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Novel 2-amino-4-aryl-6-pyridopyrimidines and *N*-alkyl derivatives: Synthesis, characterization and investigation of anticancer, antibacterial activities and DNA/BSA binding affinities

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ABSTRACT

A series of new 2-amino-4-aryl-6-pyridopyrimidines, and their *N*-alkyl bromide derivatives were designed and synthesized by employing methyl substituted azachalcones. These novel compounds were evaluated and compared to the well-known chemotherapeutics in terms of their anti-cancer and anti-microbial functions, and their DNA/protein binding affinities. In order for the cell proliferation, cytotoxicity and microdilution features to be observed, various cancer cell lines (Hep3B, A549, HeLa, C6, HT29, MCF7) were treated with 2-amino-4-aryl-6-pyridopyrimidines (**1-9**) and their *N*-alkyl bromide derivatives (**2a-c, 3a-c, 5a-c, 6a-c, 8a-c**, **9a-c**). Studies on the cells revealed that both pyrimidines and their alkyl derivatives (i) have a high anti-proliferative and anti-microbial activities, (ii) cause cell rounding, cytoplasmic blebs, and anomalous globular structure, and (iii) strongly bound to DNA/BSA macromolecules. Especially the length of the alkyl chain of the *N*-alkyl bromides has an increasing effect on the antiproliferative, antibacterial and cytotoxic functions, also DNA/protein binding affinity. Those results indicate the novel compounds to be promising antiproliferative agents, and their anti-cancer potential makes them candidates to be used for cancer therapy.

1. Introduction

Cancer, defined as uncontrolled growth and spread of abnormal cells, is one of the common life-threatening diseases. Today, cancer and cancer-related diseases have become the one foremost reason of death after accidents and injuries [1,2]. Although chemotherapy is the most common treatment of cancer therapy, many chemotherapy agents cause intense side effects due to their cytotoxic and mutagenic effects on healthy cells [1,2]. Therefore, it appears to be necessary for developing alternative drugs without or with minimal side effects on human body.

On the other hand, life-threatening infections caused by multidrug-resistant bacteria have increased in recent decades all around the world. The spread of antibiotic resistance has become a vital problem for infection treatment. Therefore, the synthesis of new and effective antibacterial agents is required in order to overcome drug resistance [3]. It is also known that pathogenic microbial florae are responsible of infectious diseases. However, some significant experimental findings imply that the strong relationship between pathogenic microbial flora and certain diseases such as liver, cervical, stomach, urogenital cancer, and lymph node disorder [4]. It should be indicated that these pathogenic microbial florae often cause chronic inflammations due to their toxic microbial metabolites that may lead to an increase in the risk of cancer or the formation of cancer-related diseases [5,6]. Dual-acting molecules with anticancer and antibacterial properties can be used in the treatments of both cancer and inflammation. In practice, this may allow clinicians to treat cancer and inflammation more effectively provided the intelligent dose adjustment [7]. In particular, it is important to synthesize nitrogen containing heterocyclic compounds as new anticancer and antibacterial reagent since the essential component of genetic material of DNA and RNA include nitrogen heterocyclic units called pyrimidine [1–3,8]. In this work, with the purpose of discovering a specific target such as DNA/BSA, new pyrimidine compounds were synthesized by using the medicinal chemistry approach. Although being a relatively low throughput method, it is frequently used in the manual

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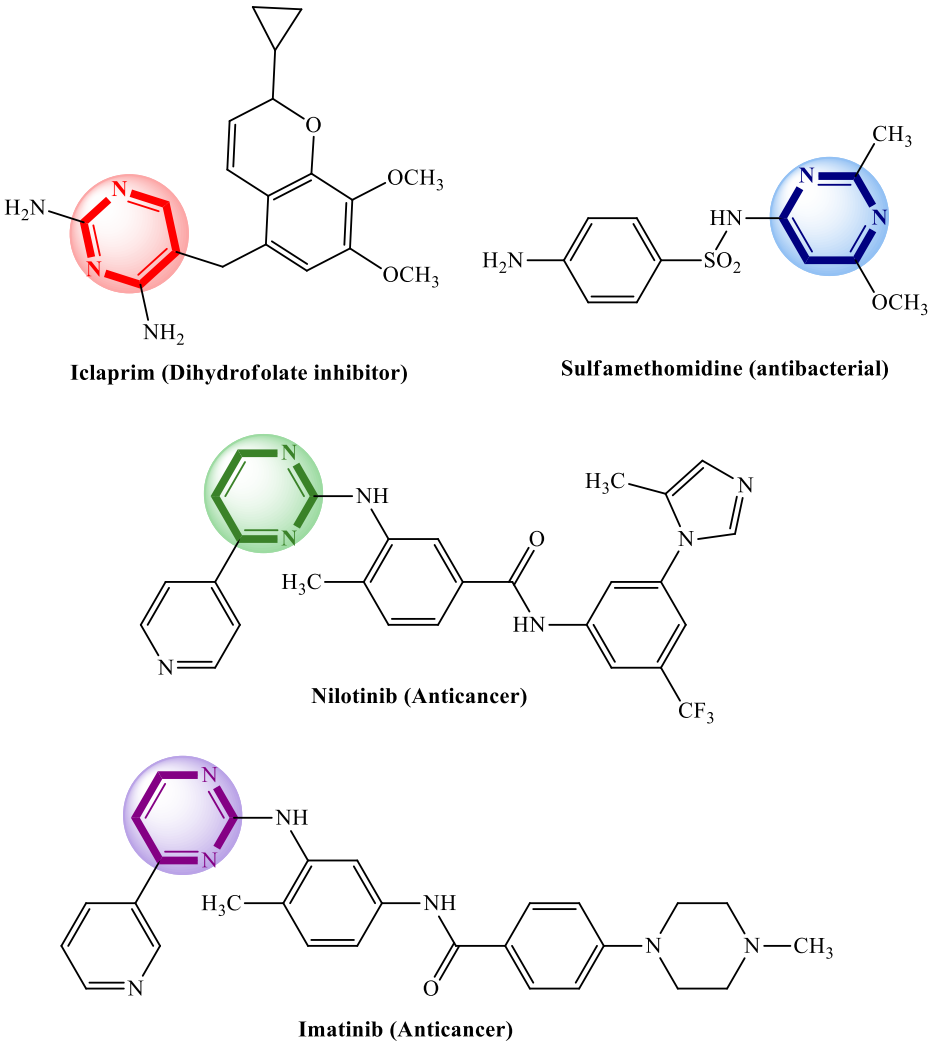


Fig. 1. Structures of some marketed drugs contain pyrimidine moiety.

synthesis of new small compounds from parent pharmacophore scaffolds. Once the chemical properties of these compounds are characterized, their pharmacological activities are tested by in vitro/*in vivo* experiments. For example, 5-fluorouracil an antimetabolite fluoropyrimidine being an analog of the nucleoside pyrimidine with anticancer activity was developed as an anticancer drug by successfully applied medicinal chemistry approach. The aim of this study is to design nitrogen-containing heterocyclic compounds that have the ability to interact with DNA/BSA, in order to be used for cancer therapy. Similar compounds were synthesized for this purpose, and some improvement were made to form an in-house library of new bioactive small molecules. The compounds were tested for their anti-microbial and anticancer activities. Pyrimidine motifs are prevalent heterocyclic moieties present in natural products (Meridianins, Variolin B *etc.*), drugs (Sulphadiazine, Imatinib, Dasatinib, Iclaprim, Nilotinib, Sulfamethomidine, Zalcitabine, Voriconazole, Capecitabine *etc.*), functional materials (organic light emitting devices and molecular wires), biological systems (vitamins, coenzymes, nucleic acids and purines *etc.*), and agrochemicals with pharmaceutical and chemical properties [1,9]. Some commercial drugs containing pyrimidine moiety are presented in Fig. 1. Pyrimidine derivatives also demonstrate several biological functions such as antibacterial [9–11], antiproliferative and antitumor [9,11], anti-inflammatory [9], antileishmanial [9,12], antiviral [1], antifungal [13], analgesic [14], anticonvulsant [15], antitubercular [10], antimalarial [16–18], enzyme inhibition [19], anti-parkinson [20], antihistaminic [21], and antioxidant activities [1]. Moreover, it has been revealed that some pyrimidine derivatives behave as an attractive agent for the treatment of neurological disorders and metabolic abnormalities [9,20].

Literature surveys reveal that the activities of pyrimidines can be associated to the diversity of linked substituents especially on the C-2, C-4 and C-6 positions [22,23]. Particularly $-NH_2$ group on the second carbon of pyrimidine core enhances the antibacterial properties of pyrimidines [1]. Due to the importance and broad biological activity scale of the pyrimidine moieties, a large number of pyrimidine synthesis methods have been reported [16–18,24–32]. Synthetic pathways can be classified into three types, according to the basic structure of the reactants which can be combined together to produce the pyrimidine core (Fig. 2). The most extensive pyrimidine synthesis belongs to Type 1 (Fig. 2) [31]. Chalcones are the most widely used starting materials in the synthesis of bioactive substituted pyrimidines according to the Type 1. Substituted chalcones and their guanidine salts can easily react under

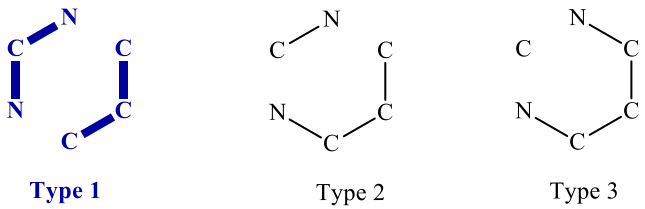
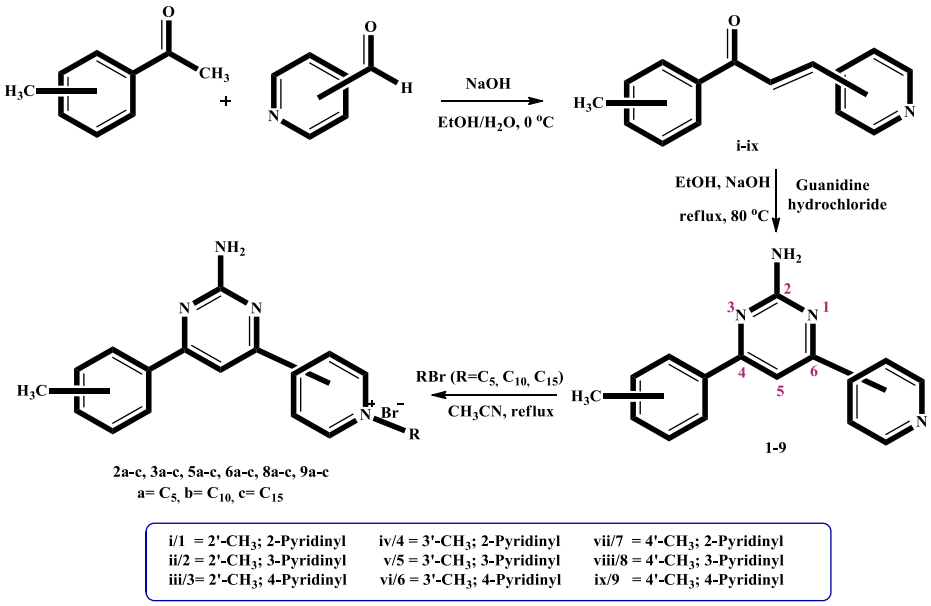


Fig. 2. Types of synthetic pathways for pyrimidine core.



Scheme 1. Consecutive synthesis of 2-amino-4-aryl-6-pyridopyrimidines (**1-9**) and their *N*-alkylpyridinium bromides (**2a-c**, **3a-c**, **5a-c**, **6a-c**, **8a-c**, **9a-c**).

basic conditions to give 2-amino-4-aryl-6-pyridopyrimidines [11,16–18,21,29,32–34].

In recent years, *N*-alkyl substituted heterocyclic salts have acquired much attention for their comprehensive inclusion in chemical, biological, and materials science studies [35–39]. Especially, *N*-alkylated pyridopyrimidines have showed excellent anticancer and antibacterial activities [11].

Prompted by aforementioned facts and based on the biological results of our previous work [11], in the present study the synthesis of a series of new 2-amino-4-aryl-6-pyridopyrimidine derivatives and their *N*-alkylpyridinium salts were planned, and with regard to the expected biological activities, their anticancer and antibacterial activities, and DNA/BSA binding affinities were evaluated.

2. Results and discussion

2.1. Chemistry

The detailed synthetic routes to synthesize 2-amino-4-aryl-6-pyridopyrimidines (**1-9**) and their *N*-alkyl bromide derivatives (**2a-c**, **3a-c**, **5a-c**, **6a-c**, **8a-c**, **9a-c**) are represented in Scheme 1.

In the first part of the work, the desired 2-amino-4-aryl-6-pyridopyrimidines (**1-9**) were synthesized by condensation of the methyl substituted azachalcones with guanidium salt under basic conditions [11,15–18,24,28,29]. In the following step, the newly designed *N*-alkylpyridinium bromides (**2a-c**, **3a-c**, **5a-c**, **6a-c**, **8a-c**, **9a-c**) were produced through the S_N2 reaction between pyridinyl nitrogen and alkyl bromide [36–40]. The purities of all compounds were checked by TLC, and if necessary, they were purified by column chromatography and their structures were established spectroscopically.

The characteristic two bands for pyrimidine ring were observed in the regions of 1659–1605 cm^{-1} and 1367–1351 cm^{-1} in the FT-IR spectra [21]. These bands belong to −C=N− vibrations and appeared after the intermolecular cyclization between azachalcone and guanidine. The formation of the pyrimidine core was also supported by the disappearing of the carbonyl group absorption band at about 1680 cm^{-1}. The three bands seen in the FT-IR spectra of the pyrimidines and *N*-alkylated pyrimidines in the regions of 3491–3345 cm^{-1}, 3393–3260 cm^{-1} and 3198–3143 cm^{-1} were the evidence for the free and H-bonded $-NH_2$ absorptions [21].

The ^1H NMR spectra of all compounds displayed further support for the 2-aminopyrimidine ring, since they showed an additional broad singlet or distinct singlet derived from $-NH_2$ at δ = 5.4–5.2 ppm in pyrimidines and δ = 5.9–5.4 ppm in alkylated forms. As seen in chemical shifts, the ionic structure of alkylated compounds caused an evident shift to low field. Further, the peaks observed at δ = 8.2–7.2 ppm in ^1H NMR spectra and δ = 109.1–103.8 ppm ^{13}C NMR spectra that belong to the characteristic H-5 and C-5 peaks of pyrimidine ring [11,21]. Therefore, these peaks confirm the formation of pyrimidine structure. All the other aromatic carbons of 2-amino-4-aryl-6-pyridopyrimidines gave peaks in the range of 170.1–121.1 ppm in the ^{13}C NMR spectra.

LC-MS/MS spectra of all compounds were characterized by high or medium intensity molecular ions peaks at the appropriate *m/z* values and they showed the corresponding molecular ion peak $[M+1]^+$or $[M+Na]^+$ in pyrimidines and $[M-^{79}Br/^{81}Br]^+$ in alkylated pyrimidines as the base peaks.

In the second part of the study, *N*-pentyl/decyl/pentadecylpyridinium bromides of pyrimidines were synthesized by S_N2 reaction as mentioned before and the attached alkyl chain to the pyridine nitrogen was confirmed by spectroscopic data [11]. The triplet peak seen at about 5.1–4.7 ppm in ^1H NMR and the carbon peak at about 62.5–61.8 ppm in ^{13}C NMR spectra of *N*-alkyl pyridinium bromides were characteristic to the newly formed $-N-CH_2-$ bonds. There were alternative nitrogen atoms for the alkylation of the structure. However, the strong pK_b value of pyridine (8.77) versus the pyrimidine (11.3) [11,20] caused the alkylation on the pyridine nitrogen. Also, the electron density of the free amino group was decreased by mesomeric effect, and thus the group behaved as a poor nucleophile. All the evidences showed that alkylation position of the compounds was pyridine nitrogen [41]. Moreover, chemical shifts of pyridine protons prominently shifted to low field after alkylation. Comparing ^1H NMR spectra data of compounds **9** and **9c** as an example, it was clearly seen that chemical shifts of the pyridine protons of **9c** shifted to low field while there was almost no change in pyrimidine and methylphenyl protons (δ (**9**/**9c**, ppm) = 7.5/7.7 (H-5), 8.1/8.1 (H-2′/6′), 7.3/7.3 (H-3′/5′), 7.9/8.7 (H-2″/6″), and 8.8/9.4 (H-3″/5″). Additionally, alkylated forms of compounds **1**, **4** and **7** could not be obtained because of the steric hindrance of pyridine nitrogen although different reaction conditions were tried and example studies are present in literature about this situation [32–35,38,39].

The characterization of all the newly synthesized compounds were

done by ^{1}H, ^{13}C, APT, COSY, ACD-NMR, FT-IR, LC-MS/MS, and elemental analysis methods, and all the obtained data were in full agreement with the structures proposed. Also, some spectra of compounds **9** and **9c** were given in supplementary information.

2.2. Biological evaluation

2.2.1. Evaluation of anticancer properties of the compounds

The annoying effect of cancer, which still causes more deaths than tuberculosis, AIDS, and malaria, on society is increasing. Unfortunately, most of the cancer patients living in the countries with low and middle income are unable to be treated by effective chemotherapy services. Therefore, intensive studies are being conducted for the discovery of effective, cheap and easily obtainable anticancer drugs. For this purpose, 2-amino-4-aryl-6-pyridopyrimidines (**1-9**) and their *N*-alkyl bromide derivatives bearing different length carbon chains (**2a-c, 3a-c, 5a-c, 6a-c, 8a-c**, **9a-c**) were newly synthesized by our research group, and the anticancer effects of those 27 compounds were evaluated according to the MTT protocol. IC_{50} inhibition values were generally used for inhibition studies. GI_{50}, TGI, and LC_{50} parameters were determined according to NCI recommendations from the absorbance data obtained from MTT test. The two frequently used anticancer drugs cisplatin -a DNA-alkylating agent- and 5-flurouracil (5-FU) -a pyrimidine analog- were used as control [42,43]. Cisplatin and 5-FU are commonly used anticancer reference drugs in research utilizing MTT test and various cancers cell lines including those used in this study [44–48]. When TGI and IC_{50} values of the 2-amino-4-aryl-6-pyridopyrimidines and their *N*-alkyl bromide derivatives were examined, it was concluded that all of those molecules have strong antitumor effect on the C6 cell line (IC_{50}: 1.8 ± 0.1–192 ± 11 μM; TGI: 1.8 ± 0.1–119 ± 6 μM) (Table 1). When the anticancer effects of the compounds were perused in HeLa cells, compounds **3**, **5** and **8** amongst pyrimidines were detected to be effective whereas *N*-alkyl bromide derivatives displayed a very strong anticancer effect on the HeLa cell line (IC_{50}: 1.8 ± 0.1–157 ± 12 μM; TGI: 1.8 ± 0.1–130 ± 11 μM) (Table 1). On HT29 cells, compound **3** amongst pyrimidines showed a potent effect, and all *N*-alkyl bromide derivatives exhibited high antitumoral properties (IC_{50}: 4.2 ± 0.2–42 ± 3 μM; TGI: 4.6 ± 0.4–17.17 μM) (Table 1). While only compound **9** amongst the 2-amino-4-aryl-6-pyridopyrimidines reached sufficient antiproliferative activity on MCF7 cells, *N*-alkyl bromide derivatives except compound **2a** and **3a** caused strong antitumor effect at the desired level (IC_{50}: 6.2 ± 0.3–82 ± 6 μM; TGI values ranging from 6.3 ± 0.3–90 ± 7 μM) (Table 2). Interestingly, none of the pyrimidines at high concentrations -such as 1906 μM displayed significant antiproliferative effects when compared to the control group (Table 2) on the A549 cell line which is an important cancer cell line on which the tests were conducted with the new compounds. However, on the A549 cell line, **b** and **c** series of alkyl derivatives showed strong anticancer properties (IC_{50}: 7.4 ± 0.5–34 ± 2 μM; TGI: 8.1 ± 0.4–39 ± 3 μM) (Table 2). Compound **6** and **9** amongst pyrimidines, and **2b-c**, **3b-c**, **5a-c**, **6b-c**, **8a-c**, and **9a-c** of alkyl derivatives displayed high antiproliferative properties on the Hep3B cell line (IC_{50}: 6.1 ± 0.4–199 ± 10 μM; TGI: 6.2 ± 0.5–207 ± 11 μM) (Table 2). When the anticancer effects of the compounds on FL cells were examined, it was seen that compounds **3**, **5**, **6**, and **9** amongst pyrimidines, and **2b-c**, **3b-c**, and **5a-c** amongst *N*-alkyl bromide derivatives showed strong anticancer properties (IC_{50}: 1.8 ± 0.1–147 ± 8 μM; TGI 1.8 ± 0.1–151 ± 9 μM) (Table 3). The low GI_{50} values (1–30 μM) exhibited by the new test compounds examined by NCI's screening method revealed their pharmacological importance (Tables 1 and 2). However, the values of lethal concentration (LC_{50}) exhibited by the new test compounds examined by NCI's screening method differ from each other. Only some of the new compounds that were examined have reasonable LC_{50} values. The LC_{50} range of 234– > 1906 μM of compounds **1**, **2**, **4**, **6**, **7**, **9**, and **3a** on the C6 cell line in terms of lethal concentration values can be ideal for pharmacological studies (Table 1). Likewise, the LC_{50} values of the compounds **3**, **2a**, and **3a** on HeLa and HT29 cell lines (LC_{50} 359– > 1906 μM and 267– > 1906 μM, respectively) were very close to the NCI criteria (Table 1). When the compounds **9**, **2c**, **5a**, **5c**, **6a**, **8a**,

Table 1
GI_{50}, TGI, LC_{50} and IC_{50} values for **1-9** and **2a-c**, **3a-c**, **5a-c**, **6a-c**, **8a-c**, **9a-c** against **C6**, **HeLa**, and **HT29**.

Compounds (μM)	C6				HeLa				HT29			
	GI_{50}	TGI	LC_{50}	IC_{50}	GI_{50}	TGI	LC_{50}	IC_{50}	GI_{50}	TGI	LC_{50}	IC_{50}
1	33 ± 2	119 ± 6	925 ± 63	192 ± 11	> 1906	> 1906	> 1906	> 1906	4.7 ± 0.2	> 1906	> 1906	1516 ± 98
2	10 ± 1	50 ± 3	> 1906	129 ± 6	> 1906	> 1906	> 1906	> 1906	5.6 ± 0.4	1430 ± 84	> 1906	311 ± 19
3	13 ± 1	30 ± 3	117 ± 7	41 ± 5	32 ± 3	92 ± 5	459 ± 32	129 ± 4	4.8 ± 0.2	23 ± 4	> 1906	15 ± 2
4	11 ± 1	35 ± 4	405 ± 27	65 ± 4	> 1906	> 1906	> 1906	> 1906	20 ± 3	> 1906	> 1906	> 1906
5	5.3 ± 0.5	9.3 ± 0.7	42 ± 3	13 ± 1	3.8 ± 0.2	3.9 ± 0.2	6.5 ± 0.4	4.0 ± 0.2	5.6 ± 0.3	> 1906	> 1906	> 1906
6	13 ± 1	46 ± 2	720 ± 46	92 ± 5	> 1906	> 1906	> 1906	> 1906	6.6 ± 0.7	> 1906	> 1906	> 1906
7	6.2 ± 0.6	31 ± 2	> 1906	108 ± 5	> 1906	> 1906	> 1906	> 1906	15 ± 2	> 1906	> 1906	> 1906
8	6.1 ± 0.6	15 ± 1	177 ± 4	26 ± 2	10 ± 1	28 ± 2	202 ± 9	47 ± 2	5.6 ± 0.7	> 1906	> 1906	374 ± 29
9	12 ± 1	45 ± 2	888 ± 51	92 ± 5	> 1906	> 1906	> 1906	> 1906	5.2 ± 0.7	> 1906	> 1906	1204 ± 77
2a	13 ± 1	31 ± 2	119 ± 5	45 ± 3	54 ± 4	110 ± 5	267 ± 13	122 ± 6	7.2 ± 0.8	42 ± 3	> 1213	34 ± 3
2b	2.0 ± 0.3	2.1 ± 0.2	3.8 ± 0.3	2.2 ± 0.2	2.0 ± 0.2	2.1 ± 0.2	3.7 ± 0.2	2.2 ± 0.2	4.3 ± 0.2	9.4 ± 0.6	49 ± 3	8.6 ± 0.7
2c	1.8 ± 0.1	1.8 ± 0.1	2.3 ± 0.2	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	3.1 ± 0.2	1.9 ± 0.1	4.9 ± 0.3	11 ± 1	60 ± 4	11 ± 1
3a	29 ± 2	70 ± 5	234 ± 17	90 ± 6	58 ± 5	130 ± 11	359 ± 21	157 ± 12	6.2 ± 0.4	37 ± 2	> 1213	29 ± 2
3b	2.0 ± 0.2	2.1 ± 0.2	4.3 ± 0.2	2.3 ± 0.2	2.0 ± 0.2	2.1 ± 0.2	3.9 ± 0.2	2.2 ± 0.2	4.0 ± 0.2	9.3 ± 0.8	60 ± 4	8.4 ± 0.6
3c	1.8 ± 0.1	1.8 ± 0.1	2.3 ± 0.2	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	2.3 ± 0.2	1.8 ± 0.1	4.0 ± 0.2	9.9 ± 0.7	67 ± 5	8.9 ± 0.6
5a	2.4 ± 0.2	2.5 ± 0.2	4.5 ± 0.2	2.6 ± 0.2	13 ± 1	28 ± 2	81 ± 4	32 ± 2	5.0 ± 0.3	12 ± 1	83 ± 5	10 ± 1
5b	2.0 ± 0.2	2.0 ± 0.2	2.9 ± 0.1	2.1 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	3.2 ± 0.2	2.1 ± 0.2	3.5 ± 0.2	7.3 ± 0.8	43 ± 3	6.7 ± 0.4
5c	1.8 ± 0.1	1.8 ± 0.1	2.1 ± 0.2	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	2.8 ± 0.2	1.9 ± 0.1	3.8 ± 0.2	8.3 ± 0.6	41 ± 3	7.8 ± 0.4
6a	7.4 ± 0.5	16 ± 2	57 ± 4	22 ± 3	30 ± 3	59 ± 4	134 ± 6	65 ± 4	6.0 ± 0.3	17 ± 2	169 ± 8	15 ± 1
6b	2.0 ± 0.3	2.0 ± 0.1	2.5 ± 0.2	2.0 ± 0.2	2.0 ± 0.1	2.0 ± 0.2	2.9 ± 0.2	2.1 ± 0.2	3.3 ± 0.2	6.3 ± 0.4	29 ± 2	5.8 ± 0.4
6c	1.8 ± 0.1	1.8 ± 0.1	2.2 ± 0.2	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	2.9 ± 0.2	1.9 ± 0.1	2.5 ± 0.2	4.6 ± 0.4	22 ± 2	4.2 ± 0.2
8a	2.5 ± 0.2	3.0 ± 0.2	9.9 ± 0.5	3.7 ± 0.2	6.2 ± 0.3	14 ± 1	60 ± 4	20 ± 2	5.3 ± 0.2	14 ± 1	113 ± 5	12 ± 1
8b	2.0 ± 0.2	2.2 ± 0.1	5.8 ± 0.3	2.5 ± 0.2	2.0 ± 0.2	2.1 ± 0.2	4.1 ± 0.2	2.2 ± 0.2	3.6 ± 0.2	7.5 ± 0.4	37 ± 3	6.8 ± 0.3
8c	1.8 ± 0.1	1.9 ± 0.2	4.0 ± 0.3	2.0 ± 0.2	1.8 ± 0.1	1.8 ± 0.1	2.3 ± 0.2	1.8 ± 0.1	3.1 ± 0.2	6.5 ± 0.4	35 ± 3	5.9 ± 0.3
9a	13 ± 1	29 ± 2	99 ± 5	38 ± 3	38 ± 3	71 ± 5	151 ± 6	75 ± 5	5.3 ± 0.3	14 ± 1	134 ± 9	13 ± 1
9b	2.0 ± 0.3	2.0 ± 0.1	3.3 ± 0.2	2.1 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	2.9 ± 0.2	2.1 ± 0.2	3.8 ± 0.2	8.1 ± 0.5	42 ± 3	7.4 ± 0.4
9c	1.8 ± 0.1	1.8 ± 0.1	3.0 ± 0.2	1.9 ± 0.1	1.8 ± 0.1	1.9 ± 0.1	3.7 ± 0.2	2.0 ± 0.2	2.7 ± 0.2	5.1 ± 0.3	22 ± 2	4.7 ± 0.2

Percent inhibition noted is mean values ± SDs of three independent measures.

Table 2
GI_{50}, TGI, LC_{50} and IC_{50} values for **1-9** and **2a-c**, **3a-c**, **5a-c**, **6a-c**, **8a-c**, **9a-c** against **MCF7**, **A549**, and **Hep3B.**

Compounds (μM)	MCF7				A549				Hep3B			
	GI_{50}	TGI	LC_{50}	IC_{50}	GI_{50}	TGI	LC_{50}	IC_{50}	GI_{50}	TGI	LC_{50}	IC_{50}
1	6.4 ± 0.3	> 1906	> 1906	> 1906	56 ± 4	> 1906	> 1906	> 1906	17 ± 2	> 1906	> 1906	> 1906
2	7.4 ± 0.4	> 1906	> 1906	> 1906	31 ± 3	> 1906	> 1906	> 1906	14 ± 1	> 1906	> 1906	> 1906
3	9.6 ± 0.7	> 1906	> 1906	> 1906	25 ± 2	> 1906	> 1906	> 1906	13 ± 1	1679 ± 98	> 1906	1483 ± 95
4	5.7 ± 0.4	> 1906	> 1906	> 1906	> 1906	> 1906	> 1906	> 1906	37 ± 3	> 1906	> 1906	> 1906
5	8.1 ± 0.5	> 1906	> 1906	> 1906	75 ± 5	> 1906	> 1906	> 1906	20 ± 2	374 ± 16	> 1906	357 ± 17
6	6.8 ± 0.3	384 ± 19	> 1906	317 ± 21	28 ± 3	> 1906	> 1906	> 1906	11 ± 1	63 ± 5	> 1906	62 ± 5
7	8.6 ± 0.5	> 1906	> 1906	> 1906	858 ± 61	> 1906	> 1906	> 1906	15 ± 1	> 1906	> 1906	> 1906
8	6.4 ± 0.3	> 1906	> 1906	> 1906	59 ± 5	> 1906	> 1906	> 1906	13 ± 1	708 ± 44	> 1906	645 ± 41
9	5.6 ± 0.3	25 ± 3	> 1906	23 ± 3	17 ± 2	> 1906	> 1906	> 1906	14 ± 1	207 ± 11	> 1906	199 ± 10
2a	5.5 ± 0.3	> 1213	> 1213	> 1213	199 ± 18	> 1213	> 1213	> 1213	6.7 ± 0.3	> 1213	> 1213	> 1213
2b	4.9 ± 0.2	14 ± 1	153 ± 9	14 ± 1	9.5 ± 0.5	39 ± 3	614 ± 43	34 ± 2	5.5 ± 0.3	15 ± 1	131 ± 9	15 ± 1
2c	4.2 ± 0.2	12 ± 1	137 ± 8	11 ± 1	6.3 ± 0.3	18 ± 1	129 ± 11	16 ± 1	4.6 ± 0.2	13 ± 1	130 ± 9	13 ± 1
3a	5.4 ± 0.3	> 1213	> 1213	> 1213	889 ± 64	> 1213	> 1213	> 1213	7.8 ± 0.4	> 1213	> 1213	> 1213
3b	4.1 ± 0.2	10 ± 1	105 ± 9	10 ± 1	6.7 ± 0.3	21 ± 2	209 ± 10	18 ± 2	5.2 ± 0.2	15 ± 1	156 ± 11	15 ± 1
3c	6.3 ± 0.3	41 ± 3	> 905	39 ± 2	5.9 ± 0.3	18 ± 1	180 ± 11	16 ± 1	5.3 ± 0.2	26 ± 2	> 905	26 ± 2
5a	8.4 ± 0.5	54 ± 4	> 1213	52 ± 4	20 ± 2	764 ± 56	> 1213	424 ± 21	8.3 ± 0.4	52 ± 4	> 1213	51 ± 4
5b	4.8 ± 0.2	14 ± 1	177 ± 8	14 ± 1	7.0 ± 0.3	26 ± 2	434 ± 24	22 ± 2	5.5 ± 0.2	16 ± 1	162 ± 13	16 ± 1
5c	4.2 ± 0.2	13 ± 1	173 ± 9	12 ± 1	5.7 ± 0.3	16 ± 1	128 ± 10	14 ± 1	5.3 ± 0.2	16 ± 1	190 ± 14	16 ± 1
6a	6.0 ± 0.3	90 ± 7	> 1213	82 ± 6	31 ± 3	> 1213	> 1213	> 1213	6.6 ± 0.3	588 ± 44	> 1213	531 ± 43
6b	4.2 ± 0.2	11 ± 1	98 ± 7	10 ± 1	9.3 ± 0.5	27 ± 2	165 ± 11	25 ± 2	4.9 ± 0.2	13 ± 1	112 ± 7	13 ± 1
6c	2.9 ± 0.2	6.6 ± 0.3	53 ± 4	6.5 ± 0.2	3.9 ± 0.2	8.8 ± 0.4	46 ± 4	8.0 ± 0.4	3.7 ± 0.2	8.3 ± 0.4	45 ± 4	8.3 ± 0.5
8a	5.2 ± 0.3	21 ± 2	1030 ± 51	20 ± 2	13 ± 1	333 ± 27	> 1213	209 ± 13	8.7 ± 0.5	39 ± 3	1084 ± 67	38 ± 3
8b	4.2 ± 0.2	11 ± 1	145 ± 9	11 ± 1	8.4 ± 0.3	33 ± 2	497 ± 29	28 ± 2	5.6 ± 0.2	15 ± 1	139 ± 10	15 ± 1
8c	3.9 ± 0.2	11 ± 1	118 ± 9	10 ± 1	4.8 ± 0.2	12 ± 1	72 ± 5	10 ± 1	5.3 ± 0.3	15 ± 1	127 ± 8	15 ± 1
9a	5.6 ± 0.3	30 ± 2	> 1213	29 ± 2	54 ± 4	> 1213	> 1213	> 1213	7.8 ± 0.5	76 ± 6	> 1213	74 ± 5
9b	3.5 ± 0.2	7.5 ± 0.4	48 ± 4	7.4 ± 0.4	5.1 ± 0.2	11 ± 1	59 ± 4	10 ± 1	3.9 ± 0.2	9.0 ± 0.7	59 ± 4	8.9 ± 0.5
9c	2.8 ± 0.2	6.3 ± 0.3	54 ± 4	6.2 ± 0.3	3.7 ± 0.2	8.1 ± 0.4	40 ± 3	7.4 ± 0.5	3.1 ± 0.2	6.2 ± 0.5	30 ± 2	6.1 ± 0.4

Percent inhibition noted is mean values ± SDs of three independent measures.

and **9a** of the test molecules were evaluated, they showed good performance with LD_{50} values ranging from 173 to > 1906 μM on the MCF7 cell line (Table 2). In terms of lethal concentration values of these compounds; **2b, 3b-c**, **5b**, and **8b** in the A549 cell line, **6**, **9**, **3c**, **5a**, **5c**, **8a**, and **9a** in the Hep3B cell line, and **3**, **5**, **6**, **9**, **5a**, **6a**, **8a**, and **9a** in the FL cell line showed high LC_{50} values in the range of 180–614 μM, 190 to > 1906 μM, and 432 to > 1906 μM, respectively (Tables 2 and 3). Lethal concentrations were higher than positive controls, indicating that the cytotoxic effects of the test substances were lower; making them more desirable (Table 4). The GI_{50} and TGI values from NCI criteria being lower than the positive controls indicate that the tumoricidal effects of the test substances are greater and are also desirable. We demonstrated by MTT assay that a significant proportion of our newly synthesized compounds have better or similar NCI criteria than anticancer drugs used as positive controls, cisplatin and 5FU (Table 4). However, MTT method measures cell death by evaluating the mitochondrial activity of living cells. Mitochondrial activity may be relatively less in normal cells so that the method can provide false-negative results. This was supported by the finding that inhibition percentage of normal cells was higher than of cancer cells (Tables 1–4). To eliminate the possible false negativity, ELISA BrdU-based proliferation assay was also used as a comparison with MTT assay. As a result of this precise measurement, it was seen that the newly synthesized compounds were not highly antiproliferative (GI_{50} values of about 2 μM, TGI values of about 4 μM, LC_{50} values of about 10 μM) on FL normal cell line (Tables 5 and 6). However, ELISA test showed that all of the alkyl derivatives containing 5 and 15 carbon chains (**2b-c**, **3b-c**, **5b-c**, **6b-c**, **8b-c**, **9b-c**) were highly antiproliferative on the C6 and

Table 3
GI_{50}, TGI, LC_{50} and IC_{50} values for **1-9** and **2a-c**, **3a-c**, **5a-c**, **6a-c**, **8a-c**, **9a-c** against FL.

Compounds (μM)	FL				Compounds (μM)	FL			
	GI_{50}	TGI	LC_{50}	IC_{50}		GI_{50}	TGI	LC_{50}	IC_{50}
1	12 ± 1	> 1906	> 1906	> 1906	**5a**	10 ± 1	41 ± 4	649 ± 45	41 ± 4
2	6.9 ± 0.3	539 ± 34	> 1906	483 ± 29	**5b**	5.3 ± 0.3	13 ± 1	86 ± 7	13 ± 1
3	8.8 ± 0.4	44 ± 4	> 1906	43 ± 4	**5c**	4.0 ± 0.2	9.4 ± 0.7	55 ± 6	9.4 ± 0.7
4	28 ± 2	> 1906	> 1906	> 1906	**6a**	11 ± 1	151 ± 9	> 1213	147 ± 8
5	13 ± 1	62 ± 5	1582 ± 98	61 ± 5	**6b**	3.8 ± 0.1	8.4 ± 0.6	49 ± 4	8.3 ± 0.5
6	13 ± 1	107 ± 6	> 1906	104 ± 7	**6c**	1.9 ± 0.1	2.7 ± 0.1	21 ± 2	2.7 ± 0.1
7	15 ± 1	> 1906	> 1906	> 1906	**8a**	6.5 ± 0.3	23 ± 2	422 ± 34	23 ± 2
8	10 ± 1	344 ± 19	> 1906	320 ± 19	**8b**	4.4 ± 0.2	10 ± 1	70 ± 6	10 ± 1
9	10 ± 1	33 ± 3	432 ± 31	32 ± 3	**8c**	3.0 ± 0.1	6.6 ± 0.3	47 ± 5	6.5 ± 0.3
2a	10 ± 1	204 ± 14	> 1213	196 ± 14	**9a**	7.4 ± 0.3	47 ± 4	> 1213	46 ± 4
2b	5.7 ± 0.3	14 ± 1	96 ± 7	14 ± 1	**9b**	2.0 ± 0.1	2.1 ± 0.1	5.4 ± 0.4	2.0 ± 0.1
2c	3.1 ± 0.2	6.8 ± 0.4	48 ± 4	6.7 ± 0.3	**9c**	1.8 ± 0.1	1.8 ± 0.1	5.0 ± 0.4	1.8 ± 0.1
3a	10 ± 1	> 1213	> 1213	> 1213					
3b	3.1 ± 0.2	6.2 ± 0.4	38 ± 3	6.1 ± 0.3					
3c	5.3 ± 0.2	13 ± 1	84 ± 5	13 ± 1					

Percent inhibition noted is mean values ± SDs of three independent measures.

Table 4
IC_{50} (μM) of positive controls in cell lines.

	HeLa	HT29	A549	MCF7	C6	Hep3B	FL
Cisplatin	167 ± 8	134 ± 7	201 ± 8	212 ± 8	110 ± 5	162 ± 7	175 ± 8
5FU	473 ± 14	501 ± 17	536 ± 15	570 ± 16	417 ± 14	483 ± 15	454 ± 15

Table 5
GI_{50}, TGI, LC_{50} and IC_{50} values for **2a**, **3a**, **5a**, **6a**, **8a**, **9a** against C6, HeLa, and FL*

Compounds (μM)	GI_{50}			TGI			LC_{50}			IC_{50}		
	C6	HeLa	FL	C6	HeLa	FL	C6	HeLa	FL	C6	HeLa	FL
2a	75 ± 6	> 1213	4.1 ± 0.3	287 ± 14	> 1213	17 ± 2	> 1213	> 1213	> 1213	841 ± 43	> 1213	11 ± 1
3a	122 ± 9	> 1213	8.0 ± 0.4	> 1213	> 1213	> 1213	> 1213	> 1213	> 1213	> 1213	> 1213	> 1213
5a	41 ± 4	53 ± 4	7.4 ± 0.4	85 ± 6	129 ± 9	29 ± 2	209 ± 11	401 ± 17	630 ± 22	104 ± 9	174 ± 12	23 ± 2
6a	74 ± 5	112 ± 8	6.5 ± 0.3	289 ± 11	456 ± 19	50 ± 4	> 1213	> 1213	> 1213	526 ± 27	380 ± 15	32 ± 3
8a	50 ± 4	79 ± 5	5.6 ± 0.3	119 ± 8	188 ± 12	15 ± 1	357 ± 16	547 ± 22	147 ± 8	156 ± 11	236 ± 12	12 ± 1
9a	91 ± 8	170 ± 12	9.0 ± 0.6	360 ± 15	> 1213	45 ± 3	> 1213	> 1213	> 1213	728 ± 41	464 ± 18	36 ± 3

Percent inhibition noted is mean values ± SDs of three independent measures.
* These parameters were determined by ELISA BrdU Assay.

Table 6
GI_{50}, TGI, LC_{50} and IC_{50} values for **2b-c**, **3b-c**, **5b-c**, **6b-c**, **8b-c**, **9b-c** against C6, HeLa, and FL*.

Compounds	GI_{50}			TGI			LC_{50}			IC_{50}		
(μM)	C6	HeLa	FL	C6	HeLa	FL	C6	HeLa	FL	C6	HeLa	FL
2b	0.02**	0.08	2.4 ± 0.1	0.1	0.3	5.1 ± 0.2	1.2 ± 0.1	2.2 ± 0.1	12 ± 1	0.3	0.7	4.7 ± 0.2
3b	1.4 ± 0.1	1.3 ± 0.1	2.4 ± 0.1	3.1 ± 0.1	3.2 ± 0.1	5.2 ± 0.2	8.0 ± 0.6	8.5 ± 0.5	12 ± 1	3.9 ± 0.1	4.3 ± 0.2	4.7 ± 0.2
5b	0.3	0.7	1.9 ± 0.1	1.0 ± 0.1	1.9 ± 0.1	4.5 ± 0.2	4.3 ± 0.2	6.1 ± 0.3	11 ± 1	1.9 ± 0.1	2.8 ± 0.1	4.0 ± 0.2
6b	0.2	0.3	1.4 ± 0.1	0.6	1.1 ± 0.1	3.7 ± 0.1	2.9 ± 0.1	4.3 ± 0.2	11 ± 1	1.1 ± 0.1	2.0 ± 0.1	3.1 ± 0.1
8b	0.1	0.2	2.4 ± 0.1	0.5	0.6	5.2 ± 0.2	2.7 ± 0.1	3.2 ± 0.1	12 ± 1	1.0 ± 0.1	1.2 ± 0.1	4.7 ± 0.2
9b	0.01	0.01	1.8 ± 0.1	0.03	0.02	3.7 ± 0.1	0.4	0.2	8.4 ± 0.5	0.1	0.1	3.6 ± 0.1
2c	1.4 ± 0.1	0.3	2.5 ± 0.1	3.2 ± 0.1	1.0 ± 0.1	5.5 ± 0.2	8.0 ± 0.6	4.3 ± 0.1	13 ± 1	4.1 ± 0.2	1.9 ± 0.1	5.1 ± 0.2
3c	2.2 ± 0.1	1.1 ± 0.1	2.1 ± 0.1	4.5 ± 0.2	2.7 ± 0.1	5.1 ± 0.2	9.6 ± 0.7	7.6 ± 0.3	14 ± 1	5.0 ± 0.2	3.7 ± 0.2	4.5 ± 0.2
5c	0.01	0.01	2.6 ± 0.1	0.03	0.02	5.6 ± 0.2	0.4	0.3	13 ± 1	0.1	0.1	5.2 ± 0.2
6c	0.01	0.01	0.6	0.02	0.03	1.7 ± 0.1	0.3	0.3	5.9 ± 0.4	0.1	0.1	1.4 ± 0.1
8c	0.1	0.01	1.3 ± 0.1	0.2	0.1	3.3 ± 0.1	1.6 ± 0.1	0.6	9.1 ± 0.7	0.5	0.2	2.8 ± 0.1
9c	0.002	0.002	0.2	0.01	0.01	0.8	0.1	0.1	3.5 ± 0.2	0.02	0.02	0.5

Percent inhibition noted is mean values ± SDs of three independent measures.
* These parameters were determined by ELISA BrdU Assay.
** If percent inhibition is smaller than 1, the SD value is < 0.1.

Table 7
% Cytotoxicity of **1**-**9** at IC_{50} concentrations against C6, HeLa, and HT29.

Compounds	C6	HeLa	HT29
1	15 ± 2	9 ± 0.8	14 ± 1
2	14 ± 1	11 ± 1	15 ± 1
3	23 ± 2	12 ± 1	26 ± 3
4	25 ± 2	10 ± 1	21 ± 2
5	23 ± 2	8 ± 0.7	23 ± 2
6	25 ± 2	9 ± 0.7	21 ± 2
7	21 ± 2	11 ± 1	15 ± 1
8	25 ± 2	12 ± 1	23 ± 2
9	20 ± 2	14 ± 1	24 ± 2

Percent cytotoxicity was noted as mean values ± SDs of three independent measures.

HeLa cell lines (Tables 5 and 6). Furthermore, it was found that the compounds **2b**, **6b**, **8b**, **9b**, **5c**, **6c**, **8c**, and **9c** were strongly antiproliferative on C6 cells at 0.02–1.1 ± 0.1 μM IC_{50}, 0.01–0.6 μM TGI, and 0.002–0.2 μM GI_{50} value intervals. Similarly, the compounds **2b**, **8b**, **9b**, **5c**, **6c**, **8c**, and **9c** acted as effective anticancer agents on HeLa cells at 0.02–1.2 ± 0.1 μM IC_{50}, 0.01–0.6 μM TGI and 0.002–0.2 μM GI_{50} value intervals. Another important test we have used in cytotoxicity studies was undoubtedly the LDH activity measurement test indirectly showing membrane damage. When LDH activity measurement results were evaluated for the synthesized compounds, it was found that **2c**, **5b-c**, and **6a** for A549 cells; **2a-c**, **3c**, **5a-b**, **6a-c**, and **8b-c** for Hep3B cells; **2a-c**, **3a-c**, **5a-c**, **6b-c**, **8a-c**, and **9a-c** for MCF7 cells; **1**, **2**, **2a**, **3a**, **5c**, **8c**, and **9c** for C6 cells; **1**-**9**, **3a**, **5a**, **6a**, **8a**, and **9a** for HeLa cells; **1**, **2**, **7**, **2a**, **3b**, **5b**, **6a**, **8a**, and **9b** for HT29 cells; and **2a**, **3a**, **5c**, **6b**, **8c**, and **9c** for FL cells caused approximately 8% to 15% membrane damage at their IC_{50} concentrations (Tables 7 and 8). The membrane damage caused by the molecules mentioned above is very close to the cytotoxicity values of our positive controls (5FU and Cisplatin) and it meets the NCI criteria in large proportion (Table 9). For this reason, it is possible to conclude that these molecules having the cytotoxicity at the ideal limit are suitable for advanced pharmacological tests.

2.2.2. *Changes in cell morphology after the treatments with new compounds*

After the GI_{50}/IC_{50} concentrations for the applied compounds on these cell lines have been determined, the next step was to find out whether these compounds were cytotoxic or cytostatic. Following treating the cells with the 2-amino-4-aryl-6-pyridopyrimidines (**1**-**9**) and their *N*-alkyl bromide derivatives bearing 5, 10 and 15 carbon chains (**2a-c, 3a-c, 5a-c, 6a-c, 8a-c**, **9a-c**) at various concentrations, cell morphologies of C6, Hela, A549, Hep3B, MCF7 and FL were visualized by phase-contrast microscopy. These compounds caused the following morphological changes in the cells: (**i**) the transformation of the cells' epithelial shape into globular, (**ii**) low cell confluence, (**iii**)

Table 8
% Cytotoxicity of **2a-c**, **3a-c**, **5a-c**, **6a-c**, **8a-c**, **9a-c** at IC_{50} concentrations against A549, Hep3B, MCF7, C6, HeLa, HT29, and FL.

Compounds	A549	Hep3B	MCF7	C6	HeLa	HT29	FL
2a	26 ± 2	15 ± 1	7 ± 0.6	15 ± 1	26 ± 2	14 ± 1	18 ± 1
2b	25 ± 2	14 ± 1	8 ± 0.6	21 ± 2	29 ± 2	27 ± 2	26 ± 2
2c	15 ± 1	14 ± 1	7 ± 0.5	27 ± 2	27 ± 2	22 ± 2	23 ± 2
3a	23 ± 2	19 ± 2	13 ± 1	12 ± 1	16 ± 1	22 ± 2	16 ± 1
3b	23 ± 2	16 ± 1	14 ± 1	24 ± 2	24 ± 2	17 ± 1	22 ± 2
3c	26 ± 2	10 ± 1	14 ± 1	25 ± 2	23 ± 2	29 ± 2	23 ± 2
5a	19 ± 2	15 ± 1	13 ± 1	23 ± 2	15 ± 1	24 ± 2	22 ± 2
5b	14 ± 1	15 ± 1	12 ± 1	21 ± 2	28 ± 2	14 ± 1	26 ± 2
5c	16 ± 1	19 ± 1	16 ± 1	14 ± 1	27 ± 2	22 ± 2	17 ± 1
6a	14 ± 1	13 ± 1	17 ± 1	29 ± 3	14 ± 1	18 ± 1	19 ± 2
6b	26 ± 2	15 ± 1	14 ± 1	29 ± 3	27 ± 2	23 ± 2	14 ± 1
6c	21 ± 2	14 ± 1	15 ± 1	27 ± 2	28 ± 2	20 ± 2	25 ± 2
8a	25 ± 2	18 ± 1	8 ± 0.6	25 ± 2	17 ± 1	16 ± 1	26 ± 2
8b	24 ± 2	16 ± 1	14 ± 1	30 ± 2	24 ± 2	29 ± 2	26 ± 2
8c	20 ± 2	16 ± 1	12 ± 1	15 ± 1	29 ± 2	21 ± 2	16 ± 1
9a	19 ± 2	18 ± 1	8 ± 0.5	22 ± 2	14 ± 1	27 ± 2	26 ± 2
9b	26 ± 2	16 ± 1	23 ± 2	25 ± 2	24 ± 2	16 ± 1	23 ± 2
9c	27 ± 2	24 ± 2	16 ± 0.1	14 ± 1	22 ± 2	25 ± 2	14 ± 1

Percent cytotoxicity was noted as mean values ± SDs of three independent measures.

Table 9
% Cytotoxicity of positive controls at IC_{50} concentrations.

	HeLa	HT29	A549	MCF7	C6	Hep3B	FL
Cisplatin	10 ± 1	11 ± 1	9 ± 0.8	11 ± 1	9 ± 0.7	8 ± 0.6	8 ± 0.6
5FU	9 ± 0.8	8 ± 0.6	9 ± 0.7	8 ± 0.6	10 ± 1	10 ± 1	8 ± 0.6

Percent cytotoxicity was noted as mean values ± SDs of three independent measures.

floating cells with weak cellular adhesion, (**iv**) bubble-like protrusions on the cell surface, (**v**) cellular and cytoplasmic shrinkage, and (**vi**) cells to clump together (Fig. 3). These morphological changes most likely indicate apoptotic process and suggest that the compounds are cytotoxic. These degenerative changes in the cells and the decrease in the number of cells adhering to the flask surface were directly proportional to the concentration of the test substance used. In other words, the cells can achieve to maintain their normal fibroblast-like appearance when the test substance was applied at low concentrations, however, the cells treated with the compounds at high concentrations could not maintain their normal appearance (Fig. 3). Our detailed morphological examination also showed that there was a narrow therapeutic window to recognize apoptosis in the cells (implies the cytotoxic effect of molecules) within the first 12 h after the molecules were applied to cells at IC_{50} concentration (data not shown). Cells may have a chance to escape from apoptosis if these molecules were replaced by fresh media or enough time for recovery was given to the cell provided that it is below this limit values. However, the escape of cells from apoptosis did not help them re-proliferate but instead encouraged them to enter to G_0 phase (implies the cytostatic effect of molecules). These findings have demonstrated the data of the real therapeutic window of apoptotic agents and also the optimal concentration of them which is significant to the clinicians.

2.2.3. Evaluation of antibacterial effects of the compounds

Since 1940, the year of antibiotics treatment, bacteria have become resistant to many antibiotics. This resistance has not only limited the efficacy of the existing antibacterial drugs but also led to the emergence of multidrug resistance in bacteria. In recent years, > 70% of pathogenic bacteria have been shown to have resistance against at least one of the available antibacterials [49]. Under the guidance of this literature information, the effects of newly synthesized compounds on some pathogenic bacteria causing disease in human body have been revealed by using the Minimum Inhibition Concentration (MIC) method. When the MIC values of today's antimicrobial drugs were used as base, the test molecules were considered to be antibacterial at 125 μg/mL and below the MIC values. When the MIC values of 2-amino-4-aryl-6-pyridopyrimidines and their *N*-alkyl bromide derivatives displayed on Gram (+) bacteria were examined, it was found that antibacterial effects of **2b-c**, **3b-c**, **5a-c**, **6b-c**, **8a-c**, and **9c** against *E. faecalis* ATCC 29212 (< 14–303 μM); **6**, **2a**, and **8b** against *E. faecalis* VRE ATCC 19433 (130–477 μM); **2a-c**, **3b-c**, **5a-c**, **6a-c**, **8a-c**, and **9a-c** against *S. aureus* MRSA ATCC 46300 (< 14–152 μM); **2a-c**, **3b-c**, **5a-c**, **6a-c**, **8a-c**, and **9a-c** against *S. aureus* ATCC 25923 and MSSA ATCC 29213 (< 14–303 μM); **2b-c**, **3a-c**, **5a-c**, **6b-c**, **8a-c**, and **9b-c** against *S. gordonii* NCTC 7870 (< 14–303 μM); **3**, **9, 2a-c**, **3a-c**, **5a-c**, **6a-c**, **8a-c**, **9a**, and **9c** against *S. mutans* ATCC 35668 (< 14–477 μM); and **2a-c**, **3b-c**, **5a-c**, **6a-c**, **8a-c**, and **9a-c** against *A. actinomycetemcomitans* ATCC 33384 (< 14–303 μM) were more or similar to the SCF antibiotic used as a positive control (Tables 10, 11 and 12). According to the MIC values exhibited by the newly synthesized compounds on Gram (−) bacteria, it was determined that **2b**, **3b**, **5b**, **6b**, and **8b** (32–65 μM) against *E. coli* ESBL ATCC 35218 strain; **6b**, **8a-b**, and **9b** (< 16 μM) against *E. coli* ATCC 25922 strain; and **2b**, **3b**, and **8b** (130 μM) against *P. aeruginosa* AGME ATCC 27853 strain caused as much sensitivity as SCF antibiotic used as positive controls (Tables 10, 11 and 12). Our test molecules showed a slightly weaker antimicrobial effect against Gram (−) bacteria described above compared to Gram (+) bacteria. When the antimicrobial activities of the tested compounds were compared, *N*-alkyl bromide derivatives (**2a-c, 3a-c, 5a-c, 6a-c, 8a-c**, **9a-c**) showed stronger pharmacological activities than their ancestor compounds 'pyrimidines' (**1-9**). A more important point for *N*-alkyl bromide derivatives is that they have a strong antimicrobial effect when compared to the control antibacterial drug SCF against resistant strains such as VRE, MRSA, ESBL and AGME. It could be concluded that these new compounds prove to be suitable for advanced pharmacological investigations thanks to the severity of the lethal effect they exhibit on some pathogens (see Table 13).

2.2.4. Examination of DNA/BSA binding properties of the compounds

To elucidate the mechanisms of action of molecules found to exhibit high pharmacological activity in *in vitro* tests is crucial to the synthesis

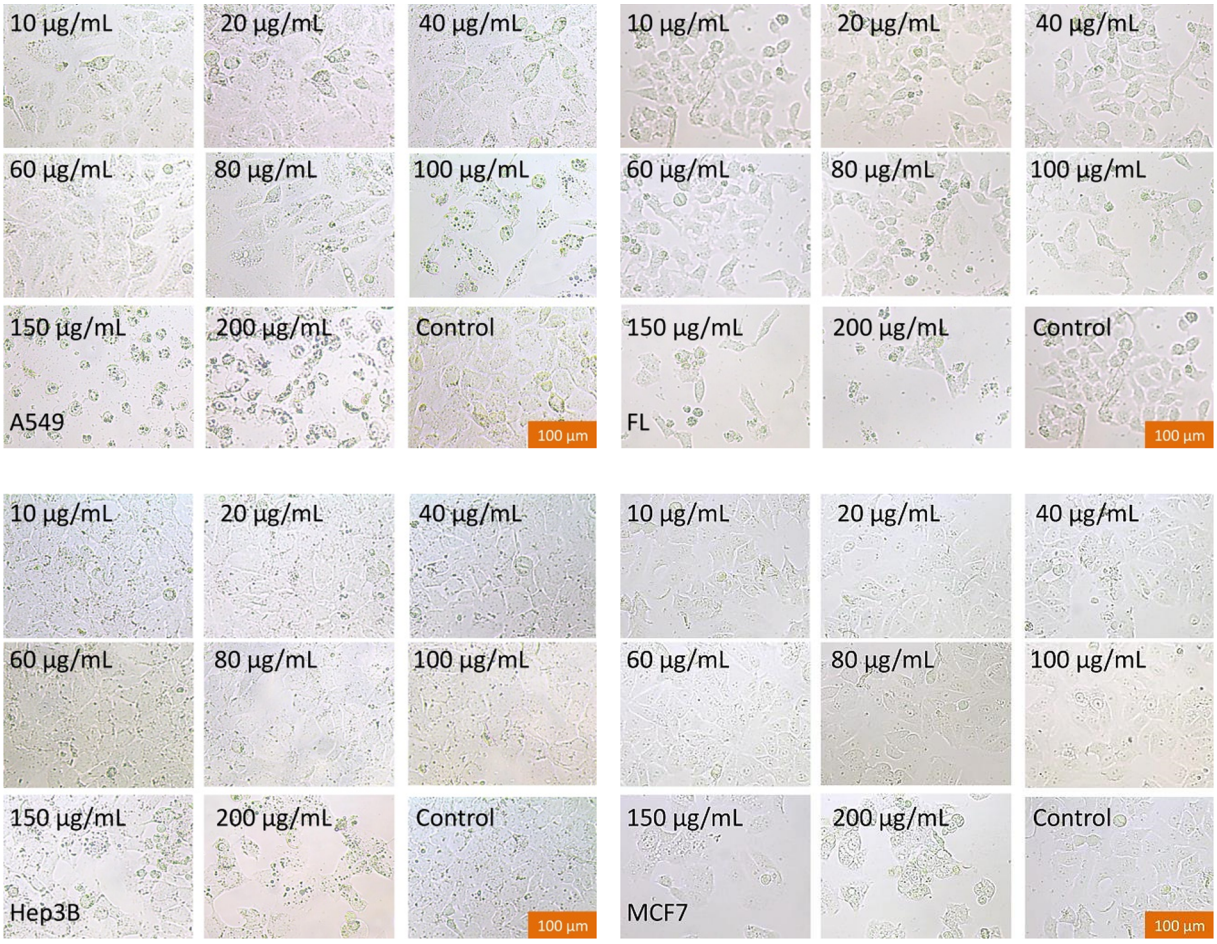


Fig. 3. Effect of ***5a*** on the morphologies of A549, FL, Hep3B and MCF7 cell lines. Exponentially growing cells were incubated overnight with various concentrations of ***5a*** at 37 °C. Control cells were treated with only DMSO. All measurements were 100 µm.

of more potent molecules. Active molecules bind to macromolecules, mostly DNA and proteins, which cause the desired changes in the organism. There are a number of methods, some of which are sophisticated, to demonstrate this interaction between the relevant macromolecules of the organism and the active molecules. Absorption spectroscopy which is easy to use and highly accurate, is one of the

Table 10
Minimum-inhibitory concentrations (MIC, in µM) of **1-9.**

Microorganisms	1	2	3	4	5	6	7	8	9	S/CF
E. faecalis **VRE** ATCC 19433	3812	953	3812	> 3812	> 3812	477	3812	3812	3812	306/277
E. faecalis ATCC 29212	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	1906	> 3812	3812	77/69
S. aureus ATCC 25923	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	306/277
S. aureus **MSSA** ATCC 29213	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	NA
S. aureus **MRSA** ATCC 46300	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	306/277
E. coli ATCC 25922	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	19/17
E. coli **ESBL** ATCC 35218	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	38/35
P. aeruginosa **AGME** ATCC 27853	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	> 3812	306/277
S. mutans ATCC 35668	1906	953	477	953	3812	1906	3812	1906	477	153/138
S. gordonii NCTC 7870	> 3812	> 3812	> 3812	> 3812	1906	1906	> 3812	> 3812	3812	153/138
A. actinomycetemcomitans ATCC 33384	> 3812	> 3812	953	> 3812	3812	1906	3812	3812	3812	77/69

S/CF: sulbactam/cefoperazone as a positive control.
NA: Not available.

Table 11
Minimum-inhibitory concentrations (MIC, in μM) of **2a-c**, **3a-c**, **5a-c.**

Microorganisms	2a	2b	2c	3a	3b	3c	5a	5b	5c	S/CF
E. faecalis **VRE** ATCC 19433	152	> 2074	905	1213	2074	1811	2426	> 2074	1811	306/277
E. faecalis ATCC 29212	607	< 16	< 14	2426	< 16	< 14	303	< 16	113	77/69
S. aureus ATCC 25923	< 19	< 16	< 14	1213	65	226	< 19	< 16	< 14	306/277
S. aureus **MSSA** ATCC 29213	152	< 16	< 14	1213	< 16	28	76	< 16	< 14	NA
S. aureus **MRSA** ATCC 46300	152	< 16	< 14	607	< 16	28	76	< 16	< 14	306/277
E. coli ATCC 25922	1213	> 2074	> 1811	1213	2074	1811	607	2074	> 1811	19/17
E. coli **ESBL** ATCC 35218	1213	32	1811	2426	65	> 1811	607	32	1811	38/35
P. aeruginosa **AGME** ATCC 27853	2426	130	> 1811	2426	130	1811	> 2426	2074	> 1811	306/277
S. mutans ATCC 35668	152	< 16	< 14	152	259	< 14	76	< 16	< 14	153/138
S. gordonii NCTC 7870	607	< 16	< 14	303	< 16	57	303	< 16	< 14	153/138
A. actinomycetemcomitans ATCC 33384	303	< 16	< 14	1213	65	113	303	< 16	< 14	77/69

S/CF: sulbactam/cefoperazone as a positive control.
NA: Not available.

most commonly used techniques to measure the affinity of any substance to DNA or protein. The interaction of molecules with DNA or protein causes significant changes in the backbone of these polymeric molecules that can be observed with spectrophotometric techniques. In general, changes in macromolecules caused by complexes arise as hyperchromic or hypochromic effects on the absorption spectra [50]. The changes occurring in this way are called as “the hypochromic effect” that causes a decrease in the UV/Vis spectral signal or as “the hyperchromic effect” that results in an increase in the UV/Vis spectral signal, and these changes are accepted as evidence of the formation of molecule-DNA adducts. In addition to the changes in the UV/Vis spectral signal, sometimes the shifts to the right (blue shift) or to the left (red shift) may occur in the absorption bands of the complexes, which may be a parameter for the stability of the complex-DNA structure. The

Table 13
The binding constants (K_b) of these compounds.

No	Kb (M^{-1})	No	Kb (M^{-1})	No	Kb (M^{-1})
1	7.9×10^4	**2a**	3.3×10^3	**6a**	5.5×10^4
2	1.0×10^4	**2b**	1.8×10^4	**6b**	2.7×10^4
3	1.2×10^3	**2c**	5.8×10^3	**6c**	2.5×10^4
4	4.8×10^3	**3a**	4.2×10^3	**8a**	0.4×10^3
5	1.6×10^4	**3b**	1.2×10^4	**8b**	1.3×10^4
6	1.4×10^4	**3c**	9.9×10^3	**8c**	1.7×10^4
7	0.9×10^4	**5a**	2.0×10^4	**9a**	4.7×10^4
8	7.4×10^4	**5b**	1.5×10^3	**9b**	1.7×10^3
9	1.0×10^4	**5c**	2.4×10^4	**9c**	2.5×10^4

Table 12
Minimum-inhibitory concentrations (MIC, in μM) of **6a-c**, **8a-c**, **9a-c.**

Microorganisms	6a	6b	6c	8a	8b	8c	9a	9b	9c	S/CF
E. faecalis **VRE** ATCC 19433	> 2426	518	> 1811	607	130	453	2426	1037	1811	306/277
E. faecalis ATCC 29212	607	< 16	226	303	< 16	< 14	607	1037	226	77/69
S. aureus ATCC 25923	76	< 16	< 14	< 19	< 16	28	152	518	28	306/277
S. aureus **MSSA** ATCC 29213	152	< 16	< 14	76	< 16	< 14	303	518	< 14	NA
S. aureus **MRSA** ATCC 46300	152	< 16	< 14	76	< 16	< 14	76	2074	< 14	306/277
E. coli ATCC 25922	607	< 16	> 1811	< 19	< 16	> 1811	607	< 16	> 1811	19/17
E. coli **ESBL** ATCC 35218	607	32	> 1811	607	32	> 1811	607	1037	> 1811	38/35
P. aeruginosa **AGME** ATCC 27853	2426	1037	> 1811	1213	130	> 1811	2426	> 2074	> 1811	306/277
S. mutans ATCC 35668	76	32	< 14	38	< 16	28	152	518	226	153/138
S. gordonii NCTC 7870	607	< 16	< 14	152	< 16	< 14	303	1037	< 14	153/138
A. actinomycetemcomitans ATCC 33384	303	< 16	57	152	< 16	< 14	303	130	57	77/69

S/CF: sulbactam/cefoperazone as a positive control
NA: Not available

DNA/BSA binding properties of the newly synthesized compounds were determined by using UV–Vis spectrophotometer, and properties of binding species as well as binding constants were explained below. A single maximum absorption peak was observed in the spectrum of the compounds obtained by UV–Vis studies, and no clear redshifts or blueshifts on this peak were observed except for compounds **3**, **5**, and **6**. When CT-DNA was added to increase amounts to the reaction medium, the absorption intensities of compounds **2**, **7**, and **8** were reduced resulting in hypochromic effect, and the increase in the absorption intensity of compounds **1**, **4**, and **9** caused hyperchromic appearance. Evaluation of compounds **3**, **5**, and **6** for their possible interaction with DNA exhibited hyperchromic properties, while compounds **3** and **5** caused redshift about 10 nm and compound **6** exhibited blueshift about 8 nm. Likewise, when CT-DNA was added in increasing amounts to the reaction mixture, compounds **2c**, **3c**, **3a**, **5c**, and **8a** caused hyperchromic effect, whereas compounds **2a**, **2b**, **3b**, **5a**, **5b**, **6a, 6b**, **6c**, **8b**, **8c**, **9a**, **9b**, and **9c** from the same group chemicals exhibited hypochromic feature. Investigating the possible interactions of the newly synthesized drug candidate molecules with BSA gives us important information about their transport pathway in blood since BSA is the standard molecule used as a carrier for many drugs and metabolites. For this reason, the spectrum bands described below will be guiding the prediction of the BSA-molecule relationship. According to the absorption spectra of 5, 10 and 15 carbons containing *N*-alkyl bromide derivatives upon increasing the concentration of BSA, compounds **2b**, **2c**, **3b**, **3c**, **5c**, **6c**, and **8c** caused hyperchromic effect, whereas only compound **18c** displayed hypochromic effect. These changes suggest that the compounds interact with BSA.

In our laboratory, absorbance data obtained by the spectrophotometric method was used to derive the binding constants (Kb), showing the affinity of the complex to DNA, of the newly synthesized complexes with the aid of the following the Wolfe–Shimmer equation [51]: [DNA]/(εa–εf) = [DNA]/(εb–εf) + 1/Kb(εb–εf), [DNA] is the concentration of DNA in the base pairs, and εa, εf and εb are the molar absorptivities of $A_{observed}$/[complex], free complex and complex-DNA solutions, respectively. Kb is the binding constant that is related to affinity between the complex and DNA and can be calculated algebraically from the line graph drawn between [DNA]/(εa-εf) and [DNA] as the slope. According to Table 11, the Kb values of pyrimidines were between 1.2 × 103 and 7.9 × 10^4 M^{-1} and Kb values of *N*-alkyl derivatives respectively were between 0.4 × 103 and 5.5 × 10^4 M^{-1}. The binding constants of the pyrimidines and alkyl derivatives were ordered from small to large in their own groups as **1** > **8** > **6a** > **9a** > **6b** > **6c** = **9c** > **5c** > **5a** > **2b** > **8c** > **5** > **6** > **8b** > **3b** > **2** = **9** > **3c** > **7** > **2c** > **4** > **3a** > **2a** > **9b** > **5b** > **3** > **8a**. The *Kb* values of the compounds-DNA adduct are mostly consistent with the IC_{50} data obtained using the MTT assay. Generally, the *N*-alkyl bromide derivative compounds exhibited equal DNA binding activity comparable to that of pyrimidines. Among other mechanisms, their DNA binding properties can help explain the effects of *N*-alkyl bromide derivatives against cell proliferation. The high Kb values suggest that the complexes were strongly bound to DNA. To compare, the Kb values of two important anticancer drugs (cisplatin and 5FU) were also examined and it was shown that these anticancer drugs had similar Kb values with newly synthesized compounds. While the binding constants of our compounds were 0.4 × 103 to 7.9 × 10^4 M^{-1}, the binding constants of the cisplatin and 5FU were reported as 5.73 × 10^4 M^{-1} and 9.7 × 10^4 M^{-1}, respectively, according to CT-DNA binding studies using these anti-cancer drugs [52–54]. The data presented in Table 11 suggest that the new compounds, particularly **1** and **8**, bind DNA much more strongly than the 5FU anticancer drug although the binding constants between new compounds and DNA were not very high but similar when compared with cisplatin.

3. Conclusion

In summary, a new series of 2-amino-4-aryl-6-pyridopyrimidines (**1-9**) and their *N*-alkyl bromide derivatives bearing different length carbon chains (**2a-c, 3a-c, 5a-c, 6a-c, 8a-c**, **9a-c**) were effectively synthesized in good yields, and characterized by using spectral techniques. In total, 27 compounds have been obtained whereof only compound **7**, **8**, and **9** are already known in the literature [28,31]. Although those mentioned compounds being known in the literature, there is no study revealing their antimicrobial and anticancer properties. In this work, the biological activities -anti-proliferative, cytotoxic, antibacterial properties and DNA/protein binding affinities- of the synthetic compounds (**1-9**, **2a-c**, **3a-c**, **5a-c**, **6a-c**, **8a-c**, **9a-c)** were explored. The majority of the synthetic compounds displayed significant anti-proliferative effects on the tested cancer cell lines**.** Especially, **b** and **c** series of alkylated derivatives showed a remarkable impact. The anticancer results revealed that the alkyl chain prolongation has a deleterious effect on cancer cell lines. On the other hand, the results of antimicrobial activities showed that the majority of the compounds were more effective against Gram (+) bacteria than Gram (−) bacteria. In particular, *N*-alkyl bromide derivatives (especially **a-c** series on G (+) and **b** series on G (−)) exhibited stronger antibacterial properties than their parent molecules (2-amino-4-aryl-6-pyridopyrimidines) (**1-9**). The evaluation of the synthetic compounds (**1-9**, **2a-c**, **3a-c**, **5a-c**, **6a-c**, **8a-c**, **9a-c)** for their DNA binding affinities showed that the new compounds; particularly compound **1** and **8**, formed a stronger bond with DNA than the 5FU anticancer drug although the binding constants between new compounds and DNA were not higher but similar when compared to cisplatin. The findings show strong evidence for new investigations involving analogous compounds and the methods to explore mechanistic aspects of anticancer activity.

4. Experimental

The materials and equipment used in this study are given in supplementary information. The procedure of the synthesis of the new compounds designed for this study and pharmacological experiments that include the preparation of cell culture, cell proliferation assay (MTT assay), cytotoxic activity assay, BrdU cell proliferation assay (BCPA), microdilution assay, and DNA binding studies are provided in supplementary information. The calculation of IC_{50} and three dose response parameters were explained in the supplementary information. All spectral data, melting points, retention factors and elemental analysis results of the obtained compounds were also given in supplemental files.

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Appendix A. Supplementary material

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