ± 0.85[b]	8.00 ± 0.82[b]	36.30 ± 2.45[a]	24.80 ± 2.90[a]
7c	100.30 ± 11.70[a]	3.50 ± 0.40[a]	5.40 ± 0.60[a]	23.50 ± 2.20[a]	39.40 ± 3.65[a,b]
7e	110.00 ± 12.90[a]	2.20 ± 0.24[a]	4.85 ± 0.62[a]	17.80 ± 0.20[a,b]	53.30 ± 4.80[b]
13d	80.00 ± 9.22[a,b]	4.30 ± 0.36[a,b]	6.80 ± 0.62[a]	34.20 ± 2.70[a]	27.80 ± 3.00[a]
17d	75.44 ± 9.20[a,b]	5.85 ± 0.60[b]	7.33 ± 0.85[b]	30.11 ± 3.32[a]	25.60 ± 2.40[a,b]

Data are expressed as means ± SE of six separate experiments. [a] and [b] are significant differences from control and cisplatin groups respectively at (p <0.05).

Table 3
Effect of the prepared compounds on the level of total protein, nucleic acids (RNA and DNA) and NO in MCF-7 treated cells

Compounds	Protein (μg/10^6 cells)	RNA (μg/10^6 cells)	DNA (μg/10^6 cells)	NO (μmol/mg protein)
Control (DMSO)	110.50 ± 12.30	15.30 ± 1.60	8.50 ± 0.80	1.90 ± 0.16
Cisplatin	33.60 ± 3.70[a]	3.40 ± 0.40[a]	2.50 ± 0.30[a]	4.20 ± 0.37[a]
7a	55.40 ± 6.00[a,b]	5.00 ± 0.48[a,b]	5.11 ± 0.55[a,b]	3.00 ± 0.33[a,b]
7b	80.20 ± 8.40[a,b]	12.00 ± 1.73[b]	7.00 ± 0.73[a,b]	2.90 ± 0.40[a,b]
7c	42.60 ± 4.72[a,b]	3.20 ± 0.28[a]	4.50 ± 0.52[a,b]	3.60 ± 0.37[a]
7e	27.50 ± 2.60[a]	2.60 ± 0.28[a,b]	3.00 ± 0.36[a]	4.40 ± 0.45[a]
13d	78.30 ± 8.60[a,b]	7.22 ± 0.60[a,b]	5.30 ± 0.71[a,b]	2.80 ± 0.70[a,b]
17d	85.80 ± 8.40[a,b]	8.80 ± 0.75[a,b]	6.10 ± 0.45[a,b]	3.00 ± 0.34[a,b]

The values are expressed as mean ± SE of six separate experiments. [a] and [b] are significant differences from control and cisplatin groups respectively at (p <0.05).

somerases, helicases) and the initiation of DNA damage via production of ROS.[53]

In summary, the present results suggest that the synthesized compounds possess significant antitumor activity comparable to the activity of commonly used anticancer drug, cisplatin and they exert their antitumor activities by modulating free radicals production through increasing the activity of SOD and depletion of intracellular reduced GSH level, CAT and GSH-Px activities, accompanied with high production of H_2O_2, NO and other free radicals causing tumor cells death, as monitored by reduction in the synthesis of protein and nucleic acids.

3. Conclusion

Three series of N^1-(4-chlorophenyl)pyrazolo[3,4-*d*]pyrimidines **7a–e**, N^1-(4-chlorophenylsulfonyl)pyrazolo[3,4-*d*]pyrimidines **13a–d** and 4-(hydrazinecarbonyl) pyrazoles **17a–d** endowed with arylidene moiety were synthesized. The new compounds were evaluated for their cytotoxic activity against MCF-7 breast cancer cells compared to cisplatin as reference drug. Some structural features were found to be beneficial to the antitumor activity of such compounds. In particular, the pyrazolo[3,4-*d*]pyrimidine containing compounds **7a–e** and **13a–d** were more potent than those possessing pyrazole ring **17a–d**. Also, substitution with N^1-(4-chlorophenyl) on the pyrazolo[3,4-*d*]pyrimidine scaffold in **7a–e** was more favorable than N^1-(4-chlorophenylsulfonyl) in **13a–d**. Meanwhile, increasing the number of methoxy groups on the arylidene substituent was accompanied by enhancement of antitumor activity and reached the highest activity with the most potent trimethoxybenzylidene derivative **7e** (IC_{50} = 7.60 μM).

Moreover, compounds **7a–c**, **7e**, **13d** and **17d** were evaluated for their ability to induce oxidative stress in cancer cells. Estimating the activities of the free-radical-metabolizing enzymes and the levels of the oxidative stress parameters suggested that the antitumor effect of the present compounds may be exerted by modulating free radicals production within the tumor cell. Although, the biological screening results of the tested compounds could offer a promising framework that may lead to discovery of potent anticancer agents, there is concern for selective production of oxidative stress in cancer cells only that initiates the need for further investigation.

4. Experimental

4.1. Chemistry

Melting points are uncorrected and determined in one end open capillary tubes using Gallen Kamp melting point apparatus MFB-595-010M (Gallen Kamp, London, England). Microanalysis was carried out at Micro-analytical Unit, Faculty of Science, Cairo University, the regional center for microbiology and biotechnology, Al-Azhar University and Organic Microanalyses Section, Central Laboratory, National Research Center. Infrared Spectra were recorded on Schimadzu FT-IR 8400S spectrophotometer (Shimadzu, Kyoto, Japan), and expressed in wave number (cm^{-1}), using potassium bromide discs. The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer. ^1H spectra were run at 300 MHz and ^{13}C spectra were run at 75.46 MHz in deuterated chloroform ($CDCl_3$) or dimethylsulfoxide (DMSO-d_6). Chemical shifts are quoted in δ and were related to that of the solvents. Mass spectra were recorded using Hewlett Packard Varian (Varian, Polo, USA) and Shimadzu Gas Chromatograph Mass spectrometer-QP 1000 EX (Shimadzu, Kyoto, Japan). TLC were carried out using Art.DC-Plastikfolien, Kieselgel 60 F254 sheets (Merck, Darmstadt, Germany), the developing solvents were benzene/methanol (4:1) and the spots were visualized at 366, 254 nm by UV Vilber Lourmat 77202 (Vilber, Marne La Vallee, France).

Compounds **3**[39] and **4**[40] were obtained according to the reported procedures.

4.1.1. 1-(4-Chlorophenyl)-6-methylpyrazolo[3,4-*d*][1,3]oxazin-4(1*H*)-one (5)

A mixture of 5-amino-1-(4-chlorophenyl)-1*H*-pyrazole-4-carboxylic acid **4** (2.37 g, 0.01 mol) and acetic anhydride (5 ml) was heated under reflux for 4 h. The reaction mixture was cooled to

room temperature. The formed solid was filtered, dried and crystallized from methanol.

Mp 147–148 °C; yield: 68%. IR ν_{max}/cm^{-1}: 3132.40 (CH aromatic), 1778.37 (C=O). ^1H NMR ($CDCl_3$) δ ppm: 2.55 (s, 3H, CH_3), 7.51 (d, 2H, H-3 and H-5 of Cl-C_6H_4, J = 8.7 Hz), 7.99 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 8.7 Hz), 8.19 (s, 1H, H-3 pyrazole). Anal. Calcd for $C_{12}H_8ClN_3O_2$ (261.66): C, 55.08; H, 3.08; N, 16.06. Found: C, 55.27; H, 3.16; N, 16.19.

4.1.2. 5-Amino-1-(4-chlorophenyl)-6-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (6)

A mixture of 1-(4-chlorophenyl)-6-methylpyrazolo[3,4-*d*][1,3] oxazin-4(1*H*)-one **5** (2.62 g, 0.01 mol) and hydrazine hydrate (0.60 ml, 0.012 mol) in *n*-butanol (20 ml) was heated under reflux for 24 h. The reaction mixture was concentrated in vacuum then cooled. The separated solid was filtered and crystallized from ethanol.

Mp 257–259 °C; yield: 52%. IR ν_{max}/cm^{-1}: 3309.85, 3275.13 (NH_2), 3101.45 (CH aromatic), 1693.50 (C=O). ^1H NMR (DMSO-d_6) δ ppm: 2.64 (s, 3H, CH_3), 5.77 (s, 2H, NH_2, exch. D_2O), 7.61 (d, 2H, H-3 and H-5 of Cl-C_6H_4, J = 8.7 Hz), 8.13 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 8.7 Hz), 8.34 (s, 1H, H-3 pyrazole). Anal. Calcd for $C_{12}H_{10}ClN_5O$ (275.69): C, 52.28; H, 3.66; N, 25.40. Found: C, 52.60; H, 3.81; N, 25.22.

4.1.3. General procedure for the synthesis of (*E*)-5-(substituted benzylideneamino)-1-(4-chlorophenyl)-6-methyl-1*H*-pyrazolo [3,4-*d*]pyrimidin-4(5*H*)-one (7a–e)

A mixture of **6** (2.75 g, 0.01 mol) and the appropriate aromatic aldehyde (0.012 mol) in glacial acetic acid (10 ml) was heated under reflux for 16 h. The reaction mixture was concentrated in vacuum then cooled. The obtained solid was filtered, dried and crystallized from ethanol.

4.1.3.1. (*E*)-5-(Benzylideneamino)-1-(4-chlorophenyl)-6-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (7a). Mp 230–232 °C; yield: 75%. IR ν_{max}/cm^{-1}: 3010.50 (CH aromatic), 1712.79 (C=O). ^1H NMR ($CDCl_3$) δ ppm: 2.69 (s, 3H, CH_3), 7.46–7.57 (m, 3H, H-3, H-4 and H-5 of C_6H_5), 7.89 (d, 2H, H-3 and H-5 of Cl-C_6H_4, J = 8.4 Hz), 8.09–8.25 (m, 4H, H-2 and H-6 of C_6H_5 and H-2 and H-6 of Cl-C_6H_4), 8.91 (s, 1H, H-3 pyrazole), 10.04 (s, 1H, N=CH). Anal. Calcd for $C_{19}H_{14}ClN_5O$ (363.80): C, 62.73; H, 3.88; N, 19.25. Found: C, 62.83; H, 3.75; N, 19.45.

4.1.3.2. (*E*)-1-(4-Chlorophenyl)-5-(4-methoxybenzylideneamino)-6-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (7b). Mp 244–246 °C; yield: 60%. IR ν_{max}/cm^{-1}: 3001.24 (CH aromatic), 2954.95, 2830.50 (CH aliphatic), 1701.22 (C=O). ^1H NMR (DMSO-d_6) δ ppm: 1.97 (s, 3H, CH_3), 3.84 (s, 3H, OCH_3), 6.85 (d, 2H, H-3 and H-5 of OCH_3-C_6H_4, J = 8.1 Hz), 7.06 (d, 2H, H-3 and H-5 of Cl-C_6H_4, J = 8.4 Hz), 7.56 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 8.4 Hz), 7.82 (d, 2H, H-2 and H-6 of OCH_3-C_6H_4, J = 8.1 Hz), 8.63 (s, 1H, H-3 pyrazole), 9.73 (s, 1H, N=CH). Anal. Calcd for $C_{20}H_{16}ClN_5O_2$ (393.83): C, 60.99; H, 4.09; N, 17.78. Found: C, 61.20; H, 4.10; N, 17.70.

4.1.3.3. (*E*)-1-(4-Chlorophenyl)-5-(4-hydroxy-3-methoxybenzylideneamino)-6-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (7c). Mp 261–263 °C; yield: 52%. IR ν_{max}/cm^{-1}: 3414.00 (OH), 3008.95 (CH aromatic), 2920.23, 2850.79 (CH aliphatic), 1708.93 (C=O). ^1H NMR (DMSO-d_6) δ ppm: δ 2.48 (s, 3H, CH_3), 3.85 (s, 3H, OCH_3), 6.94 (d, 1H, H-5 of OH-OCH_3-C_6H_3, J = 7.8 Hz), 7.35 (d, 1H, H-6 of OH–OCH_3–C_6H_3, J = 7.8 Hz), 7.55 (s, 1H, H-2 of OH-OCH_3-C_6H_3), 7.65 (d, 2H, H-3 and H-5 of Cl-C_6H_4, J = 9.0 Hz), 8.16 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 9.0 Hz), 8.38 (s, 1H, H-3 pyrazole), 8.62 (s, 1H, N=CH), 10.05 (s, 1H, OH, exch. D_2O). Anal. Calcd for $C_{20}H_{16}ClN_5O_3$ (409.83): C, 58.61; H, 3.94; N, 17.09. Found: C, 59.00; H, 4.24; N, 17.00.

4.1.3.4. (*E*)-1-(4-Chlorophenyl)-5-(3,4-dimethoxybenzylideneamino)-6-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (7d). Mp 252–254 °C; yield: 70%. IR ν_{max}/cm^{-1}: 3050.00 (CH aromatic), 3000.00, 2954.95 (CH aliphatic), 1705.07 (C=O). ^1H NMR (DMSO-d_6) δ ppm: 2.48 (s, 3H, CH_3), 3.84 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 7.13 (d, 1H, H-5 of $(OCH_3)_2$–C_6H_3, J = 8.4 Hz), 7.45 (d, 1H, H-6 of $(OCH_3)_2$–C_6H_3, J = 8.4 Hz), 7.57 (s, 1H, H-2 of $(OCH_3)_2$–C_6H_3), 7.66 (d, 2H, H-3 and H-5 of Cl-C_6H_4, J = 8.7 Hz), 8.15 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 8.7 Hz), 8.38 (s, 1H, H-3 pyrazole), 8.70 (s, 1H, N=CH). Anal. Calcd for $C_{21}H_{18}ClN_5O_3$ (423.85): C, 59.51; H, 4.28; N, 16.52. Found: C, 59.20; H, 4.55; N, 16.24.

4.1.3.5. (*E*)-1-(4-Chlorophenyl)-6-methyl-5-(3,4,5-trimethoxybenzylideneamino)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (7e). Mp 232–234 °C; yield: 55%. IR ν_{max}/cm^{-1}: 3005.10 (CH aromatic), 2943.37, 22831.50 (CH aliphatic), 1697.36 (C=O). ^1H NMR ($CDCl_3$) δ ppm: 2.75 (s, 3H, CH_3), 3.94 (s, 3H, OCH_3), 3.95 (s, 6H, 2 OCH_3), 7.14 (s, 2H, H-2 and H-6 of $(OCH_3)_3$–C_6H_2), 7.46 (d, 2H, H-3 and H-5 of Cl-C_6H_4, J = 8.7 Hz), 8.12 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 8.7 Hz), 8.74 (s, 1H, H-3 pyrazole), 9.88 (s, 1H, N=CH). Anal. Calcd for $C_{22}H_{20}ClN_5O_4$ (453.88): C, 58.22; H, 4.44; N, 15.43. Found: C, 57.96; H, 4.28; N, 15.55.

4.1.4. General procedure for the synthesis of ethyl 5-amino-1-(4-substituted phenylsulfonyl)-1*H*-pyrazole-4-carboxylate (10a–c)

A solution of ethyl ethoxymethylenecyanoacetate **8** (5.07 g, 0.03 mol) and the proper phenylsulfonylhydrazines **9a–c** (0.03 mol) in acetonitrile (50 ml) was heated under reflux for 6 h. The reaction mixture was concentrated in vacuum then cooled. The separated solid was filtered and crystallized from acetonitrile.

4.1.4.1. Ethyl 5-amino-1-(phenylsulfonyl)-1*H*-pyrazole-4-carboxylate (10a). Mp 149–151 °C; yield: 93%. IR ν_{max}/cm^{-1}: 3437.15, 3309.85 (NH_2), 3062.96 (CH aromatic), 2985.81 (CH aliphatic), 1693.50 (C=O), 1381.03, 1134.14 (SO_2). ^1H NMR ($CDCl_3$) δ ppm: 1.31 (t, 3H, CH_2CH_3, J = 7.2 Hz), 4.21 (q, 2H, CH_2CH_3, J = 7.2 Hz), 6.55 (s, 2H, NH_2, exch. D_2O), 7.52 (d, 2H, H-3 and H-5 of C_6H_5, J = 7.5 Hz), 7.64–7.69 (m, 2H, H-4 of C_6H_5 and H-3 of pyrazole), 7.98 (d, 2H, H-2 and H-6 of C_6H_5, J = 7.5 Hz). Anal. Calcd for $C_{12}H_{13}N_3O_4S$. H_2O (313.33): C, 46.00; H, 4.83; N, 13.41. Found: C, 45.84; H, 4.62; N, 13.48.

4.1.4.2. Ethyl 5-amino-1-(4-methylphenylsulfonyl)-1*H*-pyrazole-4-carboxylate (10b). Mp 122–123 °C; yield: 90%. IR ν_{max}/cm^{-1}: 3460.30, 3267.41 (NH_2), 3132.40 (CH aromatic), 2985.81 (CH aliphatic), 1693.50 (C=O), 1365.60, 1138.00 (SO_2). ^1H NMR ($CDCl_3$) δ ppm: 1.35 (t, 3H, CH_2CH_3, J = 7.2 Hz), 2.43 (s, 3H, CH_3), 4.26 (q, 2H, CH_2CH_3, J = 7.2 Hz), 6.52 (s, 2H, NH_2, exch. D_2O), 7.35 (d, 2H, H-3 and H-5 of CH_3–C_6H_4, J = 8.1 Hz), 7.87 (d, 2H, H-2 and H-6 of CH_3–C_6H_4, J = 8.4 Hz), 8.30 (s, 1H, H-3 pyrazole). Anal. Calcd for $C_{13}H_{15}N_3O_4S$ (309.34): C, 50.47; H, 4.89; N, 13.58. Found: C, 50.92; H, 5.08; N, 13.67.

4.1.4.3. Ethyl 5-amino-1-(4-chlorophenylsulfonyl)-1*H*-pyrazole-4-carboxylate (10c). Mp 162–164 °C; yield: 84%. IR ν_{max}/cm^{-1}: 3429.43, 3294.42 (NH_2), 3066.82 (CH aromatic), 2985.81 (CH aliphatic), 1689.64 (C=O), 1384.89, 1130.29 (SO_2). ^1H NMR ($CDCl_3$) δ ppm: 1.33 (t, 3H, CH_2CH_3, J = 7.2 Hz), 4.26 (q, 2H, CH_2CH_3, J = 7.2 Hz), 6.52 (s, 2H, NH_2, exch. D_2O), 7.54 (d, 2H, H-3 and H-5 of Cl-C_6H_4, J = 9.6 Hz), 7.95 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 9.6 Hz), 8.50 (s, 1H, H-3 pyrazole). MS, m/z: 356 [M^+–CH_3]. Anal. Calcd for

$C_{12}H_{12}ClN_3O_4S$ (329.76): C, 43.71; H, 3.67; N, 12.74; S, 9.72. Found: C, 44.00; H, 4.00; N, 13.00; S, 9.52.

4.1.5. Ethyl 5-acetamido-*N*-(4-chlorophenylsulfonyl-1*H*-pyrazole-4-carboxylate (11)

A mixture of compound **10c** (3.39 g, 0.01 mol) in acetic anhydride (10 ml) was refluxed for 4 h. The reaction mixture was cooled and filtered; the obtained solid was crystallized from absolute ethanol.

Mp 210–212 °C; yield: 60%. IR ν_{max}/cm^{-1}: 3294.13 (NH), 3066.82 (CH aromatic), 2985.81 (CH aliphatic), 1710.0 (C=O), 1689.64 (C=O), 1276.88, 1114.86 (SO_2). ^1H NMR ($CDCl_3$) δ ppm: 1.38 (t, 3H, CH_2CH_3, J = 7.2 Hz), 2.30 (s, 3H, CH_3), 4.37 (q, 2H, CH_2CH_3, J = 7.2 Hz), 7.50 (d, 2H, H-3 and H-5 of Cl-C_6H_4, J = 9.6 Hz), 7.84 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 9.6 Hz), 7.88 (s, 1H, H-3 pyrazole), 9.62 (s, br, 1H, N*H*COCH$_3$ exch. D_2O). MS, *m/z*: 356 [M^+-CH_3]. Anal. Calcd for $C_{14}H_{14}ClN_3O_5S$ (371.8): C, 45.23; H, 3.80; N, 11.30; S, 8.62. Found: C, 45.54; H, 3.95; N, 11.30; S, 8.60.

4.1.6. *N*-(4-Chlorophenylsulfonyl)-4-(hydrazinecarbonyl)-1*H*-pyrazol-5-yl) acetamide (12)

A mixture of **11** (3.71 g, 0.01 mol) and hydrazine hydrate (0.60 ml, 0.012 mol) in *n*-butanol (20 ml) was refluxed for 24 h. The reaction mixture was concentrated in vacuum then cooled. The separated solid was filtered and crystallized from ethanol.

Mp 184–186 °C; yield: 65%. IR ν_{max}/cm^{-1}: 3410.15, 3302.13, 3178.89 (NH_2 and NH), 3093.82 (CH aromatic.), 2924.09, 2821.21 (CH aliphatic), 1624.06 (br, 2 C=O), 1315.45, 1149.57 (SO_2). ^1H NMR (DMSO-d_6) δ ppm: 2.42 (s, 3H, CH_3), 4.20 (s, br, 2H, CONHN*H*$_2$, exch. D_2O), 5.59 (s, br, 1H, CON*H*NH$_2$, exch. D_2O), 6.82 (d, 2H, H-3 and H-5 of Cl-C_6H_4, J = 8.7 Hz), 7.64 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 8.7 Hz), 7.84 (s, 1H, H-3 pyrazole), 8.89 (s, br, 1H, N*H*COCH$_3$ exch. D_2O). MS, *m/z*: 359 [M^++2]. Anal. Calcd for $C_{12}H_{12}ClN_5O_4S$ (357.77): C, 40.28; H, 3.38; N, 19.57; S, 8.96. Found: C, 40.28; H, 3.38; N, 19.75; S, 8.66.

4.1.7. General procedure for the synthesis of (*E*)-5-(substituted benzylideneamino)-1-(4-chlorophenylsulfonyl)-6-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (13a–d)

A mixture of **12** (3.57 g, 0.01 mol) and the appropriate aldehyde (0.012 mol) in glacial acetic acid (10 ml) were heated under reflux for 24 h. The reaction mixture was concentrated in vacuum then cooled. The obtained solid product was filtered, dried and crystallized from acetic acid.

4.1.7.1. (*E*)-5-(Benzylideneamino)-1-(4-chlorophenylsulfonyl)-6-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (13a).

Mp 190–192 °C; yield: 92%. IR ν_{max}/cm^{-1}: 3010.50 (CH aromatic), 1693.20 (C=O), 1318.13, 1149.57 (SO_2). ^1H NMR ($CDCl_3$) δ ppm: 1.56 (s, 3H, CH_3), 7.14 (d, 2H, H-3 and H-5 of Cl-C_6H_4, J = 8.7 Hz), 7.37–7.47 (m, 3H, H-3, H-4 and H-5 of C_6H_5), 7.65 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 8.5 Hz), 7.76 (s, 1H, H-3 pyrazole), 7.79–7.84 (m, 2H, H-2 and H-6 of C_6H_5), 7.87 (s, 1H, N=CH). MS, *m/z*: 427.86 [M^+-CH_3]. Anal. Calcd for $C_{19}H_{14}ClN_5O_3S$ (427.86): C, 53.34; H, 3.30; N, 16.37; S, 7.49. Found: C, 53.49; H, 3.34; N, 16.67; S, 7.81.

4.1.7.2. (*E*)-1-(4-Chlorophenylsulfonyl)-5-(4-methoxybenzylideneamino)-6-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (13b).

Mp 202–204 °C; yield: 85%. IR ν_{max}/cm^{-1}: 3035.96 (CH aromatic), 2924.09 (CH aliphatic), 1693.49 (C=O), 1303.88, 1168.86 (SO_2). ^1H NMR ($CDCl_3$) δ ppm: 1.26 (s, 3H, CH_3), 3.90 (s, 3H, OCH_3), 6.97 (d, 2H, H-3 and H-5 of OCH_3–C_6H_4, J = 9.0 Hz), 7.02 (d, 2H, H-2 and H-6 of OCH_3–C_6H_4, J = 9.0 Hz), 7.61 (d, 2H, H-3 and H-5 of Cl-C_6H_4, J = 7.8 Hz), 7.83 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 7.8 Hz), 8.61 (s, 1H, H-3 pyrazole), 9.90 (s, 1H, N=CH). MS, *m/z*: 458 [M^++1]. Anal. Calcd for $C_{20}H_{16}ClN_5O_4S$ (457.89): C, 52.46; H, 3.52; N, 15.29. Found: C, 52.18; H, 3.69; N, 15.05.

4.1.7.3. (*E*)-1-(4-Chlorophenylsulfonyl)-5-(3,4-dimethoxybenzylideneamino)-6-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (13c).

Mp 196–198 °C; yield: 65%. IR ν_{max}/cm^{-1}: 3066.82 (CH aromatic), 2931.80, 2835.36 (CH aliphatic), 1662.64 (C=O), 1311.59, 1145.72 (SO_2). ^1H NMR (DMSO-d_6) δ ppm: 1.89 (s, 3H, CH_3), 3.77 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 6.95 (d, 1H, H-5 of $(OCH_3)_2$–C_6H_3, J = 7.5 Hz), 7.15 (s, 1H, H-2 of $(OCH_3)_2$–C_6H_3), 7.64 (d, 1H, H-6 of $(OCH_3)_2$–C_6H_3, J = 7.8 Hz), 7.75 (d, 1H, H-3 and H-5 of Cl-C_6H_4, J = 8.1 Hz), 7.88 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 8.7 Hz), 8.42 (s, 1H, H-3 pyrazole), 10.72 (s, 1H, N=CH). ^{13}C NMR (DMSO) δ: 20.5, 55.4, 55.5, 108.2, 111.2, 120.13, 120.5, 127.7, 129.2, 129.9, 130.6, 140.5, 141.6, 149.0, 149.8, 160.0, 168.0. MS, *m/z*: 488 [M^+]. Anal. Calcd for $C_{21}H_{18}ClN_5O_5S$ (487.92): C, 51.69; H, 3.72; N, 14.35; S, 6.57. Found: C, 51.85; H, 3.85; N, 14.22; S, 6.61.

4.1.7.4. (*E*)-1-(4-Chlorophenylsulfonyl)-5-(3,4,5-trimethoxybenzylideneamino)-6-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (13d).

Mp 216–218 °C; yield: 90%. IR ν_{max}/cm^{-1}: 3032.10 (CH aromatic), 2939.52 (CH aliphatic), 1635.64 (C=O), 1323.17, 1126.43 (SO_2). ^1H NMR ($CDCl_3$) δ ppm: 1.77 (s, 3H, CH_3), 3.85 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.89 (s, 2H, H-2 and H-6 of $(OCH_3)_3$-C_6H_2), 7.48 (d, 1H, H-3 and H-5 of Cl-C_6H_4, J = 8.1 Hz), 7.82 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 8.7 Hz), 7.93 (s, 1H, H-3 pyrazole), 9.88 (s, 1H, N=CH).) Anal. Calcd for $C_{22}H_{20}ClN_5O_6S$ (517.94): C, 51.02; H, 3.89; N, 13.52. Found: C, 51.25; H, 4.06; N, 13.08.

4.1.8. (*E*)-1-(4-Chlorophenylsulfonyl)-5-(3,4,5-trimethoxybenzamido)-6-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (14)

A mixture of 3,4,5-trimethoxybenzoic acid (2.12 g, 0.01 mol) and thionyl chloride (5 ml) was refluxed gently for 1 h. The excess thionyl chloride was removed under vacuum in water-bath. The residue was azeotroped three times with dry benzene to remove the last traces of thionyl chloride. The crude acid chloride was dissolved in dry benzene (20 ml) and was added drop-wise with stirring to a buffered solution of the hydrazide derivative **12** (3.57 g, 0.01 mol) in dry benzene (10 ml), keeping the temperature at 10 °C. After addition was complete, stirring was continued at room temperature for 3 h. The reaction mixture evaporated in vacuum and the residue was triturated with ice-water. The obtained solid product—crude benzamide derivative **14**—was filtered, washed several times with water, dried and crystallized from absolute ethanol.

Mp 228–229 °C; yield: 81%. IR ν_{max}/cm^{-1}: 3317.56 (NH), 3066.82 (CH aromatic), 2939.52 (CH aliphatic), 1681.93 (C=O), 1327.03, 1157.29 (SO_2). ^1H NMR (DMSO-d_6) δ ppm: 1.25 (s, 3H, CH_3), 3.72 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 6.89 (d, 2H, H-3 and H-5 of Cl-C_6H_4, J = 9.0 Hz), 7.25 (s, 2H, H-2 and H-6 of $(OCH_3)_3$-C_6H_2), 7.88 (d, 2H, H-2 and H-6 of Cl-C_6H_4, J = 8.4 Hz), 8.85 (s, 1H, H-3 pyrazole), 10.48 (s, 1H, NH, exch. D_2O). ^{13}C NMR (DMSO) δ: 20.5, 55.8, 56.0, 60.0, 104.9, 106.5, 128.5, 129.3, 129.5, 129.9, 137.8, 139.0, 140.5, 141.5, 152.5, 152.6, 153.8, 165.5, 166.85). MS, *m/z*: 517 [M^+-CH_3]. Anal. Calcd for $C_{22}H_{20}ClN_5O_7S$ (533.94): C, 49.49; H, 3.78; N, 13.12. Found: C, 49.29; H, 3.56; N, 13.18.

4.1.9. Ethyl 5-acetamido-1*H*-pyrazole-4-carboxylate (15)[42]

A mixture of compound **10a** or **10b** (0.01 mol) and acetic anhydride (5 ml) was heated under reflux for 4 h. The reaction mixture was cooled to room temperature. The formed solid was filtered, dried and crystallized from ethanol.

Mp 201–203 °C (reported mp 204 °C); yield: 68%. IR ν_{max}/cm^{-1}: 3340.71, 3251.98 (2NH), 2978.09 (CH aliphatic), 1681.93, 1604.77 (C=O). ^1H NMR ($CDCl_3$) δ ppm: 1.38 (t, 3H, CH_2CH_3, J = 7.2 Hz), 2.29 (s, 3H, CH_3), 4.37 (q, 2H, CH_2CH_3, J = 7.2), 7.77 (s, 1H, H-3 pyrazole), 9.57 (s, 1H, N*H*$COCH_3$, exch. D_2O), 11.85 (s, 1H, NH pyrazole, exch. D_2O).

4.1.10. *N*-[4-(Hydrazinecarbonyl)-1*H*-pyrazol-5-yl]acetamide (16)

A mixture of **15** (1.97 g, 0.01 mol) and hydrazine hydrate (0.60 ml, 0.012 mol) in *n*-butanol (20 ml) was heated under reflux for 24 h. The reaction mixture was concentrated in vacuum then cooled. The separated solid was filtered and crystallized from ethanol.

Mp 236–238 °C; yield: 60%. IR ν_{max}/cm^{-1}: 3402.43, 3339.85, 3159.40 (NH_2 and NH), 2974.23, 2804.50 (CH aliphatic), 1627.92 (br, 2C=O). ^1H NMR (DMSO-d_6) δ ppm: 4.17 (s, 3H, CH_3), 5.63 (s, 2H, br, CONHN*H*$_2$, exch. D_2O), 7.71 (s, 1H, H-3 pyrazole), 8.90 (s, 2H, CON*H*NH_2 and N*H*$COCH_3$, exch. D_2O), 11.68 (s, br, 1H, NH pyrazole, exch. D_2O). Anal. Calcd for $C_6H_9N_5O_2$ (183.17): C, 39.34; H, 4.95; N, 38.23. Found: C, 39.64; H, 5.01; N, 37.98.

4.1.11. General procedure for the synthesis of (*E*)-N-[4-(2-substituted benzylidenehydrazinecarbonyl)-1*H*-pyrazol-5-yl]acetamide (17a–d)

A mixture of **16** (1.83 g, 0.01 mol) and the appropriate aldehyde (0.012 mol) in glacial acetic acid (10 ml) was heated under reflux for 24 h. The precipitate formed was filtered, dried and crystallized from absolute ethanol.

4.1.11.1. (*E*)-*N*-[4-(2-Benzylidenehydrazinecarbonyl)-1*H*-pyrazol-5-yl]acetamide (17a). Mp 247–248 °C; yield: 76%. IR ν_{max}/cm^{-1}: 3290.56, 3190.26, 3143.97 (3 NH), 3024.38 (CH arom.), 2927.94 (CH aliph.), 1693.50, 1643.35 (C=O). ^1H NMR (DMSO-d_6) δ ppm: (mixture of tautomers) 2.16 (s, 3H, CH_3), 7.39–7.46 (m, 3H, H-3, H-4 and H-5 of C_6H_5), 7.65–7.71 (m, 2H, H-2 and H-6 of C_6H_5), 8.11 (s, br, 1H, H-3 pyrazole), 8.33 (s, br, 1H, N=CH), 10.37 (s, br, 1H, N*H*$COCH_3$, exch. D_2O), 11.15 (s, br, 1H, CONH–N=, exch. D_2O), 11.47 (s, br, 1H, NH pyrazole, exch. D_2O). Anal. Calcd for $C_{13}H_{13}N_5O_2$ (271.27): C, 57.56; H, 4.83; N, 25.82. Found: C, 57.50; H, 4.60; N, 25.53.

4.1.11.2. (*E*)-*N*-{4-[2-(4-Methoxybenzylidene)hydrazinecarbonyl]-1*H*-pyrazol-5-yl}acetamide (17b). Mp 242–243 °C; yield: 86%. IR ν_{max}/cm^{-1}: 3305.99, 3278.99, 3140.11 (3NH), 3035.96 (CH aromatic), 2939.52, 2839.22 (CH aliphatic), 1689.64, 1639.49 (C=O). ^1H NMR (DMSO-d_6) δ ppm: (mixture of tautomers) 2.19 (s, 3H, CH_3), 3.80 (s, 3H, OCH_3), 7.03 (d, 2H, 2H, H-3 and H-5 of OCH_3–C_6H_4, J = 7.2 Hz), 7.64 (d, 2H, H-2 and H-6 of OCH_3-C_6H_4, J = 7.8 Hz), 7.95, 8.04 (2s, 1H, H-3 pyrazole), 8.23, 8.26 (2s, 1H, N=CH), 10.37, 10.64 (2s, 1H, N*H*COCH3, exch. D_2O), 11.10, 11.37 (2s, 1H, CONH–N=, exch. D_2O), 12.84, 13.12 (2s, 1H, NH pyrazole, exch. D_2O). MS, m/z: 301.10 [M^+]. Anal. Calcd for $C_{14}H_{15}N_5O_3$ (301.30): C, 55.81; H, 5.02; N, 23.24. Found: C, 55.56; H, 4.75; N, 23.50.

4.1.11.3. (*E*)-*N*-{4-[2-(3,4-Dimethoxybenzylidene)hydrazinecarbonyl]-1*H*-pyrazol-5-yl}acetamide (17c). Mp 232–233 °C; yield: 80%. IR ν_{max}/cm^{-1}: 3309.85, 3282.84, 3248.13 (NH), 3051.39 (CH aromatic), 2997.38, 2873.94 (CH aliphatic), 1693.50, 1649.49 (C=O). ^1H NMR (DMSO-d_6) δ ppm: (mixture of tautomers) 2.20 (3H, s, CH_3), 3.81 (s, 6H, 2 OCH_3), 7.02 (d, 1H, H-5 of $(OCH_3)_2$–C_6H_3, J = 7.1 Hz), 7.19 (d, 1H, H-6 of $(OCH_3)_2$–C_6H_3, J = 7.4 Hz), 7.32 (s, 1H, H-2 of $(OCH_3)_2$-C_6H_3), 7.95, 8.10 (2s, 1H, H-3 pyrazole), 8.23, 8.27 (2s, 1H, N=CH), 10.26, 10.64 (2s, 1H, N*H*$COCH_3$, exch. D_2O), 11.20, 11.42 (2s,1H, CONH–N=, exch. D_2O), 12.95, 13.14 (2s, 1H, NH of pyrazole, exch. D_2O). Anal. Calcd for $C_{15}H_{17}N_5O_4$ (331.33): C, 54.38; H, 5.17; N, 21.14. Found: C, 54.68; H, 4.88; N, 21.42.

4.1.11.4. (*E*)-*N*-{4-[2-(3,4,5-Trimethoxybenzylidene)hydrazinecarbonyl]-1*H*-pyrazol-5-yl}acetamide (17d). Mp 271–272 °C; yield: 91%. IR ν_{max}/cm^{-1}: 3294.42, 3132.40 (3 NH), 3089.96 (CH aromatic), 2943.37, 2839.22 (CH aliphatic), 1689.64, 1651.07 (C=O). ^1H NMR (DMSO-d_6) δ ppm: (mixture of tautomers) 2.20 (s, 3H, CH_3), 3.70 (s, 3H, OCH_3), 3.83 (s, 6H, 2 OCH_3), 7.00 (s, 2H, H-2 and H-6 of $(OCH_3)_3$–C_6H_2), 7.99, 8.19 (2s, 1H, H-3 pyrazole), 8.27, 8.35 (2s, 1H, N=CH), 10.40, 10.60 (2s, 1H, N*H*$COCH_3$, exch. D_2O), 11.40, 11.54 (2s, 1H, CONH–N=, exch. D_2O), 12.88, 13.15 (2s, 1H, NH pyrazole, exch. D_2O). MS, m/z: 362 [M^+]. Anal. Calcd for $C_{16}H_{19}N_5O_5$ (361.35): C, 53.18; H, 5.30; N, 19.38. Found: C, 52.95; H, 5.27; N, 19.64.

4.2. Biological evaluation

4.2.1. Materials and methods

4.2.1.1. Chemicals. Dimethylsulfoxide (DMSO), cisplatin and Sulforhodamine-B stain (SRB) were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA).

4.2.1.2. Cell culture. The MCF-7 cancer cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in humidified atmosphere containing 5% CO_2. MCF-7 cells at a concentration of 0.50×10^6 were grown in a 25 cm^2 flask in 5 ml of complete culture medium.

4.2.2. In vitro cytotoxic assay[43]

The cytotoxic activity was measured in vitro using the SRB colorimetric assay using the method of Skehan.[43] Cells were inoculated in 96-well microtiter plate (10^4 cells/ well) for 24 h before treatment with the compound(s) to allow attachment of cell to the wall of the plate. Test compounds were dissolved in DMSO and diluted with saline to the appropriate volume. Different concentrations of the compound under test (0.1, 2.5, 5, and 10 mmol/ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compound(s) for 48 h. at 37 °C and in 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. 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. The relation between surviving fraction and drug concentration is plotted to get the survival curve for breast tumor 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 Table 1.

4.2.3. Biochemical assays

The cells in culture medium were treated with 20 μl of 1/10 of IC_{50} values of the compounds or the standard reference drug, cisplatin, then incubated for 24 h at 37 °C, in a humidified 5% CO_2 atmosphere. The MCF-7 cells were harvested and homogenates were prepared in saline using a tight pestle homogenizer until complete cell disruption for further biochemical analysis.

4.2.3.1. Antioxidant enzyme assays. The supernatant obtained after centrifugation of cell homogenates was used for the determination of enzymes activities of SOD, CAT and GSH-Px as described by Paglia and Valentine,[54] Aebi,[55] Marklund and Marklund,[56] respectively.

4.2.3.2. Oxidative stress assays. The levels of H_2O_2, NO and reduced GSH were determined by the methods of Wolf,[57] Montgomery and Dymock,[58] and Ellman,[59] respectively.

4.2.3.3. Estimation of nucleic acids and protein. Nucleic acids (DNA and RNA) and total protein were precipitated and measured in cell homogenates. Total DNA was extracted and assayed according to the method described by Zhou et al.,[60] total RNA was extracted and assayed according to the method adopted from that provided by Hybaid/AGS (Germany), and total cellular protein was assayed by the method of Lowry et al.[61]

4.2.4. Statistical analysis

The results are reported as mean ± standard error (SE) for at least three experiments in case of cytotoxic activity and six experiments in case of biochemical assays. Statistical differences were analyzed according to one way ANOVA test followed by student's *t* test wherein the differences were considered to be significant at $p < 0.05$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.09.036.

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