

Design and Synthesis of a New Class of Pyridine-Based *N*-Sulfonamides Exhibiting Antiviral, Antimicrobial, and Enzyme Inhibition Characteristics

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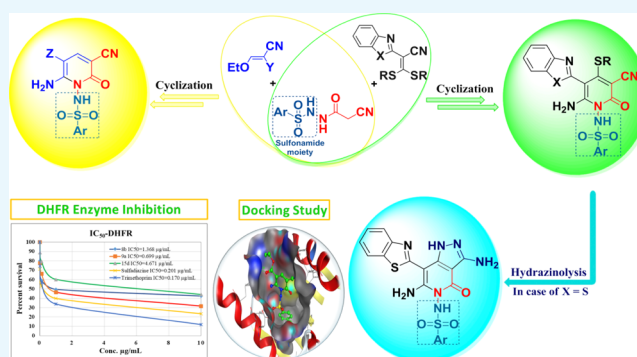


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ABSTRACT: A new strategy for designing and assembling a novel class of functionalized pyridine-based benzothiazole and benzimidazole incorporating sulfonamide moieties was developed. The synthesis was carried out by reacting *N*-cyanoacetoarylsulfonylhydrazide with various electrophiles such as 2-(benzo[*d*]thiazol-2-yl)-3,3-bis(alkylthio)acrylonitriles and 2-(benzo[*d*]imidazol-2-yl)-3,3-bis(methylthio)acrylonitriles, as well as 2-ethoxyl acrylonitrile derivatives. The synthesized compounds were tested for their antiviral and antimicrobial potency. Two of the synthesized compounds, **15c** and **15d**, showed more than 50% viral reduction against HSV-1 and CBV4, with significant IC_{50} and CC_{50} values. The two potent compounds **15c** and **15d** have also shown inhibitory activity against Hsp90 α protein with IC_{50} values of 10.24 and 4.48 $\mu\text{g}/\text{mL}$, respectively. A combination of **15c** and **15d** with acyclovir has led to IC_{50} values that are lower than that of acyclovir alone. Molecular modeling studies were used to identify the interactions between the **15c** and **15d** compounds and the active site of Hsp90 α enzyme. The antimicrobial investigation of the new compounds has also shown that **8b** and **15d** exhibited a higher inhibition zone (IZ) than sulfadiazine and gentamicin against *Klebsiella pneumoniae*, whereas **9a** showed higher IZ than ampicillin against *Staphylococcus aureus*. According to the enzyme assay study on dihydrofolate reductase, **9a** was shown to be the most potent compound among all examined compounds.



1. INTRODUCTION

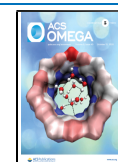
Recently, we conducted numerous research investigations to develop different innovative synthetic methods for the preparation of *N*-sulfonamido and *N*-sulfonyl-based heterocyclic compounds that have come into application as new forms for antiviral and antimicrobial agents.^{1–4} Series of *N*-sulfonylpyrazole, synthesized by our group,^{5,6} were evaluated against the enzyme cathepsin B.16 and breast adenocarcinoma MCF-7 cell line.^{7,8} Similarly, the *N*-arylsulfonylpyrazole series was also identified to be active as inhibitors of the NS2B-NS3 enzyme.⁹ These promising results led our research team to further investigate new approaches for the synthesis of alternative scaffolds for use as promising chemotherapeutics. In 1990, we have reported the first synthesis of structurally simple 2-arylbenzothiazoles (**A**), by the reaction of *o*-aminothiophenol with arylmethylenecyanothioacetamide,¹⁰ whose derivatives displayed thereafter interesting pharmacological properties suggesting their potential as anticancer, antiviral, and antimicrobial agents, **Figure 1**. The aniline derivative, compound (**B**), had excellent *in vitro* cytotoxicity in nanomolar concentrations against breast cancer cell line.¹¹ Compound (**C**) has demonstrated a superior *in vivo* efficacy against breast cell line; however, metabolic instability

prevented its development as a chemotherapeutic agent, **Figure 2**. To overcome this problem, fluorinated analog (**D**) had been developed.¹² Compound (**E**) showed excellent growth inhibition of kidney cancer cell line A498,^{13,14} whereas compound (**F**), naphthalimidearylbenzothiazole, was found to possess potent cytotoxicity against human hepatocarcinoma cell line SMMC-7721.¹⁵ These findings were later on filed as patents. As a result of these interesting findings, we plan in this study to complement our previous work on 2-arylbenzothiazoles by replacing the benzene ring with a pyridine ring-tagged sulfonamide moiety. The synthesis was carried out by reacting *N*-cyanoacetoarylsulfonylhydrazide with various electrophiles such as 2-(benzo[*d*]thiazol-2-yl)-3,3-bis(alkylthio)acrylonitriles and 2-(benzo[*d*]imidazol-2-yl)-3,3-bis(methylthio)acrylonitriles, as well as 2-ethoxyl acrylonitrile derivatives. Antiviral and antimicrobial activities, as well as the toxicity effect of the

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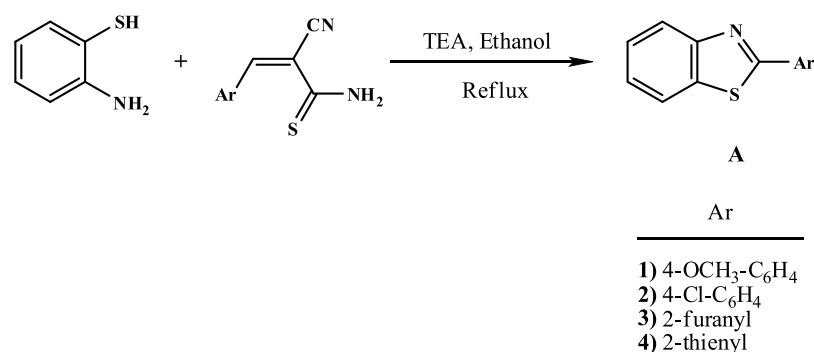


Figure 1. Chemical reaction illustrating the first method for the synthesis of 2-arylbenzothiazole.

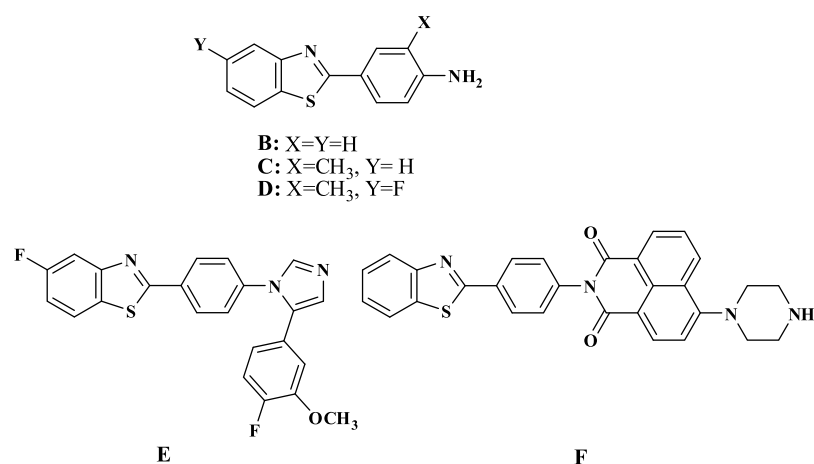


Figure 2. Chemical structure of drugs containing 2-arylbenzothiazole nucleus A–F.

synthesized compounds have all been evaluated. Many of the synthesized compounds showed interesting activities when compared to that of current antimicrobial drugs.

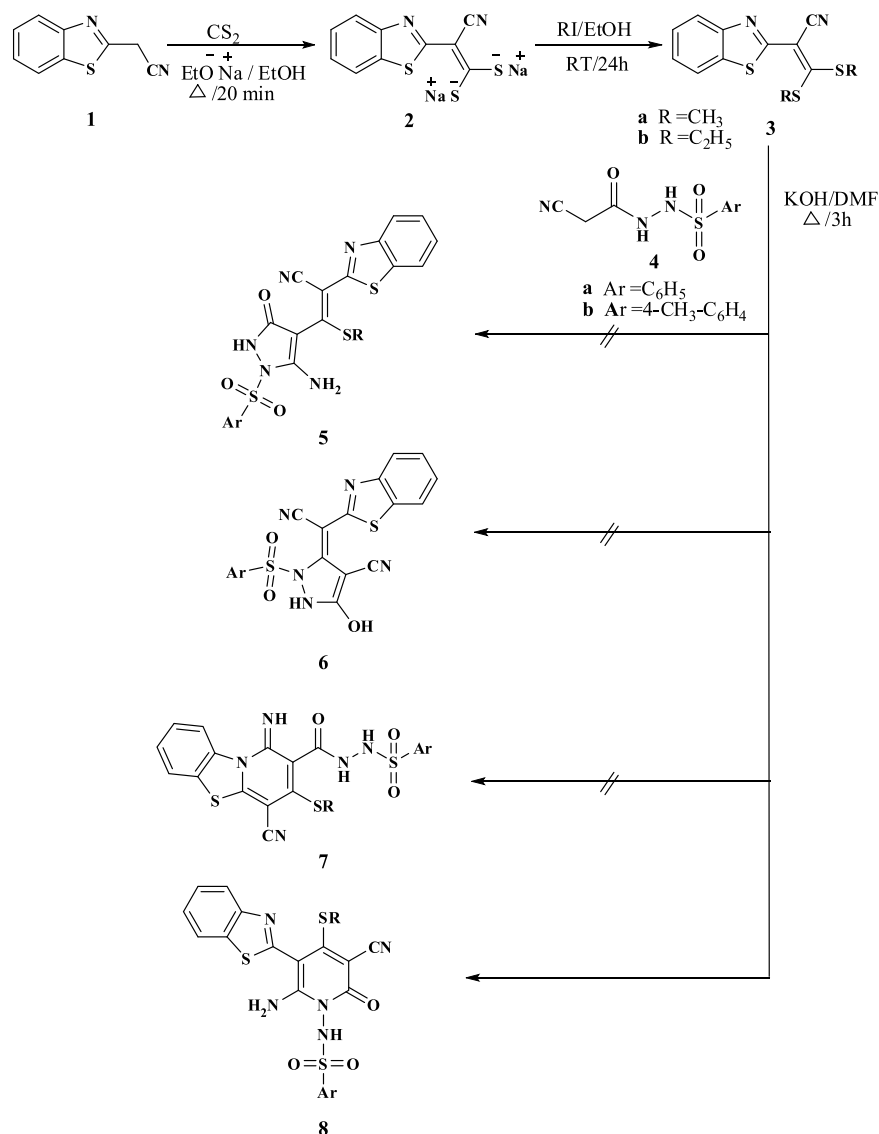
2. RESULTS AND DISCUSSION

2.1. Chemistry. The synthetic strategies adopted for producing the target pyridine-based benzothiazole and benzimidazole compounds are represented in Schemes 1–4. This work focused on the key compound bearing sulfonamide moiety, which was used as a potential precursor for the synthesis of the novel series of *N*-sulfonylaminopyridones tagged either a benzothiazole or benzoimidazole ring. The key compound, *N*-cyanoacetoarylsulfonylhydrazide was afforded *via* a convenient reaction that was developed earlier of cyanoacetohydrazide with arylsulfonyl chloride.⁶ The reaction of *N*-cyanoacetoarylsulfonylhydrazide **4a,b** with 2-(benzo[*d*]-thiazol-2-yl)-3,3-bis(alkylthio)-acrylonitrile **3** in the presence of potassium hydroxide in dry dimethylformamide (DMF) produced an adduct for which four possible isomeric structures were considered, as shown in Scheme 1. The X-ray crystal structure of **8b** confirmed the presence of the compound in the solid state.¹⁶ Addition of the active methylene carbon atom of **4a,b** to the double bond of **3a,b**, followed by the elimination of RSH and the subsequent cyclization *via* the addition of the NH group to the cyano group of benzothiazole led to the formation of the thermodynamically controlled and kinetically favored product **8a–d**. The ¹H NMR spectra of **8b** revealed the presence of an amino group at 8.83 ppm and a pyridine methylthio group at 2.45 ppm. In addition, NH₂ stretching

band was confirmed in the infrared (IR) spectra at a range of 3200–3208 cm⁻¹.

Furthermore, treatment of thioalkylpyridone derivatives **8a–d** with hydrazine hydrate in the presence of a catalytic amount of piperidine in DMF resulted in the formation of the corresponding 1*H*-pyrazolo[4,3-*c*]pyrid-2-ones **9a,b**, Scheme 2. This reaction occurred with the loss of the alkylthio group by nucleophilic attack of hydrazine and the subsequent cyclization through the attack on the cyano group in the pyridone ring to afford pyrazolopyridones **9a,b**. The structure of compounds **9a,b** was confirmed by their basic analysis spectroscopic data [Fourier transform infrared spectroscopy (FTIR), ¹H NMR and mass]. The absence of cyano stretching band in the IR spectra as well as the disappearance of the SCH₃ protons in ¹H NMR validated the conversion of **8a–d** to **9a,b**. In addition, the ¹H NMR spectra showed two peaks at 11.92–11.95 and 6.10–6.11 ppm and thus indicating the presence of NH₂ and NH, respectively.

The target benzoimidazole derivatives **13a,b** was synthesized using the reaction sequence illustrated in Scheme 3. They are formed by the reaction of *N*-cyanoacetoarylsulfonylhydrazide **4a,b** with 2-(1*H*-benzo[*d*]imidazole-2-yl)-3,3-bis(methylthio)-acrylonitrile derivatives **12** at room temperature. The structure of **13a,b** was characterized by their basic analysis and spectroscopic data (¹H NMR and IR). For example, ¹H NMR spectra of **13a** revealed singlet peaks at 2.35 and 2.90 ppm, which are assigned to CH₃ and SCH₃ protons, respectively, and multiplet peaks at range of 8.30–7.40 ppm for aromatic protons.

Scheme 1. Synthesis of *N*-(5-(Benzo[*d*]thiazol-2-yl)-4-(alkylthio)-2-oxypyridin-1(2*H*)-yl)arylsulfonamide

Moreover, our investigation was extended to include reacting *N*-cyanoacetoarylsulfonylhydrazide **4a,b** with various acrylonitrile derivatives, Scheme 4. The reaction of *N*-cyanoacetoarylsulfonylhydrazide **4a,b** with 2-ethyl acrylonitrile derivatives **14a,b** in ethanolic sodium ethoxide afforded the corresponding *N*-arylsulfonylpyridones **15a–d**. The synthetic route to target compounds **15a–d** is assumed to occur *via* the addition of the active methylene group of **4a,b** to the ylidene bond in **14a,b** followed by the elimination of one ethanol molecule and the cyclization *via* the addition of the NH group to the cyano group. The elemental analysis and spectral data validated the proposed individual structures of compounds **15a–d**. IR showed NH₂ absorption band at a range of 3308–3286 cm⁻¹. In addition, a singlet peak in ¹H NMR at range of 7.95–7.94 ppm confirmed the presence of pyridine-H. In the case of compounds **15c,d**, ¹H NMR spectrum showed a triplet peak at 1.28 ppm and quartet at 4.24 ppm for CH₂ and CH₃, respectively, of the COOCH₂CH₃ group. Furthermore, ¹H NMR spectra revealed the presence of an amino group at the range of 7.68–8.02 ppm.

2.2. Biological Results. **2.2.1. Antiviral Evaluation.** The antiviral activities of the newly synthesized were evaluated *in*

vitro against a wide variety of viruses such as herpes simplex virus type 1 (HSV-1), coxsackievirus B4 (CBV4), hepatitis A virus HM 175 (HAV HM 175), ED-43/SG-Feo (VYG) replicon of hepatitis C virus genotype 4a (HCVcc), and adenovirus type 7 (HAdV7). As reported, no specific medications are available for HCVgenotype4, CBV4, and HAdV7 viruses and the commercial drugs are only used to treat the symptoms but not the illness itself except for HSV-1, acyclovir is used.^{17–19} In order to study their antiviral activities, the newly synthesized compounds were first subjected to a cytotoxicity evaluation as shown in the Supporting Information document using cell line FRHK-4, Hep2, BGM, Vero, and Huh 7.5 as the specific hosts to the various studied viruses. For comparison, acyclovir was used as standard drug against HSV-1. No significant difference was observed between the nontoxic doses of the different synthesized compounds, which ranged between 90 and 120 μg/mL. The synthesized compounds showed an apparent effect on viruses having different types of genome, that is, either RNA such as CBV4, HAV, and HCV or DNA such as HAdV7 and HSV-1, Table 1. Two compounds in particular, **15c** and **15d**, showed interesting antiviral effects that exceed 50% reduction against three of the studied viruses,

Scheme 2. Synthesis of *N*-(7-(Benzo[*d*]thiazol-2-yl)-4-oxo-1*H*-pyrazolo[4,3-*c*]pyridin-5(4*H*)-yl)arylsulfonamide

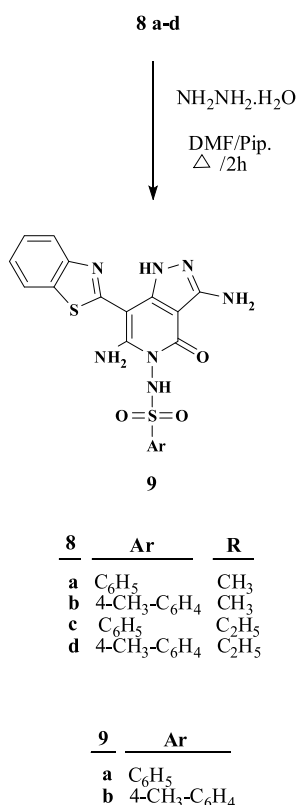
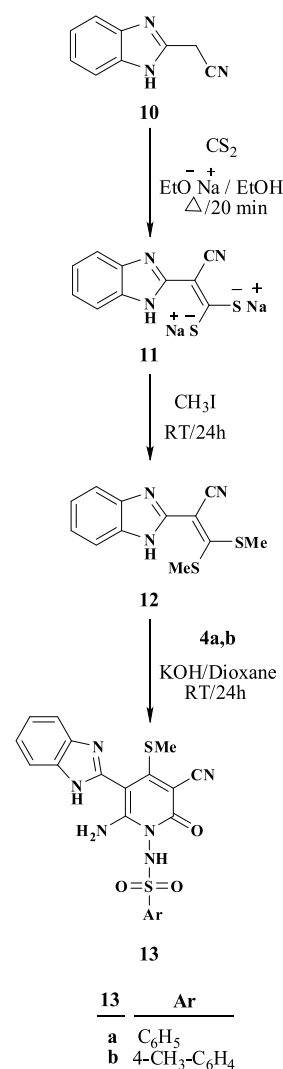


Figure 3. Error bars in the Figure 3 represent standard division of the measured data. The 50% maximum cytotoxicity concentration (CC₅₀), defined as the concentration in μg/mL required to reach 50% cytotoxicity of the treated uninfected cells, and the 50% maximal inhibitory concentration (IC₅₀), defined as the concentration in μg/mL required to inhibit 50% of the tested viruses, were both evaluated for the two compounds, **15c** and **15d**, that exhibited greater than 50% viral reduction against the aforementioned tested viruses. In addition, the selectivity index (SI) of the promising compounds was calculated by evaluating the CC₅₀/IC₅₀ ratio, Table 2.

The two compounds, **15c** and **15d**, showed, however, moderate levels of activity against HSV-1, CBV4, and HAV. In the case of HSV-1, both compounds **15c** and **15d** exhibited IC₅₀ values of 90 μg/mL, whereas their CC₅₀ were 250 and 220 μg/mL, respectively. Both compounds showed 50% viral reduction against HSV-1 while acyclovir showed 99.6% viral reduction. Additionally, compound **15c** showed 50% reduction for CBV4 and 53% reduction for HAV, with IC₅₀ 88 μg/mL and SI 2.95 for both viruses while compound **15d** showed reduction 50% for CBV4 alone with IC₅₀ 90 μg/mL and SI 2.66.

2.2.1.1. Structure Activities Relationship. Based on the results of the tested activities against HSV-1, CBV4, and HAV, as shown in Tables 1 and 2, the structure–activity relationships (SARs) have been established. We can observe that both compounds **15c** and **15d** possessing the ethoxycarbonyl group at C5 of the pyridine ring have almost equal activities toward HSV-1 and CBV4. However, replacement of the ethoxycarbonyl group with a cyano group led to the other two compounds, **15a** and **15b**, which showed lower to no activity

Scheme 3. Synthesis of *N*-(5-(1*H*-Benzo[*d*]imidazol-2-yl)-2-oxopyridin-1(2*H*)-yl)arylsulfonamide

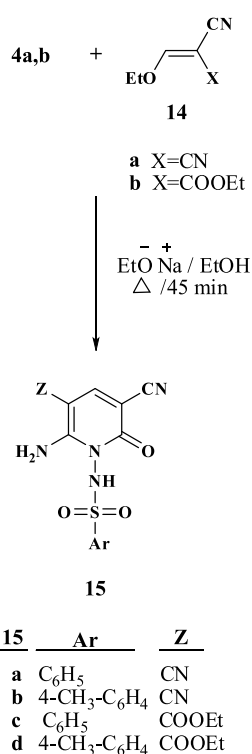


against the aforementioned viruses. Alternatively, the presence of a benzothiazole ring at C5 and an alkylthio group at C4 of the pyridine ring, compounds **8a–d**, resulted in lower activities for these compounds than those of the compounds containing ethoxycarbonyl group at C5, **15c** and **15d**.

2.2.1.2. Hsp90α Inhibition Assay. Enzyme Assay: It is well-known that the protein “heat shock protein 90” (Hsp90α), which exists in almost all cell types, is quite important in the viral protein processes such as protein folding, protein assembly, and protein replication.²⁰ During infection, HSV-1 uses the protein Hsp90α as part of the viral polymerase process.²¹ Therefore, an inhibitor of Hsp90α would naturally be an inhibitor of the HSV-1 infection. This has encouraged us to examine the possibility that our newly synthesized compounds **15c** and **15d** that showed high potency against HSV-1 might act as novel Hsp90α inhibitors.

The Hsp90α (C-terminal) inhibitor screening assay kit was used to examine the effect of **15c** and **15d** against Hsp90α. A combination of **15c** and **15d** with the well-known drug acyclovir (standard drug), in 1:1 ratio, were also investigated. The IC₅₀, the concentration of tested compounds that is required to inhibit 50% of the virus cell population, was evaluated, Table 3.

Scheme 4. Synthesis of 2-Oxopyridin-1(2H)-yl-benzenesulfonamide



It is interesting to report that both compounds had promising inhibitory effect against Hsp90 α as measured in $\mu\text{g}/\text{mL}$. According to the calculated IC₅₀, compound 15d with IC₅₀ value of 4.48 $\mu\text{g}/\text{mL}$ showed more potency as the Hsp90 α inhibitor than both acyclovir with IC₅₀ value of 4.78 $\mu\text{g}/\text{mL}$ and 15c with IC₅₀ values of 10.24 $\mu\text{g}/\text{mL}$. Combining 15c as well as 15d with acyclovir has resulted in IC₅₀ values of 5.22 and 2.26 $\mu\text{g}/\text{mL}$, respectively, which is a major reduction from those of the individual compounds. It is clear that the combination of both compounds 15c and 15d with acyclovir has increased their potency. The resultant data clearly indicates that compound 15c and its combination with acyclovir act as potential inhibitors for Hsp90 α and accordingly inhibitors for

Table 1. Antiviral Activity of Nontoxic Doses of Synthesized Compounds against Herpes Simplex Virus, CBV4, HAV HM 175, HCVcc Genotype 4 & Adenovirus Type 7

compd no. ^c	mean % of reduction				
	herpes simplex virus	CBV4	HAV HM 175	HCVcc genotype 4	adenovirus type 7
3b	26.7 ± 0.20	20.0 ± 0.20	40.0 ± 0.30	20.0 ± 0.25	30.0 ± 0.30
8a	30.0 ± 0.25	^a		20.0 ± 0.10	
8b	33.3 ± 0.25	30.0 ± 0.15	36.7 ± 0.30	26.7 ± 0.25	20.0 ± 0.20
8c	10.0 ± 0.15	13.3 ± 0.10	10.0 ± 0.15	16.7 ± 0.15	10.0 ± 0.15
8d					
9a	20.0 ± 0.15	13.3 ± 0.10	10.0 ± 0.10	13.3 ± 0.10	10.0 ± 0.10
9b					
13a	20.0 ± 0.30	20.0 ± 0.10	13.3 ± 0.10	20.0 ± 0.15	10.0 ± 0.10
15a					
15b	30.0 ± 0.30	40.0 ± 0.20	20.0 ± 0.10	33.3 ± 0.10	13.3 ± 0.50
15c	50.0 ± 0.35	53.3 ± 0.30	50.0 ± 0.30	20.0 ± 0.15	36.7 ± 0.30
15d	50.0 ± 0.30	50.0 ± 0.35	36.7 ± 0.25	20.0 ± 0.20	30.0 ± 0.10
acyclovir	99.6 ± 2.80	NT ^b	NT	NT	NT

^aNo activity. ^bNT = not tested. ^cAll data were taken as the average of three independent measurements.

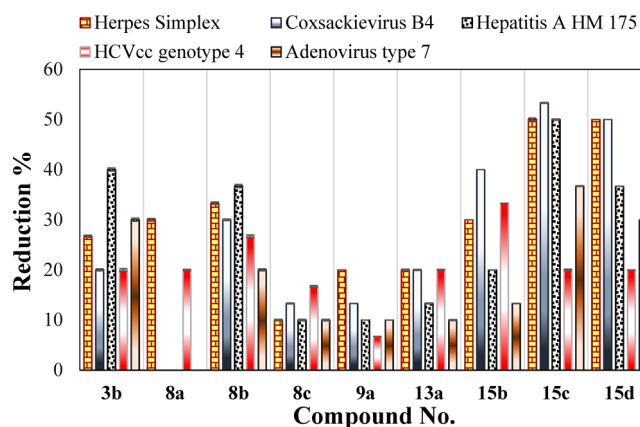


Figure 3. Comparison between the percent of viral load reduction of synthesized compounds.

Table 2. Antiviral Activity in Terms of CC₅₀, IC₅₀, ($\mu\text{g}/\text{mL}$) and SI

compd no. ^a	CC ₅₀ ($\mu\text{g}/\text{mL}$)	IC ₅₀ ($\mu\text{g}/\text{mL}$)	SI
Herpes Simplex Virus			
15c	250	90	2.77
15d	220	90	2.44
acyclovir	2.8	0.7	4.00
CBV4			
15c	260	88	2.95
15d	240	90	2.66
HAV HM 175			
15c	260	88	2.95

^aAll data were taken as the average of three measurements.

HSV-1 as was clearly illustrated from the antiviral reduction investigation and are, therefore, highly recommended.

2.2.1.3. Molecular Modeling and Docking Study. To further understand the underlying mechanism of the anti-HSV-1 action of the potent compounds with viral reduction of 50% or more, 15c and 15d were tested by docking them onto the crystal structure of the Hsp90 α protein (PDB ID: 3B25) obtained from protein data bank (PDB). To prepare the models, the bound ligand, B2K (4-methyl-6-(toluene-4-

Table 3. IC₅₀ Values of the Compounds 15c, 15d, and Acyclovir on the Hsp90 α Protein

compd no. ^a	100 ($\mu\text{g/mL}$)	10 ($\mu\text{g/mL}$)	1 ($\mu\text{g/mL}$)	0.1 ($\mu\text{g/mL}$)	IC ₅₀ ($\mu\text{g/mL}$)
15c	72.742%	48.686%	26.135%	8.4154%	10.25 \pm 0.30
15d	89.892%	57.424%	25.873%	10.459%	4.48 \pm 0.12
acyclovir	84.063%	52.339%	35.451%	11.242%	4.78 \pm 0.18
15c/acyclovir	85.554%	60.166%	26.411%	3.432%	5.22 \pm 0.14
15d/acyclovir	87.314%	62.996%	44.340%	18.436%	2.26 \pm 0.16

^aAll data were taken as the average of three measurements.

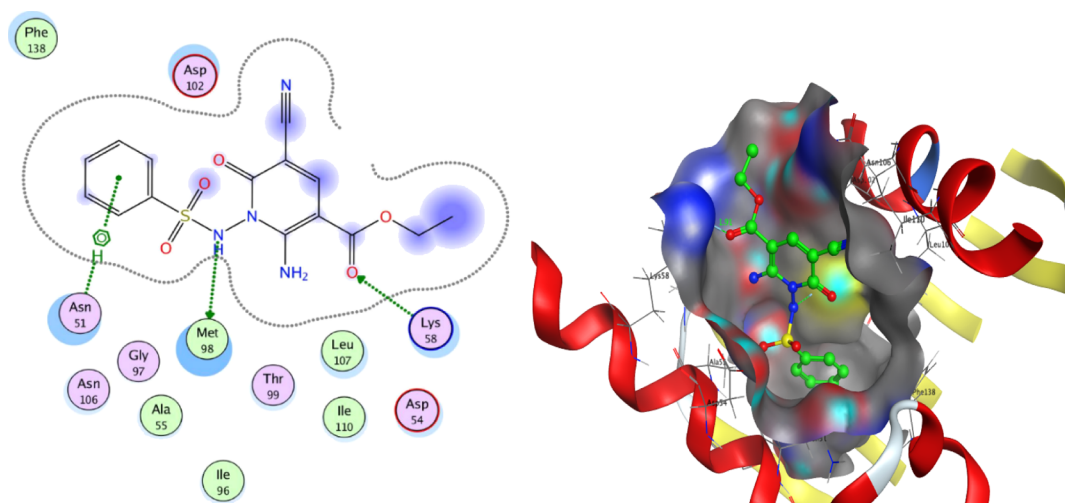


Figure 4. Best docked pose of 15c inside the binding pocket of Hsp90 α (PDB id 3B25).

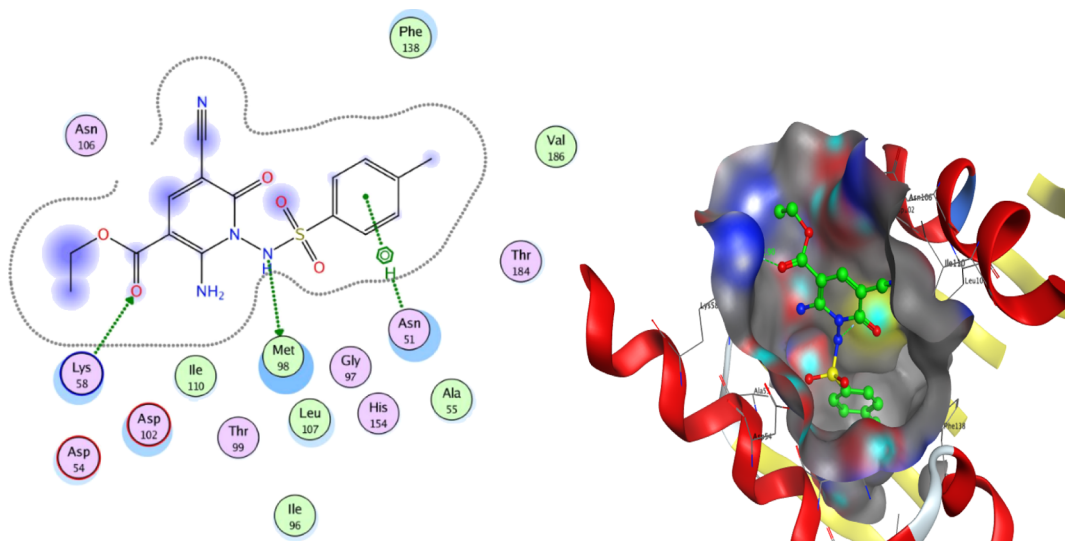


Figure 5. Best docked pose of 15d inside the binding pocket of Hsp90 α (PDB id 3B25).

sulfonyl)-pyrimidin-2-ylamine) was removed to uncover the binding pattern of these compounds with the receptor. The molecular modeling environment (MOE) was used to perform the docking study. Three-dimensional molecular structures were then readied and docked onto the enzyme active site. The docking study showed that the compounds 15c and 15d had binding energies of -5.7789 and -6.3783 , respectively. The benzene ring of 15c and 15d showed hydrophobic interaction with Asn 51, Figures 4 and 5. Furthermore, both 15c and 15d displayed two hydrogen bonding interactions with one interaction present between the nitrogen atom of the sulfonamide group and residue Met 98, and the other one

present between the oxygen atom of the acetyl group and residue Lys 58, Figures 4 and 5. The bond length between the sulfonamide group and residue Met 98 of compounds 15c and 15d is 3.39 and 3.37 cÅ, respectively, while the bond length between the acetyl group and residue Lys 58 is 1.91 and 2.90 cÅ, respectively. The docking study revealed that the investigated compounds occupy the protein binding pockets of the Hsp90 α inhibitor and thus suggests its inhibitory action of Hsp90 α .

2.2.2. Antimicrobial Evaluation. The newly synthesized target compounds were evaluated for their *in vitro* antibacterial activity against *Escherichia coli*, *Klebsiella pneumonia*, and

Table 4. Antimicrobial IZ in mm \pm Standard Deviation of Synthesized Compounds

compd no.	diameter of the IZ (mm)					
	Gram (–ve) bacteria			Gram (+ve) bacteria		fungi
	<i>E. coli</i> (ATCC:3008)	<i>K. pneumonia</i> (ATCC:4415)	<i>P. aeruginosa</i> (ATCC:27853)	<i>S. aureus</i> (ATCC:6538)	<i>S. mutans</i> (ATCC:25175)	<i>C. albicans</i> (ATCC:10231)
3b	^a					
8a		19.0 \pm 0.5		25.8 \pm 0.5	19.8 \pm 1.0	
8b		32.2 \pm 2.5		19.3 \pm 1.0	10.7 \pm 0.5	
8c				24.5 \pm 0.7	21.3 \pm 1.1	
8d				29.9 \pm 0.6	12.5 \pm 1.0	
9a				34.0 \pm 1.0	13.9 \pm 0.5	
9b	15.0 \pm 0.5	21.3 \pm 1.0				24.3 \pm 0.5
13a				30.0 \pm 1.0		22.0 \pm 0.5
13b				28.9 \pm 0.7		21.0 \pm 0.1
15a						19.6 \pm 2.0
15b						
15c						
15d	19.6 \pm 1.5	35.3 \pm 1.5		11.3 \pm 1.1	9.0 \pm 1.0	
gentamicin	25.0 \pm 0.1	32.0 \pm 0.1	35.0 \pm 0.1	NT	NT	NT
ampicillin	NT ^b	NT	NT	32.0 \pm 0.5	22.0 \pm 0.5	NT
nystatin	NT	NT	NT	NT	NT	28.0 \pm 0.2
sulfadiazine	19.8 \pm 0.4	20.4 \pm 0.9	24.0 \pm 1.4	31.3 \pm 2.1	16.3 \pm 1.1	NA

^aNo activity. ^bNot tested.

Pseudomonas aeruginosa as examples of Gram-negative bacteria and *Staphylococcus aureus* and *Streptococcus pyogenes* as examples of Gram-positive bacteria. They were also evaluated for their *in vitro* antifungal potential against *Candida albicans* fungal strain. The agar-diffusion method was used for the determination of the preliminary antibacterial and antifungal activities. Gentamicin, ampicillin, and nystatin were used as standard drugs against Gram negative bacterial, Gram positive bacterial, and fungal strains, respectively. Sulfadiazine as one of sulfonamide antibiotics with pyrimidine ring bearing the sulfamide group at position 2 has been also tested. The results were recorded for each tested compound as the average diameter of inhibition zones (IZs) of the microbial growth around the disks in mm \pm SD, as summarized in Table 4.

The minimum inhibitory concentration (MIC) measurements were determined for the most active compounds **8b**, **9a**, and **15d** by using twofold serial dilution method (as explained later) and the results are all summarized in Table 5.

In general, thiomethylpyridone derivatives **8a** and **8b** showed improved inhibitory effectiveness against the tested Gram-positive bacteria. Compound **8b** was identified as the most potent agent among the thiomethylpyridone series. It was equipotent to gentamicin against *K. pneumonia* strain (IZ 32.2 \pm 2.5 mm, MIC 125 μ g/mL). On other hand, 1*H*-pyrazolopyridones **9a** (IZ 34 \pm 1 mm, MIC 125 μ g/mL) was shown to be the most potent derivative among all synthesized compounds against *S. aureus*, Table 1. Pyrazolopyridone **9b** had the highest antifungal activity among all of the synthesized compounds investigated in this study. Pyridone derivatives **13a,b** revealed moderate antifungal activities against *C. albicans* and strong bacterial activities against *S. aureus*. Pyridone derivative **13a** was more active than **13b** against both *S. aureus* and *C. albicans*. Regarding the antibacterial activities of sulfonylpyridones **15a–d**, pyridone **15d** was the only compound that showed some activities against most of tested antibacterial strains. Compound **15d** exhibited higher potency than gentamicin against *K. pneumonia* (IZ 35.3 \pm 1.5 mm, MIC 1000 μ g/mL). Compound **15a**

Table 5. MIC (μ g/mL) of the Most Active Compounds **8b**, **9a**, and **15d**

selected organism	MIC μ g/mL			standards	
	8b	9a	15d	sulfadiazine	gentamicin
<i>E. coli</i> (ATCC:3008)	NT ^a	NT	1000	250	31.25
<i>K. pneumonia</i> (ATCC:4415)	125	NT	62.5	250	62.5
<i>P. aeruginosa</i> (ATCC:27853)	NT	NT	NT	250	125
selected organism	MIC μ g/mL			standards	
	8b	9a	15d	sulfadiazine	ampicillin
<i>S. aureus</i> (ATCC:6538)	1000	125	NT	500	62.5
<i>S. mutans</i> (ATCC:25175)	250	1000	NT	125	62.5
selected organism	MIC μ g/mL			standards	
	8b	9a	15d	nystatin	
<i>C. albicans</i> (ATCC:10231)	NT	NT	NT	NT 31.25	

^aNot test.

showed a moderate antifungal activity against *C. albicans*. Both **15b** and **15c** have no activity to all tested bacterial and fungal strains. In addition, all of the synthesized compounds showed no activity against *P. aeruginosa*.

2.2.2.1. Structure Activities Relationship. The study of SAR of the synthesized compounds demonstrated that unsubstituted benzene sulfonamide pyridone derivatives **8a**, **8c**, and **9a** were more potent than the *p*-tolyl pyridone derivatives **8b**, **8d**, and **9b** against *S. mutans* while *p*-tolyl pyridone derivatives **8b**, **9b**, and **15d** were more active against Gram-negative bacteria specially *K. pneumonia* as compared to the unsubstituted benzene sulfonamide pyridone derivatives **8a**, **9a**, and **15a**. Moreover, the unsubstituted benzene sulfonamide pyridone derivatives **8a**, **9a**, and **13a** were more potent than the *p*-tolyl pyridone derivatives **8b**, **9b**, and **13b** against *S. aureus*. In addition, both **8b**- and **15d**-bearing *p*-tolyl group showed a higher potency than the reference drug, gentamicin, against *K.*

Table 6. IC₅₀ Values (the Drug Concentrations That Inhibited 50% of Cell Proliferation) of the Compounds on the DHFR^a

compd no.	10 (μg/mL)	1 (μg/mL)	0.1 (μg/mL)	0.01 (μg/mL)	IC ₅₀ (μg/mL)
8b	57.653%	50.383%	40.816%	19.897%	1.368 ± 0.04
9a	68.367%	53.571%	33.800%	22.576%	0.699 ± 0.02
15d	56.122%	39.923%	20.662%	11.096%	4.673 ± 0.13
sulfadiazine	76.658%	60.204%	46.811%	28.699%	0.201 ± 0.01
trimethoprim	88.070%	66.114%	43.022%	25.511%	0.170 ± 0.01

^aAll data were taken as the average of three measurements.

pneumonia. Compound **9a** showed higher potency than ampicillin against *S. aureus*.

Comparing the antimicrobial activity of synthesized compounds (as reported above in terms of its IZ) with sulfadiazine, which is considered to be a well-known antibacterial sulfonamide drug indicated that some of the synthesized compounds showed superior activities. For example, *p*-tolyl pyridone derivatives **8b**, **9b**, and **15d** showed a much higher potency against Gram-negative bacteria, *K. pneumoniae*, than sulfadiazine (IZ 20.4 ± 0.9 mm). In addition, both **8a** and **8c**, unsubstituted benzene sulfonamide pyridone derivatives, were highly potent than sulfadiazine (IZ 16.3 ± 1.1 mm) against *S. mutans*. Furthermore, 1*H*-pyrazolopyridones **9a** was highly effective while pyridone derivatives **8d**, **13a**, and **13b** showed a relatively lower activity than sulfadiazine (IZ 31.3 ± 2.1 mm) against *S. aureus*. Although, sulfadiazine had no activity against *C. albicans*, some of synthesized compounds such as **9b**, **13a**, **13b**, and **15a** showed some activity.

These remarkable findings indicate the influence of the synthesized compounds and the effectiveness of these newly synthesized chemical structures in effectively inhibiting and combating the mentioned hazardous bacterial strains, confirming the appropriate approach of this work in envisaging and generating new effective antimicrobial drugs.

2.2.2.2. Dihydrofolate Reductase Activity Assay. Folate inhibitors are well-known agents in provoking the synthesis of folic acid and therefore are usually used for treating protozoal, bacterial, and fungal infections. This class of antimicrobial drugs may contain compounds from the subclasses of sulfonamides or dihydrofolate reductase (DHFR) inhibitors or a combination of both drugs.²² Generally, sulfonamide drugs inhibit conversion of *p*-aminobenzoic acid PABA to dihydrofolate (DHF) through the action of the enzyme dihydropteroate synthase, which can be converted to tetrahydrofolic acid through the action of the enzyme DHFR. DHFR inhibitors are commonly used for fighting malaria and other protozoal infections, as well as for treating fungal, bacterial, and mycobacterial infections.²³ Trimethoprim is used as the DHFR inhibitor, in the case of bacterial infections, because it competes with the pteridine moiety of DHF in binding with the DHFR enzyme. It has been shown that drugs containing pyrimidine ring such as trimethoprim in association with sulfonamide drug are commonly used as an antibacterial drug because of its ability to bind to the active site of the enzyme.²⁴ Inasmuch, it has been also reported that drugs such as trimetrexate having pyridopyrimidines inhibit the DHFR enzyme's function during the development of the cancer cells.²⁵ Therefore, it would be quite interesting to perform an enzyme assay on the DHFR enzyme. In these regards, the highly potent antimicrobial compounds, **8b**, **9a**, and **15d** were tested in terms of their inhibitory activities toward the enzyme using the DHFR inhibitor screening kit. For the purpose of comparison, both sulfadiazine, as one of

sulfonamide antibiotics, and trimethoprim were used as standard drugs, Table 6.

The inhibitory potency IC₅₀ values of tested compounds, **8b**, **9a**, and **15d**, were compared to that of sulfadiazine. All tested compounds showed IC₅₀ values higher than that of sulfadiazine (IC₅₀ = 0.201 μg/mL) and trimethoprim (IC₅₀ = 0.170 μg/mL), Figure 6. It was determined that compound **9a** had IC₅₀

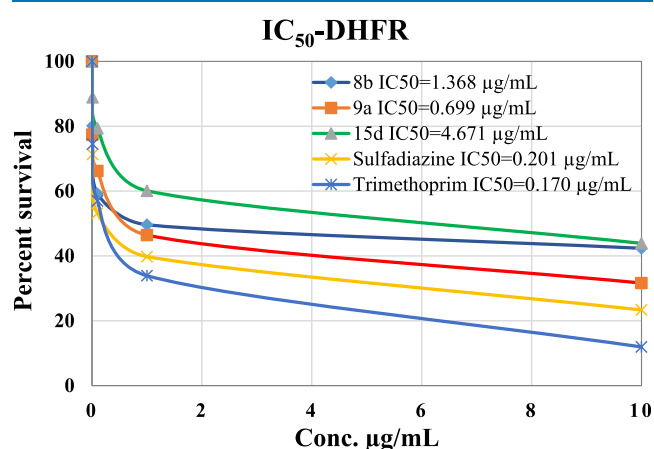


Figure 6. Percent cell survival vs the concentration of sulfadiazine, trimethoprim, **8b**, **9a**, and **15d**.

value of 0.699 μg/mL followed by **8b** with IC₅₀ value of 1.368 μg/mL and **15d** with IC₅₀ value of 4.671 μg/mL making **9a** the most potent agent against DHFR among the newly synthesized compounds.

3. CONCLUSIONS

The work in this article is focused on synthesizing a new class of functionalized benzothiazole and benzimidazole-based pyridine incorporating sulfonamide moieties, with remarkable antiviral and antimicrobial potency. The synthesis was carried out by reacting *N*-cyanoacetarsylsulfonylhydrazide with various electrophiles such as 2-(benzo[*d*]thiazol-2-yl)-3,3-bis(alkylthio)acrylonitriles and 2-(benzo[*d*]imidazol-2-yl)-3,3-bis(methylthio)-acrylonitriles as well as 2-ethoxyl acrylonitrile derivatives. The structure of the different synthesized compounds was confirmed by basic spectroscopic data and elemental analysis. The antiviral activities of the newly synthesized compounds were evaluated *in vitro* against a wide variety of viruses such as HSV-1, CVB4, HAV HM 175, HCVcc genotype **4a**, and HAdV7. The CC₅₀, IC₅₀, and SI were also evaluated for the promising compounds. Two of the synthesized compounds, **15c** and **15d** showed more than 50% viral reduction against HSV-1 and CBV4, with significant IC₅₀ and CC₅₀ values. Even though these compounds have also shown inhibitory activity against Hsp90α protein, their combination with the known drug acyclovir has resulted in a

very high potency against the Hsp90 α and consequently against the HSV-1 virus. To evaluate the underlying principles behind the action of these new compounds in inhibiting HSV-1, a molecular docking study was performed with focus on compounds that showed the greatest potency as HSV-1 virus. The study showed that the investigated compounds occupied the protein binding pockets of Hsp90 α with strong binding interactions. The newly synthesized target compounds were also evaluated for their *in vitro* antimicrobial activity against *E. coli*, *K. pneumonia*, *P. aeruginosa*, *S. aureus*, and *S. mutans*, as well as *C. albicans*. In general, the thiomethylpyridone derivatives showed improved inhibitory effectiveness against the tested Gram-positive bacteria while 1*H*-pyrazolopyridones showed potency more than ampicillin against *S. aureus*. According to the enzyme assay study on DHFR, compound **9a** was the most potent among the tested potent compounds.

4. EXPERIMENTAL PART

4.1. Chemistry. Melting points were determined on digital melting point apparatus, SMP3, using one end open capillary tubes and are uncorrected. IR spectra were recorded on FTIR plus 460 or using KBr pellets. ¹H NMR and ¹³C NMR were carried out in the Center of Drug Discovery Research at Ain Shams University and spectra were recorded on a Bruker ADVANCE (III) model (400 MHz) spectrometer in DMSO-*d*₆ as a solvent using tetramethylsilane as an internal standard and chemical shifts are reported as δ_{ppm} units. The elemental analyses were done at the microanalytical data unit at Cairo University and performed on a Vario EI III Elemental CHNS Analyzer. Progress of the reactions was monitored by thin-layer chromatography (TLC) using aluminum sheet coated with silica gel Merck 60F and was visualized by a UV lamp. The reagents and solvents were purchased in commercially available grade purity.

4.1.1. General Procedure for the Synthesis of 3b. A mixture of sodium ethoxide (0.08 mol) and 2-cyanomethylbenzothiazole (0.04 mol) in absolute ethanol (100 mL) was refluxed for 20 min. After cooling, carbon disulfide (0.04 mol) was added gradually and the solution was warmed for 20 min. The reaction mixture was stirred overnight at room temperature after the addition of methyl iodide (0.08 mol). The solution was poured onto ice water and the solid product formed was filtered. After drying, the solid product was dissolved in hot petroleum ether and then filtrated. The precipitate formed after evaporation of the solvent recrystallized from DMF.

4.1.1.1. 2-(Benzo[d]thiazol-2-yl)-3,3-bis(ethylthio)acrylonitrile (3b). Pale yellow crystals, mp 93–95 °C, yield 72%; IR (KBr, cm⁻¹): ν 3056 (ArCH), 2969 (CH₂, CH₃), 2213 (CN), 1502 (C=N); ¹H NMR (300 MHz, DMSO-*d*₆): δ 1.27–1.34 (m, 6H, 2SCH₂CH₃), 3.168–3.23 (m, 4H, 2SCH₂CH₃), 7.41–7.57 (m, 2H, benzothiazole-H), 8.03–8.17 (m, 2H, benzothiazole-H); Anal. Calcd for C₁₄H₁₄N₂S₃ (306.47): C %, 54.87; H %, 4.60; N %, 9.14. Found: C %, 54.85; H %, 4.58; N %, 9.16.

4.1.2. General Procedure for the Synthesis of 8a–d. 2-(Benzo[d]thiazol-2-yl)-3,3-bis(alkylthio)acrylonitrile (0.01 mol) was added to a solution of *N*-cyanoacetoarylsulfonylhydrazides (0.01 mol) in dry DMF (30 mL) containing pulverized potassium hydroxide (0.01 mol). The reaction mixture was refluxed with stirring for 2 h (TLC monitoring). After cooling, the reaction mixture poured onto ice-cold water and neutralized with HCl. The solid product was filtered,

washed with water, and dried. Further purification was done using a hot mixture of petroleum ether: ethyl acetate (50:50; v/v). The remaining solid compound was crystallized from DMF.

4.1.2.1. *N*-(6-Amino-5-(benzo[d]thiazol-2-yl)-3-cyano-4-(methylthio)-2-oxopyridin-1(2*H*)-yl)benzenesulfonamide (8a). Yellow crystals; (DMF), yield (80%), mp 211 °C; IR (KBr, cm⁻¹): ν 3392, 3200 (NH, NH₂), 3067 (ArCH), 2925 (CH₃), 2210 (CN), 1675 (CO), 1594 (C=N), 1353, 1172 (O=S=O); ¹H NMR (400 MHz, DMSO-*d*₆): δ 2.51 (s, 3H, SCH₃), 7.49 (t, *J* = 7.6 Hz, 1H, benzothiazole-H), 7.54–7.63 (m, 3H, 3Ar-H), 7.73 (t, *J* = 8 Hz, 1H, benzothiazole-H), 7.83 (d, *J* = 7.6 Hz, 2H, Ar-H), 8.05 (d, *J* = 8 Hz, 1H, benzothiazole-H), 8.13 (d, *J* = 8 Hz, 1H, benzothiazole-H), 8.87 (br, 2H, NH₂), 11.52 (s, 1H, NH); Anal. Calcd for C₂₀H₁₅N₅O₃S₃ (469.56): C %, 51.16; H %, 3.22; N %, 14.91. Found: C %, 51.09; H %, 3.20; N %, 14.94.

4.1.2.2. *N*-(6-Amino-5-(benzo[d]thiazol-2-yl)-3-cyano-4-(methylthio)-2-oxopyridin-1(2*H*)-yl)-4-methylbenzenesulfonamide (8b).¹⁶ Yellow crystals; (DMF), yield (78%), mp 221 °C; IR (KBr, cm⁻¹): ν 3393, 3208 (NH, NH₂), 3072 (ArCH), 2922 (CH₃), 2210 (CN), 1677 (CO), 1594 (C=N), 1350, 1170 (O=S=O); ¹H NMR (400 MHz, DMSO-*d*₆): δ 2.42 (s, 3H, CH₃), 2.45 (s, 3H, SCH₃), 7.42 (d, *J* = 8 Hz, 2H, Ar-H), 7.49 (t, *J* = 7.6 Hz, 1H, benzothiazole-H), 7.56 (t, *J* = 7.6 Hz, 1H, benzothiazole-H), 7.71 (d, *J* = 7.2 Hz, 2H, Ar-H), 8.06 (d, *J* = 8 Hz, 1H, benzothiazole-H), 8.13 (d, *J* = 8 Hz, 1H, benzothiazole-H), 8.84 (br, 2H, NH₂), 11.44 (s, 1H, NH); Anal. Calcd for C₂₁H₁₇N₅O₃S₃ (483.59): C %, 52.16; H %, 3.54; N %, 14.48. Found: C %, 52.11; H %, 3.48; N %, 14.50.

4.1.2.3. *N*-(6-Amino-5-(benzo[d]thiazol-2-yl)-3-cyano-4-(ethylthio)-2-oxopyridin-1(2*H*)-yl)benzenesulfonamide (8c). Yellow crystal; (EtOH), yield (77%), mp 226–229 °C; IR (KBr, cm⁻¹): ν 3395, 3206 (NH₂), 3062 (ArCH), 2923 (CH₂, CH₃), 2208 (CN), 1677 (CO), 1593 (C=N) 1352, 1176 (O=S=O); ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.18 (t, *J* = 6.8 Hz, 3H, SCH₂CH₃), 2.95 (q, *J* = 6.8 Hz, 2H, SCH₂CH₃), 7.48 (t, *J* = 7.6 Hz, 1H, benzothiazole-H), 7.53–7.63 (m, 3H, Ar-H), 7.74 (t, *J* = 7.6 Hz, 1H, benzothiazole-H), 7.82 (d, *J* = 8 Hz, 2H, Ar-H), 8.05 (d, *J* = 8 Hz, 1H, benzothiazole-H), 8.12 (d, *J* = 8 Hz, 1H, benzothiazole-H), 11.53 (s, 1H, NH); Anal. Calcd for C₂₁H₁₇N₅O₃S₃ (483.59): C %, 52.16; H %, 3.54; N %, 14.48. Found: C %, 52.19; H %, 3.50; N %, 14.46.

4.1.2.4. *N*-(6-Amino-5-(benzo[d]thiazol-2-yl)-3-cyano-4-(ethylthio)-2-oxopyridin-1(2*H*)-yl)-4-methylbenzenesulfonamide (8d). Yellow crystal; (EtOH), yield (71%), mp 255 °C; IR (KBr, cm⁻¹): ν 3407, 3201 (NH, NH₂), 3068 (ArCH), 2927 (CH₂, CH₃), 2209 (CN), 1676 (CO), 1592 (C=N), 1350, 1170 (O=S=O); ¹H NMR (400 MHz, MeOH-*d*₄): δ 1.30 (t, *J* = 8 Hz, 3H, SCH₂CH₃), 2.48 (s, 3H, CH₃), 3.05 (q, *J* = 6.8 Hz, 2H, SCH₂CH₃), 7.41 (d, *J* = 8 Hz, 2H, C₆H₄), 7.48 (t, *J* = 7.2 Hz, 1H, benzothiazole-H), 7.56 (t, *J* = 7.2 Hz, 1H, benzothiazole-H), 7.77 (d, *J* = 8 Hz, 2H, Ar-H), 8.00–8.03 (m, 2H, benzothiazole-H); ¹³C NMR (100 MHz, DMSO-*d*₆): 14.7, 21.6 (2CH₃), 30.9 (CH₂), 112.8 (CN), 89.9, 98.4, 144.7, 155.3, 162.9 (pyridone-C), 107.9, 122.2, 122.9, 124.1, 126.0, 126.7, 128.4, 129.9, 135.7, 136.5, 156.8 (Ar-C); Anal. Calcd for C₂₂H₁₉N₅O₃S₃ (497.61): C %, 53.10; H %, 3.85; N %, 14.07. Found: C %, 53.14; H %, 3.81; N %, 14.09.

4.1.3. General Procedure for the Synthesis of 9a,b. A mixture of **8a–d** (0.01 mol) and hydrazine hydrate (0.1 g, 0.02 mol) in dry DMF containing a catalytic amount of piperidine (3 drops) was heated for 2 h (TLC monitoring). The mixture

was then concentrated under reduced pressure to afford a solid residue. Ethanol was added to solid and then filtered off and washed with ethanol. The solid product was recrystallized from methanol.

4.1.3.1. *N*-(3,6-Diamino-7-(benzo[d]thiazol-2-yl)-4-oxo-1*H*-pyrazolo[4,3-*c*]pyridin-5(4*H*)-yl)benzenesulfonamide (9a). White solid; (MeOH), yield (62%), mp 299 °C; IR (KBr, cm^{-1}): ν 3365, 3200 (NH, NH_2), 3066 (ArCH), 1678 (CO), 1596 (C=N), 1339, 1165 (O=S=O); ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 6.10 (br, 2H, NH_2), 7.26 (t, $J = 7.6$ Hz, 1H, benzothiazole-H), 7.42 (t, $J = 8$ Hz, 1H, Ar-H), 7.57–7.60 (m, 2H, Ar-H), 7.70 (t, $J = 7.6$ Hz, 1H, benzothiazole-H), 7.80–7.83 (m, 3H, 2Ar-H & benzothiazole-H), 7.98 (d, $J = 8$ Hz, 1H, benzothiazole-H), 10.99 (s, 1H, NH), 11.95 (s, 1H, NH); Anal. Calcd for $\text{C}_{19}\text{H}_{15}\text{N}_7\text{O}_3\text{S}_2$ (453.50): C %, 50.32; H %, 3.33; N %, 21.62. Found: C %, 50.30; H %, 3.32; N %, 21.60.

4.1.3.2. *N*-(3,6-Diamino-7-(benzo[d]thiazol-2-yl)-4-oxo-1*H*-pyrazolo[4,3-*c*]pyridin-5(4*H*)-yl)-4-methylbenzenesulfonamide (9b). White solid; MeOH, yield (58%), mp 322 °C; IR (KBr, cm^{-1}): ν 3410, 3351 (NH, NH_2), 3184 (ArCH), 2922 (CH_3), 1678 (CO), 1616 (C=N), 1337, 1157 (O=S=O); ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 2.41 (s, 3H, CH_3), 6.11 (br, 2H, NH_2), 7.27 (t, $J = 8$ Hz, 1H, benzothiazole H), 7.37–7.42 (m, 3H, 2Ar-H & benzothiazole-H), 7.71 (d, $J = 7.2$ Hz, 2H, Ar-H), 7.82 (d, $J = 7.6$ Hz, 1H, benzothiazole-H), 7.99 (d, $J = 7.6$ Hz, 1H, benzothiazole-H), 10.85 (s, 1H, NH), 11.92 (s, 1H, NH); ^{13}C NMR (100 MHz, $\text{DMSO-}d_4$): 21.6 (CH_3), 81.4, 89.6, 144.2, 152.0, 164.1 (pyridone-C), 120.2, 121.6, 123.1, 126.2, 128.1, 129.9, 133.3, 137.3, 126.7, 149.9, 151.3, 156.6 (Ar-C); Anal. Calcd for $\text{C}_{20}\text{H}_{17}\text{N}_7\text{O}_3\text{S}_2$ (467.52): C %, 51.38; H %, 3.67; N %, 20.97. Found: C %, 51.39; H %, 3.65; N %, 20.96.

4.1.4. General Procedure for the Synthesis of 13a,b. To a stirred solution of *N*-cyanoacetoarylsulfonylhydrazides (0.01 mol) in dry dioxane (30 mL) containing pulverized potassium hydroxide (0.01 mol), 2-(1*H*-benzo[d]imidazol-2-yl)-3,3-bis-(methylthio)acrylonitrile (0.01 mol) was added. The reaction mixture was left to stir overnight at room temperature (TLC monitoring) and then poured onto ice-cold water and neutralized with HCl. The separated product was filtered and washed with water. The crude product was further purified by using a hot mixture of petroleum ether/ethyl acetate (80:20; v/v) and the residual precipitate was recrystallized from ethyl acetate.

4.1.4.1. *N*-(6-Amino-5-(1*H*-benzo[d]imidazol-2-yl)-3-cyano-4-(methylthio)-2-oxopyridin-1(2*H*)-yl)-benzenesulfonamide (13a). Orange solid; (ethyl acetate), yield (69%), mp 226–230 °C; IR (KBr, cm^{-1}): ν 3441 (NH, NH_2), 3143 (ArCH), 2209 (CN), 1687 (CO), 1590 (C=N), 1370, 1170 (O=S=O); ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 2.34 (s, 3H, SCH_3), 7.22–7.86 (m, 7H, 5Ar-H & 2benzoimidazole-H); 7.97 (d, $J = 8$ Hz, 1H, benzoimidazole-H), 8.27 (d, $J = 8$ Hz, 1H, benzoimidazole-H); Anal. Calcd for $\text{C}_{20}\text{H}_{16}\text{N}_6\text{O}_3\text{S}_2$ (452.51): C %, 53.08; H %, 3.56; N %, 18.57. Found: C %, 53.11; H %, 3.53; N %, 18.56.

4.1.4.2. *N*-(6-Amino-5-(1*H*-benzo[d]imidazol-2-yl)-3-cyano-4-(methylthio)-2-oxopyridin-1(2*H*)-yl)-4-methylbenzenesulfonamide (13b). Orange solid; (ethyl acetate), yield (68%), mp 289 °C; IR (KBr, cm^{-1}): ν 3411, (NH, NH_2), 3143 (ArCH), 2926 (CH_3), 2199 (CN), 1687 (CO), 1590 (C=N), 1370, 1170 (O=S=O); ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 2.34 (s, 3H, CH_3), 2.50 (s, 3H, SCH_3), 7.43 (d, $J = 8$ Hz, 2H, C_6H_4), 7.42–7.62 (m, 2H, benzoimidazole-H), 7.67 (d, $J = 8$

Hz, 1H, benzoimidazole-H), 7.89 (d, $J = 8$ Hz, 2H, Ar-H), 8.29 (d, $J = 8$ Hz, 1H, benzoimidazole-H); Anal. Calcd for $\text{C}_{21}\text{H}_{18}\text{N}_6\text{O}_3\text{S}_2$ (466.54): C %, 54.06; H %, 3.89; N %, 18.01. Found: C %, 54.09; H %, 3.90; N %, 18.01.

4.1.5. General Procedure for the Synthesis of 15a–d. 2-(Ethoxymethylene)malononitrile or (*Z*)-ethyl 2-cyano-3-ethoxyacrylate (0.01 mol) was refluxed with *N*-cyanoacetoarylsulfonylhydrazides in sodium ethoxide solution (30 mL) for 30–45 min (TLC monitoring). After completion of the reaction, the mixture was concentrated under reduced pressure to afford a solid residue. Ethanol was added to solid and then filtered off and washed with ethanol. The solid product was recrystallized from proper solvents.

4.1.5.1. *N*-(6-Amino-3,5-dicyano-2-oxopyridin-1(2*H*)-yl)-benzenesulfonamide (15a). White solid; (EtOH), yield (77%), mp 339–340 °C; IR (KBr, cm^{-1}): ν 3383, 3286 (NH, NH_2), 2925 (ArCH), 2221 (CN), 1607 (CO), 1265, 1132 (O=S=O); ^1H NMR (400 MHz, $\text{DMSO-}d_6$): 7.36–7.38 (m, 3H, Ar-H), 7.69–7.71 (m, 2H, Ar-H), 7.95 (s, 1H, pyridone-H); Anal. Calcd for $\text{C}_{13}\text{H}_9\text{N}_5\text{O}_3\text{S}$ (315.31): C %, 49.52; H %, 2.88; N %, 22.21. Found: C %, 49.50; H %, 2.89; N %, 22.25.

4.1.5.2. *N*-(6-Amino-3,5-dicyano-2-oxopyridin-1(2*H*)-yl)-4-methylbenzenesulfonamide (15b). White solid; EtOH, yield (75%), mp 332 °C; IR (KBr, cm^{-1}): ν 3383, 3308, 3202 (NH, NH_2), 3065 (ArCH), 2927 (CH_3), 2218 (CN), 1682 (CO), 1341, 1131 (O=S=O); ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 2.33 (s, 3H, CH_3), 7.16 (d, $J = 7.6$ Hz, 2H, Ar-H), 7.59 (d, $J = 7.6$ Hz, 2H, Ar-H), 7.94 (s, 1H, pyridone-H); ^{13}C NMR (100 MHz, $\text{DMSO-}d_4$): 21.3 (CH_3), 116.9, 117.9 (2CN), 72.0, 87.2, 145.3, 145.9, 159.8 (pyridone-C), 126.5, 128.5, 136.3, 139.3 (Ar-C); Anal. Calcd for $\text{C}_{14}\text{H}_{11}\text{N}_5\text{O}_3\text{S}$ (329.33): C %, 51.06; H %, 3.37; N %, 21.27. Found: C %, 51.09; H %, 3.34; N %, 21.23.

4.1.5.3. Ethyl 2-Amino-5-cyano-6-oxo-1-(phenylsulfonamido)-1,6-dihydropyridine-3-carboxylate (15c). Off-white solid; EtOH, yield (74%), mp 246 °C; IR (KBr, cm^{-1}): ν 3585, 3411, 3271 (NH, NH_2), 3059 (ArCH), 3981 (CH_2 , CH_3), 2221 (CN), 1.679, 1641 (2CO), 1586 (C=N), 1381, 1134 (O=S=O); ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 1.28 (t, $J = 7.2$ Hz, 3H, SCH_2CH_3), 4.21 (q, $J = 7.2$ Hz, 2H, SCH_2CH_3), 7.37–7.71 (m, 6H, 5Ar-H & NH), 8.02 (s, 1H, pyridone-H), 8.63 (s, 2H, NH_2); Anal. Calcd for $\text{C}_{15}\text{H}_{14}\text{N}_4\text{O}_5\text{S}$ (362.36): C %, 49.72; H %, 3.89; N %, 15.46. Found: C %, 49.70; H %, 3.92; N %, 15.45.

4.1.5.4. Ethyl 2-Amino-5-cyano-1-(4-methylphenylsulfonamido)-6-oxo-1,6-dihydropyridine-3-carboxylate (15d). Off-white solid; EtOH, yield (71%), mp 239–241 °C; IR (KBr, cm^{-1}): ν 4580, 3410, 3276 (NH, NH_2), 3053 (ArCH), 2980 (CH_3 , CH_2), 2221 (CN), 1680, 1642 (2CO), 1587 (C=N), 1375, 1133 (O=S=O); ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 1.28 (t, $J = 7.2$ Hz, 3H, SCH_2CH_3), 2.33 (s, 3H, CH_3), 2.21 (q, $J = 7.6$ Hz, 2H, SCH_2CH_3), 7.16 (d, $J = 7.6$ Hz, 2H, Ar-H), 7.59 (d, $J = 7.6$ Hz, 2H, Ar-H), 7.64 (br, 1H, NH), 8.03 (s, 1H, pyridone-H), 8.63 (s, 2H, NH_2); ^{13}C NMR (100 MHz, $\text{DMSO-}d_4$): 14.6, 21.3 (2 CH_3), 60.5 (CH_2), 118.5 (CN), 86.3, 89.8, 144.2, 158.7, 165.3 (pyridone-C), 126.5, 128.5, 139.3, 145.4, 160.4 (Ar-C); Anal. Calcd for $\text{C}_{16}\text{H}_{16}\text{N}_4\text{O}_5\text{S}$ (376.39): C %, 51.06; H %, 4.38; N %, 14.89. Found: C %, 51.05; H %, 4.35; N %, 14.85.

4.2. Antiviral Activity. **4.2.1. Cytotoxicity Test.** Measuring of the antiviral activities were carried out at the National Research Center, Giza, Egypt. The antiviral experiments were

done according to the literature.^{3,26,27} First, 50 mg of each sample was dissolved in 1 mL of DMSO. Decontamination of samples was done by adding 0.024 mL of 100× of antimycotic–antibiotic mixture to 1 mL of each sample. To estimate the nontoxic dose of the tested samples, bi-fold dilutions were done to 0.1 mL of original dissolved samples and 0.1 mL of each dilutions were inoculated in Hep-2, Vero, BGM, FRHK4, and Huh 7.5 cell lines, which obtained from the Holding Company for Biological Products & Vaccines VACSERA, Egypt, and previously cultured in 96 multi well plates (Greiner Bio-One, Germany). Cytotoxicity assay was done using cell morphology evaluation by inverted light microscope and cell viability test applying the trypan blue dye exclusion method.

4.2.2. Cell Morphology Evaluation by Inverted Light Microscopy. Vero, FRHK4, BGM, Hep-2, and Huh 7.5 cell cultures (2×10^5 cells/mL) were prepared individually in 96-well tissue culture plates (Greiner Bio-One, Germany). The cell cultures were then incubated for 24 h at 37 °C in a humidified 5% (v/v) CO₂ atmosphere cell monolayers. The medium was then removed from each well and replenished with 0.1 mL of bi-fold dilutions of different samples tested and prepared in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL). 0.1 mL of DMEM without samples was added as cell control. Subsequently, all cultures were incubated for 72 h at 37 °C in a humidified 5% (v/v) CO₂ atmosphere. Cell morphology was observed daily for microscopically detectable morphological alterations, such as cell rounding, loss of confluence and shrinking, and vacuolization and cytoplasm granulation. Morphological deviations were recorded.²⁶

4.2.3. Cell Viability Assay. This assay was done by the trypan blue dye exclusion method.²⁷ Vero, FRHK4, BGM, Hep-2, and Huh 7.5 cell cultures (2×10^5 cells/mL) were grown in 12-well tissue culture plates (Greiner Bio-One, Germany). After 24 h of incubation, the same procedure described above for tested sample cytotoxicity was carried out by applying 0.1 mL of tested sample dilutions (bifold dilutions) per well. The medium was removed after 72 h and cells were trypsinized and an equal volume of 0.4% (w/v). Trypan blue dye aqueous solution was added to the cell suspension. By using the phase contrast microscope, viable cells were counted.

4.2.4. Determination of Adenovirus 7, HAV HM 175, CBV4, and HSV-1 Titers Using Plaque Assay. Nontoxic dilutions were mixed (0.1 mL) with 0.1 mL of different doses of HSV-1, HAdV7, HAV HM 175, and CBV4 (1×10^5 , 1×10^6 , 1×10^7). The mixture was incubated in 37 °C for 1/2 h. The inoculation of (0.1 mL) 10-fold dilutions of treated and untreated CBV4, adenovirus 7, HSV-1, and HAV HM 175 was added separately into BGM, Hep-2, Vero, and FRHK4 cell lines, respectively, in 12 multi well plates. The plates were left for 1 h of incubation for adsorption at 37 °C in a 5% CO₂–water vapor atmosphere without constant rocking. Afterward, the plates were rocked occasionally to keep the cells from drying. After adsorption, 1 mL of 2× media DMEM, Gibco-BRL and 1 mL 1% agarose were added to each well. The plates were incubated at 37 °C in a 5% CO₂–water vapor atmosphere. After incubation, the cells were stained with 0.4% crystal violet after formalin fixation, and the number of plaques were counted. The viral titers were then calculated, and expressed as plaque-forming units per milliliter (pfu/mL).²⁸ CC₅₀ and IC₅₀ were done for the promising compounds that showed viral reduction of 50% or more. The 50% cytotoxic concentration

(CC₅₀) of the test extract was defined as the concentration that reduce the OD₄₉₂ of treated uninfected cells to 50% of that of untreated uninfected cells. IC₅₀ is the concentration at which the compound plaque reduction rate reaches halfway between the baseline and the maximum. All data were taken as the average of three measurements (triplicate).

4.2.5. Antiviral Bioassay of Tested Compounds against ED-43/SG-Feo (VYG) Replicon of HCV genotype 4a. ED-43/SG-Feo (VYG) replicon of HCV genotype 4a was treated with the nontoxic dose of the tested compounds. HCV RNA was quantified in algal extracts treated Huh 7.5 infected cells using qRT-PCR (TaqMan probe kit, QIAGEN) and according to the manufacturer's instructions to show a dose-dependent decrease in sub genomic RNA copies according to the literature.²⁹

4.3. Hsp90α (C-Terminal) Inhibitor Screening Assay. The Hsp90α (C-terminal) inhibitor screening assay was carried out using Hsp90α (C-terminal) Inhibitor Screening Assay Kit actalog 50317, size: 384 reactions, according to the method described in the catalog and literature.³ Enzyme assay experiments were carried out at the Tissue Culture Unit, the Egyptian Company for Production of Vaccines, Sera and Drugs (VACSERA), Giza, Egypt. All samples and controls were tested in triplicate. First, both Hsp90α and PPID were thawed on ice. Aliquot protein into single use aliquots. Store remaining undiluted protein in aliquots at –80 °C immediately. Note: Hsp90α and PPID proteins are very sensitive to freeze/thaw cycles. Dilute 3× Hsp90α assay buffer 2 with water to 1× Hsp90α assay buffer 2. Dilute Hsp90α in 1× Hsp90α assay buffer 2 at 1.5 ng/μL. Add 4 μL of diluted HSPα protein to each well designated for the “Positive Control”, “Test Inhibitor”, and “Blank”. To the wells labeled “Substrate Control”, add 4 μL of 1× Hsp90 assay buffer 2. Discard any unused diluted protein after use. Add 2 μL of inhibitor solution to each well-designated “Test Inhibitor”. For the “Positive Control”, “Substrate Control”, and “Blank”, add 2 μL of the same solution without inhibitor (inhibitor buffer). Dilute PPID in 1× Hsp90α assay buffer 2 at 10 ng/μL. Keep diluted protein on ice until use. Add 4 μL of 1× Hsp90α assay buffer 2 to the well-designated “Blank”. Initiate reaction by adding 4 μL of diluted PPID to each well designated for the “Substrate control”, “Positive Control”, and “Test Inhibitor”. Incubate at room temperature for 30 min. Dilute 3× detection buffer with water to 1× detection buffer. Dilute glutathione acceptor beads 250-fold with 1× detection buffer. Add 10 μL per well. Shake plate briefly. Incubate at room temperature for 30 min. Dilute streptavidin-conjugated donor beads 125-fold with 1× Detection Buffer. Add 10 μL per well. Incubate at room temperature for 1 h. Read Alpha-counts. The percentage inhibition was calculated for the different concentrations tested against the control, and the IC₅₀ values against Hsp90α protein were calculated from the concentration inhibition response curve.

4.4. Molecular Modeling. Docking study was performed using the crystal structure of Hsp90α (PDB code: 3B25) in complex to B2K (4-methyl-6-(toluene-4-sulfonyl)-pyrimidin-2-ylamine).³⁰ The PDB file was downloaded from the PDB. Structure of chain A was processed using the structure preparation application in MOE (Molecular Operating Environment, 2014). The ligand molecule was removed from the protein active site. Then, the missing hydrogens was added using the Protonate three-dimensional application of MOE and properly assign the ionization states. To discover the favorable

binding conformation, the default procedure in the MOE Dock application was used. Primary placement poses created by the alpha triangle matcher were rescored and filtered using the London dG scoring method to pick exhibiting maximal hydrophobic, ionic, and hydrogen-bond contacts to the protein. This was followed by a refinement stage. The generated poses were energy minimized using the MMFF94x force field. Finally, the optimized poses were ranked using the GBVI/WSA DG free-energy estimates. Docking poses were inspected and interactions with binding pocket residues were analyzed.

4.5. Antimicrobial Activity. Antimicrobial assessment and the MIC performed at the Microbiology Unit in the Biochemistry Central Laboratory, Faculty of Science, Cairo University, Cairo, Egypt. The synthesized compounds were individually tested against Gram-positive and Gram-negative bacterial pathogens and fungi. Agar well diffusion method was used to determine the activity of tested compounds.^{31,32} The compounds were tested at a concentration of 15 mg/mL against both bacterial and fungal strains. Microbial suspension was prepared in sterilized saline equivalent to McFarland 0.5 standard solution (1.5×10^5 CFU mL⁻¹). Its turbidity was adjusted to the optical density equals to 0.13 using spectrophotometer at 625 nm. After adjusting the turbidity of the inoculum suspension within 15 min, a sterile cotton swab was dipped into the adjusted suspension and was flooded on the dried agar surface. The agar was then allowed to dry for 15 min. Wells of 6 mm diameter was made in the solidified media with the help of sterile borer. The solution of the tested compound (100 μ L) was added to each well with micropipette. The plates were then incubated at 37 °C. The IZ was measured in millimeter (mm) after 24 h incubation at 30 °C in the case of bacterial plates while in the case of fungal plates, the incubation was for 48 h. This experiment was carried out in triplicate and the results were recorded for each tested compound as mm \pm SD.

4.6. MIC Measurement. Stock solutions of the tested compounds, ampicillin, gentamicin, and nystatin were prepared in DMSO at concentration of 1000 μ g/mL followed by serial twofold dilution at concentrations of (500, 250, 125, 62.5, 31.25 μ g/mL), which was then mixed with sterile nutrient agar from Sigma-Aldrich, USA, in a sterile plate, followed by the inoculation of a defined microbial inoculum onto the agar plate surface. The plates were then left to incubate at 37 °C in a humid chamber. After 24 h, the MIC endpoints were read and recorded as the lowest concentration of an antimicrobial agent that completely inhibits growth under suitable incubation conditions.³²

4.7. DHFR Inhibitor Screening Assay. Enzyme assay experiments were carried out at the Tissue Culture Unit, the Egyptian Company for Production of Vaccines, Sera and Drugs (VACSERA), Giza, Egypt. The assay for the inhibitory effect was applied as indicated in the BioVision manufacture's protocol. Dilute methotrexate 100-fold was prepared by diluting 2 μ L of methotrexate with 198 μ L of DHFR buffer. Each tested sample was then dissolved into 100x of an appropriate solvent. 2 μ L of the tested sample, diluted methotrexate or DHFR assay buffer was added into wells assigned as sample screening (S), inhibitor control (IC), or enzyme control (EC), respectively. Dilute DHFR 400-fold was then prepared by diluting 2 μ L of DHFR with 798 μ L of DHFR buffer. Enough enzyme mixture for the number of wells were also prepared to be analyzed. Diluted DHFR (98 μ L) was

added into desired well(s) having the tested samples, EC or IC with volume equivalent to 100 μ L. Background control (BC) was prepared by adding 100 μ L of DHFR assay buffer to desired well(s). A stock solution of nicotinamide adenine dinucleotide phosphate (NADPH), a 40-fold dilution was prepared by diluting 10 μ L of NADPH stock solution with 390 μ L DHFR assay buffer, vortex briefly and kept at 0 °C. Diluted NADPH (40 μ L) was added to each well containing the tested samples, EC, IC, or BC. Samples were then mixed well and incubated at room temperature for 10–15 min and was kept away from light. A 15-fold dilution of the DHFR substrate was prepared by diluting 40 μ L of DHFR stock substrate with 560 μ L of DHFR assay buffer, vortexed briefly and kept at 0 °C. Diluted DHFR substrate (60 μ L) was then added to each well containing the tested samples, EC, IC, or BC. After samples were mixed well, the total volume reached to 200 μ L. The absorbance was measured then at 340 nm in kinetic mode for 10–20 min at room temperature. Choose two time points (t_1 & t_2) in the linear range of the plot and obtain the corresponding values for the absorbance (OD1 and OD2). Calculate the slope for all test inhibitor samples [S] and [EC] by dividing the net DOD ($A_1 - A_2$) values with the time Dt ($t_2 - t_1$). Subtract the solvent control or inhibitor BC readings from its paired sample readings.

% relative inhibition

$$= (\text{slope of [EC]} - \text{slope of [S]}) / \text{slope of EC} \times 100$$

$$\% \text{ relative activity} = \text{slope of [S]} / \text{slope of EC} \times 100$$

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.0c03773>.

All spectral analysis such as IR, ¹H NMR, and ¹³C NMR spectra and nontoxic doses for the newly synthesized compounds on FRHK-4, Hep2, BGM, Vero, and Huh 7.5 cell lines (PDF)

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Notes

The authors declare no competing financial interest.

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