



Article

Development of New Pyrazolo [3,4-*b*]Pyridine Derivatives as Potent Anti-Leukemic Agents and Topoisomerase II α Inhibitors with Broad-Spectrum Cytotoxicity

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Abstract

Background/Objectives: In the current medical era, Topoisomerase II is recognized as an essential enzyme that regulates DNA topology during critical biological processes such as DNA replication, transcription, and repair. This study aimed to design, synthesize, and biologically evaluate a new series of pyrazolo[3,4-*b*]pyridines (**8a–g**, **10a–g**, and **12**) as potential anticancer agents and Topoisomerase II inhibitors. **Methods:** The synthesized compounds were subjected to in vitro anticancer screening at the National Cancer Institute (NCI, USA). Active derivatives were further evaluated through a five-dose screening to determine their antiproliferative potency. Selected compounds were examined for their effects on leukemia cell lines (K562 and MV4-11), and mechanistic studies were performed to assess DNA damage, cell cycle distribution, and apoptosis-related protein modulation. Additionally, enzyme inhibition assays were conducted to determine Topoisomerase II α (TOPII α) inhibition. **Results:** Initial single-dose screening identified several active compounds, notably **8b**, **8c**, **8e**, **8f**, **10b**, **10c**, **10e**, and **10f**. Among these, compound **8c** exhibited potent and broad-spectrum antiproliferative activity across the NCI cancer cell line panel, with a GI₅₀ MG-MID value of 1.33 μ M (range: 0.54–2.08 μ M). The synthesized molecules showed moderate to good anti-leukemic efficacy against K562 and MV4-11 cells. Mechanistic investigations revealed that compound **8c** induced DNA damage and S-phase cell cycle arrest, leading to apoptosis as evidenced by the modulation of PARP-1,

Bax, XIAP, and Caspases. Furthermore, target-based assays confirmed that compound **8c** significantly inhibited the DNA relaxation activity of TOPII α in a dose-dependent manner, comparable to etoposide. **Conclusions:** The study highlights compound **8c** as a promising pyrazolo[3,4-*b*]pyridine derivative with potent antiproliferative activity and effective inhibition of Topoisomerase II α . These findings suggest its potential as a lead scaffold for further optimization in anticancer drug development..

Keywords: TOPII α ; molecular modeling; Western blotting; apoptosis; pyrazolopyridine

1. Introduction

Cancer is still one of the leading causes of death worldwide and remains a serious public health concern [1,2]. By 2050, the global burden of cancer is anticipated to reach 19 million cancer diagnoses worldwide, with an estimated 10.5 million cancer-related deaths [3]. Among the diverse types of cancer, leukemia stands out as one of the most aggressive and potentially fatal hematological malignancies [4]. Acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), and chronic lymphocytic leukemia (CLL) are the four primary subtypes of leukemia that are clinically distinguished based on cell lineage and the rate of disease progression [5]. Each subtype exhibits distinct pathological, genetic, and clinical features, which require individualized treatment approaches. Although leukemia often responds well to chemotherapy, the range of safe and durable treatment options remains limited [6]. As a result, the development of novel, more effective therapies for leukemia remains a pressing and continuous task. Pyrazolo [3,4-*b*]pyridine is a privileged fused heterocyclic scaffold commonly found in pharmaceuticals and drug-like substances [7]. Research has shown that derivatives of pyrazolo [3,4-*b*]pyridine (Figure 1) exhibit anticancer properties through various mechanisms, including inhibition of several targets such as cyclin-dependent kinases (I–IV) [8–10], tubulin polymerization (V) [11], glycogen synthase kinase-3 (VI) [12], hematopoietic cell kinase (VII) [13], fibroblast growth factor receptor (VIII) [14], tropomyosin receptor kinases (IX) [15] and TOPII (X–XI) [16,17].

Indole is a simple chemical scaffold with a bicyclic planar structure found in many natural products [18]. The biological actions of indole derivatives include antibacterial [19], anti-inflammatory [20], anticancer [21,22], anticonvulsant [23], antiviral [24], antidiabetic [25], and antioxidant [26] properties. Regarding cancer proliferation inhibition, indole derivatives are highly effective because they strongly target multiple pathways (Figure 2), including PIM (XII) [27], CDK (XIII) [28], EGFR (XIV) [29], PI3K α (XV) [30], Bcl-2/Mcl-1 (XVI) [31], TOPII (XV–XVI) [32], etc. [33].

The endonuclease class enzymes known as DNA topoisomerases (TOPs) are widely distributed throughout all life spheres and play essential roles in fundamental cellular processes [34,35]. TOP enzymes carry out a variety of tasks linked to the maintenance of DNA metabolism and regulate DNA topology during transcription and replication [36,37]. Based on their structure, amino acid sequence, catalytic activity, and reaction mechanism, TOPs can be divided into type I and type II [38,39]. Numerous investigations have demonstrated that TOPs are overexpressed in tumor cells, and their inhibition suppressed tumor cell growth, establishing TOPs as valuable targets for the development of anticancer therapeutics [40,41]. Furthermore, leukemia's higher topoisomerase II α expression levels have been associated with increased sensitivity to agents targeting this enzyme [42]. TOPII inhibitors, such as doxorubicin and etoposide, have been extensively employed in cancer therapy. Although these compounds demonstrate clinical efficacy, their application is often

limited by toxicity and adverse side effects [43,44]. Doxorubicin, for instance, is known to induce dose-dependent cardiotoxicity primarily through the generation of reactive oxygen species (ROS) and mitochondrial dysfunction, leading to cardiomyocyte apoptosis [45,46]. Etoposide, on the other hand, is associated with myelosuppression due to its interference with DNA synthesis and repair in rapidly dividing hematopoietic cells [47]. Moreover, resistance to TOPII inhibitors in leukemia patients remains a significant clinical challenge. Mechanisms contributing to drug resistance include the deletion or downregulation of the TOPII α gene, upregulation of DNA repair enzymes such as PARP and RAD51, and enhanced drug efflux via ATP-binding cassette (ABC) transporters [48]. These adaptations reduce drug efficacy and complicate treatment outcomes, thus, the development of novel and more efficient TOPII inhibitors is the subject of extensive research [41,49].

TOPII inhibitors are known for their inflexible, coplanar aromatic structures, which serve as templates for additional structural optimization to facilitate the intercalation of stable ternary complexes with DNA. The molecular scope of inhibitor design tactics has been expanded beyond “poison inhibition” to encompass “catalytic inhibition”, “dual-targeting”, and “structure-induced blockade” [50]. As a result, new TOPII inhibitors with non-classical structures are appearing in preclinical research. A tendency towards structural and mechanistic diversity is reflected in these structures, which extend beyond conventional scaffolds and incorporate a variety of chemotypes [50]. Furthermore, the creation of dual-target compounds that integrate TOPII inhibitors with other target inhibitors, such as PARP [51], HDAC [52], and tubulin [53], results in long-lasting lethal stress and reduces resistance.

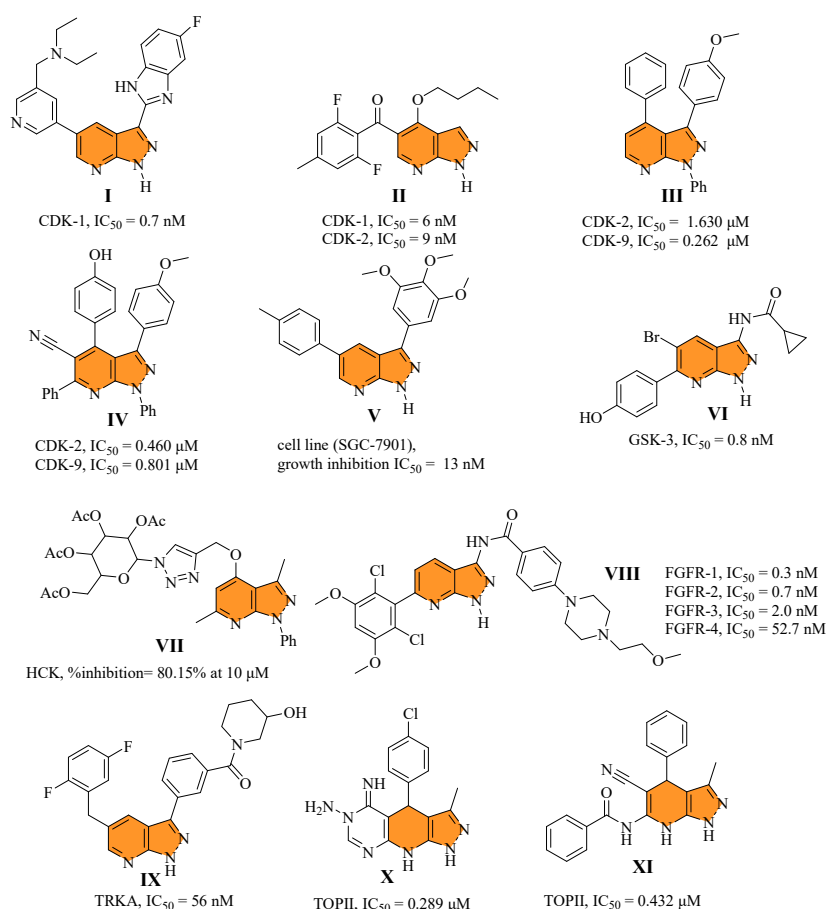


Figure 1. Structure of some reported pyrazolo [3,4-*b*]pyridine (I–XI) derivatives alongside their biological activity.

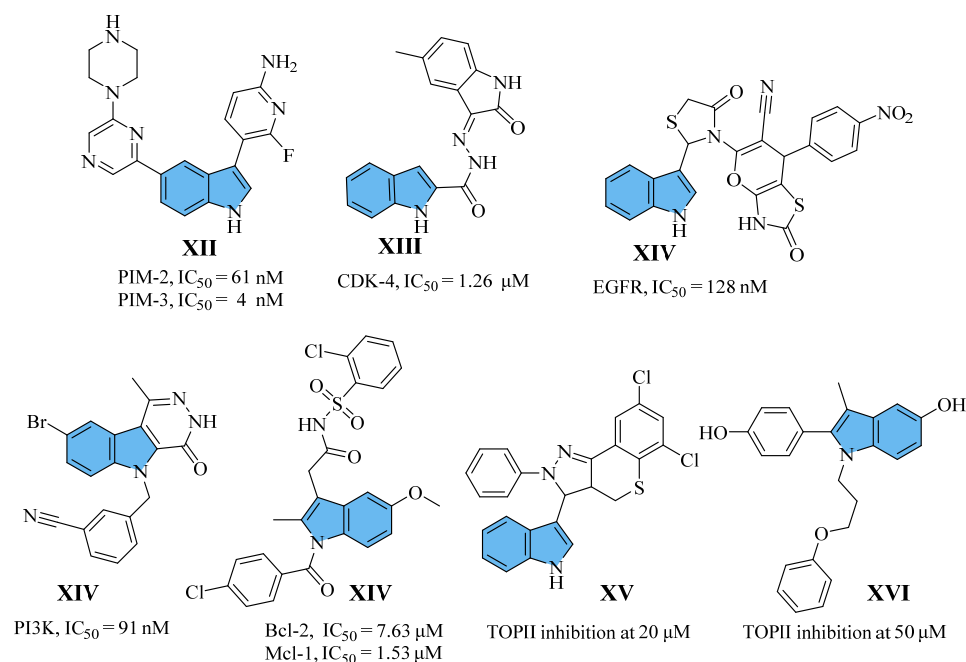


Figure 2. Structure of some reported indole (XII–XVI) derivatives alongside their biological activity.

Previous studies have highlighted planar structures as a key pharmacophoric feature of TOPII inhibitors such as the scaffolds found in compounds **X** [16] and **XI** [17], both of which contain pyrazolo [3,4-*b*]pyridine in their structure. Additionally, the indole ring, due to its planar structure, can act as an intercalation site for DNA interaction, as demonstrated by compounds **XV** and **XVI**, which showed promising TOPII inhibition activities. All things considered, the compounds in this study were based on a hybridization strategy involving pyrazolo [3,4-*b*]pyridine as the main scaffold bearing the indole moiety at three varying positions to achieve promising activity against TOPII. As a part of the structure, phenyl rings were also grafted to afford binding interaction with the lipophilic DNA groove binding regions (Figure 3). The obtained compounds were tested in a preliminary anticancer screening at the National Cancer Institute (NCI), and the most promising were subjected to biological mechanistic studies and molecular docking to explore their potential anticancer mechanisms.

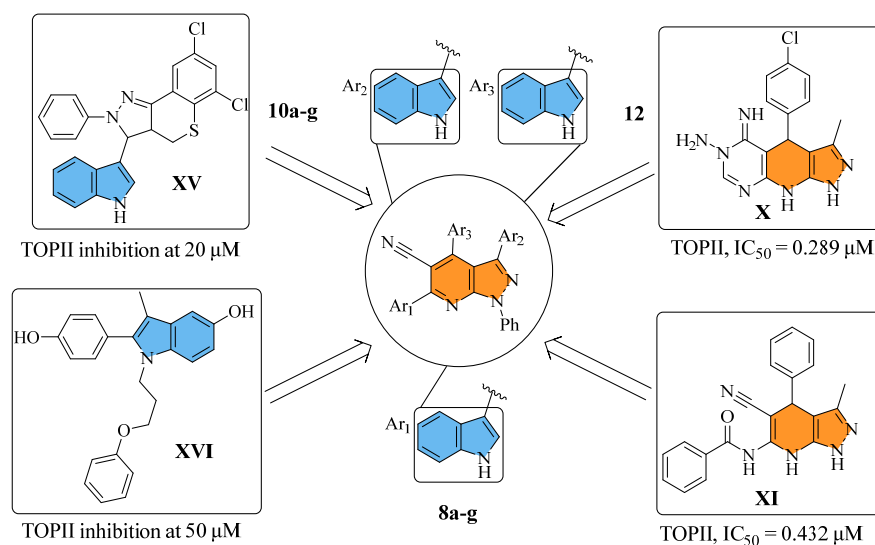
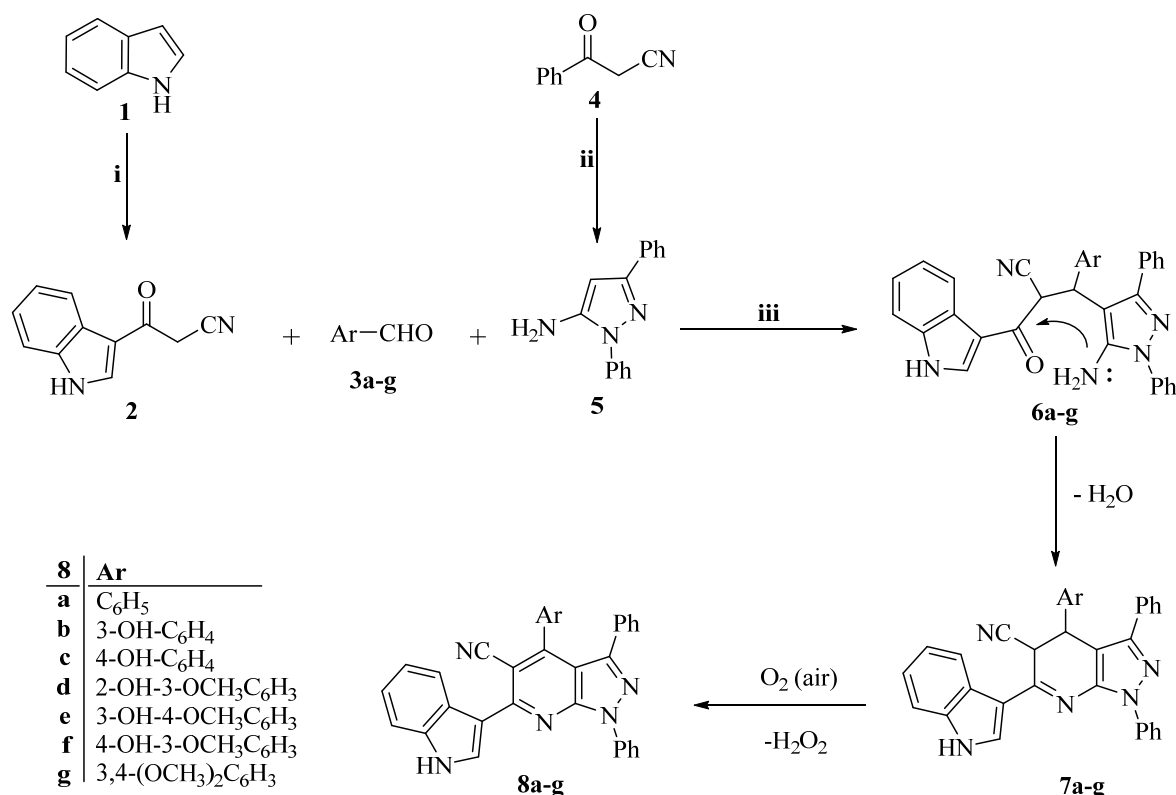


Figure 3. The adopted hybridization strategy for the design of small molecules **8a–g**, **10a–g**, and **12**.

2. Results and Discussion

2.1. Chemistry

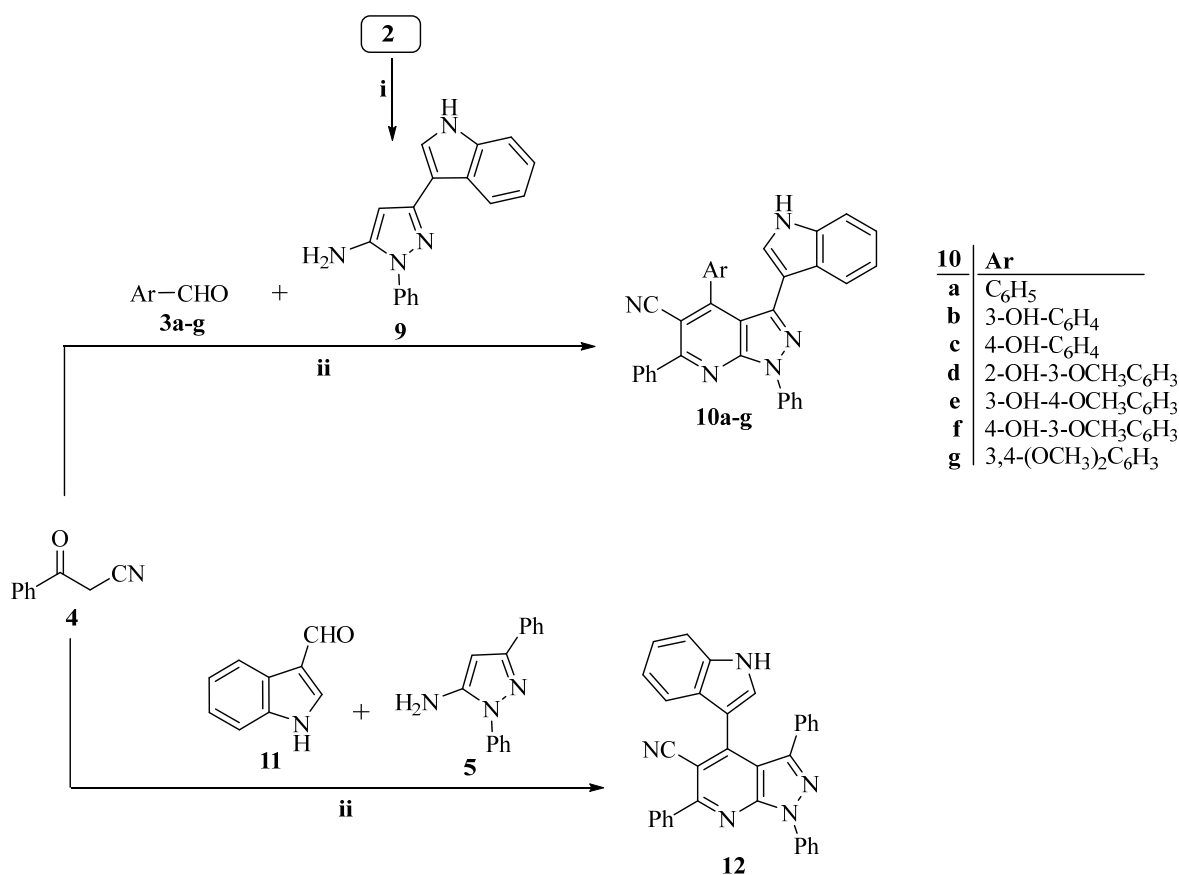
Schemes 1 and 2 show the synthetic routes taken to produce the target pyrazolo [3,4-*b*]pyridine derivatives (**8a–g**, **10a–g**, and **12**). To obtain 3-(1*H*-indol-3-yl)-3-oxopropanenitrile (**2**), indole (**1**) was heated with cyanoacetic acid, and acetic anhydride was involved [54].



Scheme 1. The synthetic route of target pyrazolo [3,4-*b*]pyridine compounds (**8a–g**). Reagents and conditions: i. Cyanoacetic acid, acetic anhydride, 85 °C 30 min; ii. *Abs.* EtOH, C₆H₅NHNH₂, reflux 1 h; iii. *Abs.* EtOH, reflux 12 h.

Additionally, 3-oxo-3-phenylpropanenitrile (**4**) and 3-(1*H*-indol-3-yl)-3-oxopropanenitrile (**2**) were condensed with phenylhydrazine in refluxing absolute ethanol to create the 3-substituted-1-phenyl-1*H*-pyrazol-5-amines (**5** and **9**) [55–60]. As shown in Schemes 1 and 2, the target pyrazolo [3,4-*b*]pyridine derivatives (**8a–g**, **10a–g**, and **12**) were then prepared using a one-pot, three-component reaction that included the relevant 3-substituted-1-phenyl-1*H*-pyrazol-5-amine (**5** or **9**), an equimolar amount of the corresponding 3-oxo-3-arylpropanenitrile (**2** or **4**), and the appropriate aldehyde (**3a–g** and **11**).

Intermediates **6a–g** are produced as part of the reaction mechanism, and they are cyclized and then water-eliminated to produce intermediates **7a–g**. The required compounds **8a–g** are subsequently obtained through a final oxidative dehydrogenation process, in which atmospheric oxygen acts as the oxidant. Nuclear magnetic resonance (NMR) spectroscopy (¹H and ¹³C) (Figures S1–S37) and high-resolution mass spectrometry (HRMS) (Figures S38–S51) validated the structures of the synthesized compounds, which were in agreement with the proposed structures.



Scheme 2. The synthetic route of target pyrazolo [3,4-*b*]pyridine compounds (10a–g and 12). Reagents and conditions: i. *Abs.* EtOH, C₆H₅NHNH₂, reflux 1 h; ii. *Abs.* EtOH, reflux 12 h.

2.2. Structure Elucidation of the Target Compounds

The ¹H NMR spectra of target compounds 8a–g, 10a–g, and 12 confirmed their structures. In particular, the absence of the aldehydic proton (CH=O) signals from aldehydes (3a–g and 11) and the active methylene protons (CH₂) from nitriles (2 and 4) around δ 3.5 ppm, as well as the disappearance of characteristic signals around δ 5.0 ppm, which correspond to the two NH₂ protons of the precursor 3-substituted-1-phenyl-1*H*-pyrazol-5-amines (5 and 9). The addition of aromatic moieties throughout the process was further supported by the spectra, which showed an increase in aromatic proton signals.

The suggested structures were further supported by the ¹³C NMR spectra, which revealed the elimination of carbon signals associated with nitrile and aldehyde carbonyl groups, typically detected at δ 190 ppm. These modifications verified that the initial materials had completely changed into the final pyrazolo [3,4-*b*]pyridine derivatives.

With variances falling within the permissible range of ±0.4%, the elemental analysis findings showed good agreement with the estimated values for the target compounds' molecular formulae. The structures were further validated by molecular ion peaks obtained from high-resolution mass spectrometry (HRMS) that matched the predicted molecular weights. The effectiveness and selectivity of the synthetic processes were further demonstrated by high-performance liquid chromatography (HPLC) analysis, which verified that all synthesized compounds had a purity higher than 95.00% (Figures S52–S66).

2.3. Biological Evaluation

The NCI 60 human cancer cell line panel was used for preliminary in vitro anticancer screening at a single dose of 10 μM.

The United States’ National Cancer Institute (NCI) conducted the first anticancer assessment of the recently synthesized indole-conjugated pyrazolo [3,4-*b*]pyridine derivatives (**8a–g**, **10a–g**, and **12**). The comprehensive NCI 60 human tumor cell line panel, which comprises leukemia, non-small cell lung, melanoma, central nervous system (CNS), ovarian, renal, prostate, and breast cancer cell lines, was first screened in vitro at a fixed concentration of 10 µM. The findings, presented as the mean percentage growth (G%) of treated cells compared to untreated controls, shed light on the cytotoxic effects (G% less than 0) as well as the cytostatic activity (G% between 0 and 100).

The single-dose activity profiles of compounds **8a–g**, **10a–g**, and **12** were examined using the COMPARE algorithm on 60 distinct cancer cell lines. The pyrazolo [3,4-*b*]pyridines assessed exhibited a broad range of cytotoxic effects on various cancer types, with anti-cancer activities ranging from weak to very potent [61]. Table 1 summarizes the growth inhibition percentages (GI%) for each target molecule, which are computed as (100–G%).

Table 1. Percentage cell growth inhibition (GI%) of an in vitro panel of 60 human tumor cell lines at 10 µM concentration of compounds **8a–g**, **10a–g**, and **12**.

Cell Lines		Growth Inhibition Percentage (GI%)														
		8a	8b	8c	8d	8e	8f	8g	10a	10b	10c	10d	10e	10f	10g	12
Leukemia	CCRF-CEM	30	80	83	☯	83	99	☯	63	L	92	80	L	89	48	56
	HL-60(TB)	12	99	93	☯	95	93	☯	18	L	L	61	L	93	☯	25
	K-562	69	95	96	☯	99	99	11	73	97	95	15	99	95	64	60
	MOLT-4	46	82	84	☯	89	94	☯	67	L	99	42	L	90	48	59
	RPMI-8226	29	L	96	15	96	98	☯	44	L	92	32	L	91	21	35
	SR	NT	NT	NT	NT	NT	NT	NT	69	L	NT	38	L	99	31	NT
Lung Cancer (NSC)	A549	22	96	L	☯	L	91	☯	39	88	93	☯	L	L	39	34
	EKVX	27	L	L	15	L	L	29	70	L	L	28	L	L	50	27
	HOP-62	29	L	L	22	L	L	12	22	L	L	☯	L	L	18	16
	HOP-92	☯	L	L	☯	82	70	☯	NT	NT	89	NT	NT	NT	NT	☯
	NCI-H226	36	L	L	48	L	L	20	42	L	L	13	L	L	23	30
	NCI-H23	15	L	L	☯	L	L	20	52	L	L	15	L	L	23	39
	NCI-H322M	11	L	L	☯	L	90	☯	19	73	98	20	90	82	26	13
	NCI-H460	61	93	L	☯	L	L	☯	75	L	L	☯	L	L	42	47
	NCI-H522	☯	90	L	☯	L	98	☯	L	L	L	21	L	L	44	19
Colon Cancer	COLO 205	13	L	L	☯	L	L	☯	37	L	L	☯	L	L	16	13
	HCC-2998	23	L	L	☯	L	L	☯	70	L	L	☯	L	L	34	25
	HCT-116	60	L	L	☯	L	L	☯	44	98	L	☯	96	L	43	52
	HCT-15	80	L	L	14	L	L	☯	56	L	L	23	L	L	35	56
	HT29	36	L	L	☯	L	L	☯	41	L	L	☯	L	L	29	46
	KM 12	40	L	L	☯	L	L	☯	69	L	L	☯	L	L	39	34
	SW-620	27	L	L	☯	L	L	☯	37	L	L	☯	L	L	11	13
CNS Cancer	SF-268	17	65	92	15	81	72	☯	41	88	56	29	L	93	31	15
	SF-295	45	L	L	22	L	L	☯	35	L	L	19	L	L	26	23
	SF-539	53	L	L	14	L	L	12	26	L	L	11	L	L	14	28
	SNB-19	32	L	L	24	L	99	11	40	L	87	22	L	90	33	16
	SNB-75	18	65	91	13	99	83	☯	25	70	47	28	85	72	26	☯
	U251	52	L	L	13	L	L	☯	38	L	L	17	L	L	30	35

Table 1. Cont.

Cell Lines		Growth Inhibition Percentage (GI%)														
		8a	8b	8c	8d	8e	8f	8g	10a	10b	10c	10d	10e	10f	10g	12
Melanoma	LOX IMVI	66	L	L	13	L	L	11	49	L	L	17	L	L	33	40
	MALME-3M	25	L	L	13	L	L	☯	58	L	L	13	L	L	66	17
	M14	☯	L	L	☯	L	L	☯	31	98	L	☯	L	92	27	18
	MDA-MB-435	16	L	L	☯	L	97	☯	44	L	L	☯	L	L	32	13
	SK-MEL-2	☯	67	L	☯	98	44	☯	40	L	29	16	L	L	21	☯
	SK-MEL-28	26	L	L	☯	L	L	☯	57	L	93	☯	L	L	60	☯
	SK-MEL-5	25	L	L	26	L	L	14	30	87	L	22	L	53	☯	42
	UACC-257	☯	59	82	☯	L	55	☯	24	60	51	☯	90	96	15	☯
	UACC-62	16	L	L	12	L	L	☯	19	66	64	13	L	55	23	☯
Ovarian Cancer	IGROV1	19	L	L	☯	L	97	☯	27	L	98	☯	L	L	17	15
	OVCAR-3	☯	L	L	☯	L	L	☯	☯	L	L	☯	L	L	☯	☯
	OVCAR-4	☯	95	99	22	93	87	☯	25	82	70	19	96	91	29	12
	OVCAR-5	☯	L	L	☯	L	L	☯	15	L	98	☯	L	92	☯	☯
	OVCAR-8	36	L	L	18	L	96	☯	28	L	99	28	L	99	28	19
	ADR-RES	40	L	L	21	L	L	☯	29	L	L	24	L	L	28	17
	SK-OV-3	☯	57	83	☯	66	33	☯	25	76	33	☯	82	78	☯	☯
Renal Cancer	786-0	64	93	L	☯	L	L	☯	41	89	L	14	99	81	28	19
	A498	☯	L	L	☯	L	L	☯	54	L	L	☯	L	L	☯	☯
	ACHN	44	L	L	☯	97	98	☯	36	L	L	☯	L	86	22	26
	CAKI-1	15	99	L	17	92	85	17	54	L	L	27	L	L	49	31
	RXF 393	L	L	L	36	L	L	31	71	L	L	21	L	L	72	49
	SN 12C	17	L	L	14	L	L	☯	48	L	L	14	L	L	29	26
	TK-10	☯	68	82	☯	63	57	☯	☯	50	45	☯	61	58	☯	☯
	UO-31	42	L	L	12	L	L	29	33	46	L	40	L	L	35	47
Prostate Cancer	PC-3	37	L	L	12	L	92	☯	NT	NT	98	NT	NT	NT	NT	58
	DU-145	21	L	L	☯	L	L	☯	42	L	L	☯	L	L	32	15
Breast Cancer	MCF7	46	L	L	16	L	L	16	56	L	L	21	L	L	41	57
	MDA-MB-231	31	L	L	☯	L	L	22	39	L	L	30	L	L	42	27
	HS 578T	32	L	L	18	L	97	☯	52	L	L	☯	L	L	63	19
	BT-549	☯	L	L	☯	L	L	11	41	83	L	☯	L	L	28	☯
	T-47D	☯	83	74	14	72	60	☯	66	L	74	31	96	99	39	48
	MDA-MB-468	☯	L	L	☯	L	L	☯	35	L	L	☯	L	98	19	41
GI Mean		24	120	154	8	138	125	3	43	126	118	15	138	122	29	23

Compounds that were not tested are indicated by “NT”. A growth inhibition percentage (GI%) of less than 10% denotes low cytotoxic activity, as shown by the symbol “☯”. Moderate anticancer activity is indicated by a GI% between 10 and 60, and substantial growth inhibition is indicated by values between 60 and 100. The letter “L” stands for GI% values higher than 100%, which suggests strong cytotoxicity and a fatal impact on the cancer cells. The effectiveness of each chemical throughout the NCI-60 cancer cell line panel can be interpreted with the use of these activity classifications.

According to the NCI criteria, derivatives **8d**, **8g**, and **10d** (mean GI% values below 20) did not exhibit significant cytotoxicity against the examined cell lines (except for **8d** towards NCI-H226 and **10d** towards CCRF-CEM, HL-60(TB), and MOLT-4) [62]. Compounds **8a** (mean GI% = 24), **10a** (mean GI% = 43), **10g** (mean GI% = 29), and **12** (mean GI% = 23)

exposed promising sensitivity towards limited representative cell lines (leukemia; K-562 and renal cancer; RXF 393) (Table 1).

The remaining eight compounds; 3-hydroxyphenyl derivatives (**8b** and its counterpart **10b**), 4-hydroxyphenyl derivatives (**8c** and its counterpart **10c**), 4-hydroxy-3-methoxyphenyl derivatives (**8e** and its counterpart **10e**) and 3-hydroxy-4-methoxyphenyl derivatives (**8f** and its counterpart **10f**) showed auspicious results in NCI preliminary screening with mean GI% = 120, 154, 138, 125, 126, 118, 138 and 122 (Table 1).

The antiproliferative activity of compounds **8a** (mean GI% = 24) and **10a** (mean GI% = 43) was considerably increased by adding a hydroxyl group to the *meta* position of one of the phenyl rings. This resulted in **8b** and **10b** derivatives, with mean growth inhibition (GI%) values of 120% and 126%, respectively. Among the 60 cell lines examined, both compounds showed the most potent anticancer effects. According to Table 1, each demonstrated fatal activity against 42 cell lines, 33 of which were shared by both, and inhibitory effects on the other lines. The GI% values for these lines ranged from 46 to 99.

When the hydroxyl group in compound **8b** was shifted from the *meta* to the *para* position, obtaining compound **8c**, the antiproliferative activity increased. Both derivatives exerted a lethal effect on 47 cell lines. The same structure modification of **10b**, yielding compound **10c**, led to a modest decrease in activity, and **10c** exerted a lethal effect only on 37 cell lines. The mean GI% values for **8c** and **10c** were 154% and 118%, respectively (Table 1).

Introducing a methoxy group at the *para* position adjacent to the hydroxyl group in compounds **8b** and **10b** resulted in enhanced antiproliferative activity, as observed in the obtained derivatives **8e** and **10e**, both of which showed a mean GI% of 138. These compounds exerted lethal effects on 39 common cancer cell lines. Lastly, compounds **8e** and **10e** showed enhanced anticancer activity with mean GI% values of 125 and 122, respectively, when the hydroxyl and methoxy groups were repositioned, with the hydroxyl in the *para* position and the methoxy in the *meta* position, as in compounds **8f** and **10f** with 34 and 35 cancer cell lines were fatally affected by these compounds, respectively.

Based on their in vitro cytotoxic characteristics against the NCI-60 human cancer cell line panel, the structure–activity relationship (SAR) of the synthesized compounds is depicted in Figure 4. The mean growth inhibition percentages (GI%) presented in Table 1 serve as the basis for this analysis. Important pharmacophoric characteristics that contribute to potency and selectivity across several cancer cell types were identified by establishing a correlation between the observed anticancer activity and the structural alterations within the pyrazolo [3,4-*b*]pyridine scaffold. This SAR analysis lays the groundwork for future structural optimization in the development of more potent anticancer drugs, providing insightful information on how specific substituents affect biological activity.

In the next step, the most potent derivatives, including 3-hydroxyphenyl derivatives (**8b** and its counterpart **10b**), 4-hydroxyphenyl derivatives (**8c** and its counterpart **10c**), 4-hydroxy-3-methoxyphenyl derivatives (**8e** and its counterpart **10e**) and 3-hydroxy-4-methoxyphenyl derivatives (**8f** and its counterpart **10f**), were screened against the 60 cancer cell lines at 10-fold dilutions and five different concentrations (0.01, 0.1, 1, 10 and 100 μ M) [61]. Following established experimental procedures, the sulforhodamine B (SRB) colorimetric assay was used to evaluate cell viability [63]. Cell density and vitality after chemical treatment are indirectly assessed by measuring cellular protein content in this experiment. The molar concentrations of the substances needed to inhibit 50% of cell proliferation (GI₅₀) and induce 50% cell death (LC₅₀) are represented by the GI₅₀ and LC₅₀ values for each cancer cell line that is tested. Table 2 summarizes these important pharmacological parameters, providing information on the cytotoxic and antiproliferative efficacy of the investigated agents [64,65]. The NCI 60 cancer cell line panel demonstrated

strong antiproliferative activity across all chosen compounds (**8b**, **8c**, **8e**, **8f**, **10b**, **10c**, **10e**, and **10f**), with efficacy ranging from sub-micromolar to low micromolar concentrations, according to the results. These results demonstrate compounds' strong growth inhibition against a wide range of cancer cell types, underscoring their considerable potential as potent anticancer agents.

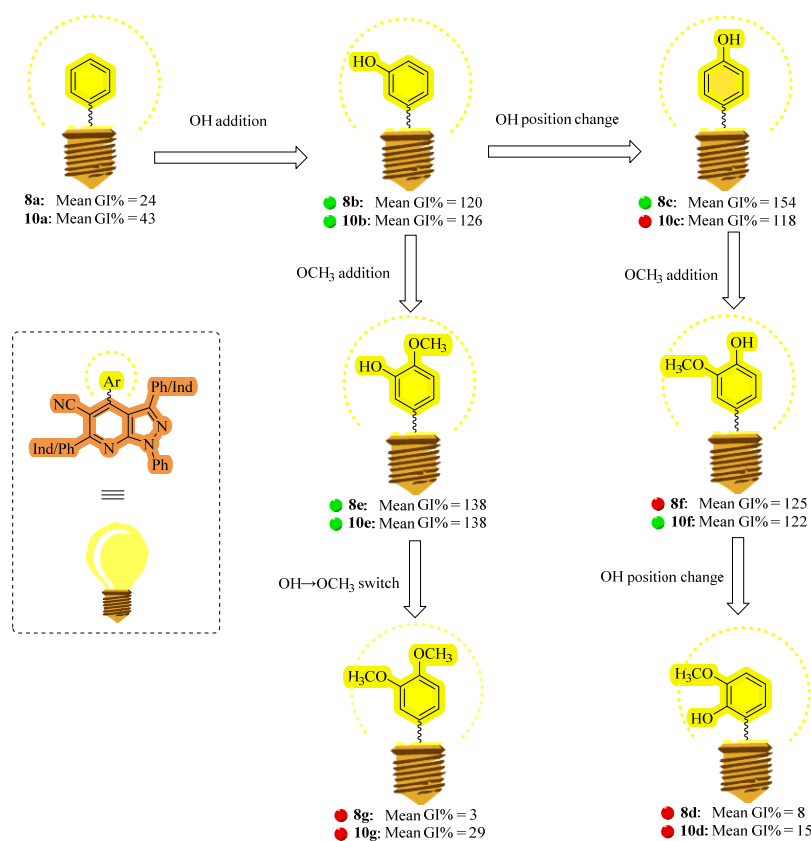


Figure 4. The target compounds' structure–activity relationship (SAR) as in vitro anticancer agents based on their mean GI% values across 60 human cancer cell lines.

Table 2. Five dose in vitro anticancer activity results expressed as GI₅₀ (μM) for compounds **8b**, **8c**, **8e**, **8f**, **10b**, **10c**, **10e**, and **10f** against all sixty cancer cell lines.

	Subpanel Cell Lines	GI ₅₀ (μM)							
		8b	8c	8e	8f	10b	10c	10e	10f
Leukemia	CCRF-CEM	2.67	0.32	0.74	2.44	4.28	2.85	2.92	2.95
	HL-60(TB)	3.47	0.65	0.99	4.77	3.56	3.04	2.92	2.47
	K-562	2.67	0.45	1.62	3.68	3.21	2.74	2.85	2.07
	MOLT-4	2.82	0.48	1.23	3.77	3.31	2.00	2.36	2.12
	RPMI-8226	2.90	0.88	0.57	3.74	4.27	☯	2.52	2.39
	SR	2.87	0.48	0.71	2.76	3.57	2.60	2.53	2.21
Lung Cancer (NSC)	A549	2.84	1.57	2.34	3.32	3.43	4.07	2.66	2.80
	EKVX	2.37	1.27	1.46	2.92	3.21	2.36	2.75	2.37
	HOP-62	2.32	1.16	3.52	2.30	2.11	3.19	1.73	2.02
	HOP-92	1.39	2.31	1.18	2.65	2.92	3.10	2.87	2.65
	NCI-H226	2.19	1.87	1.86	1.84	☯	☯	☯	4.03
	NCI-H23	2.25	1.44	1.52	2.68	1.86	2.02	1.88	1.86
	NCI-H322M	2.12	1.89	2.08	3.83	4.00	☯	3.24	2.96
	NCI-H460	2.36	1.27	2.17	2.10	2.67	3.09	1.75	1.90
	NCI-H522	2.40	1.68	2.38	2.46	2.07	1.83	1.80	2.03

Table 2. Cont.

	Subpanel Cell Lines	GI ₅₀ (μM)							
		8b	8c	8e	8f	10b	10c	10e	10f
Colon Cancer	COLO 205	1.63	2.05	2.61	2.02	1.92	2.79	1.65	1.85
	HCC-2998	2.22	1.59	1.50	1.81	1.78	2.09	1.85	1.90
	HCT-116	2.32	1.37	1.62	1.78	1.91	2.33	1.83	1.75
	HCT-15	1.97	1.16	1.26	1.70	1.74	3.41	2.39	1.90
	HT29	2.54	1.24	2.28	2.15	3.05	3.14	1.84	1.82
	KM 12	1.59	1.34	1.54	2.04	1.98	3.09	1.91	1.73
	SW-620	☯	2.26	☯	☯	2.89	3.14	1.50	3.12
CNS Cancer	SF-268	2.79	3.64	3.65	4.67	2.85	☯	2.98	2.61
	SF-295	2.08	0.23	0.37	2.02	2.61	☯	2.43	2.14
	SF-539	1.69	0.31	0.62	1.80	3.72	☯	2.15	1.82
	SNB-19	NT	NT	NT	NT	☯	NT	4.26	3.31
	SNB-75	1.84	0.23	0.44	2.13	2.04	3.43	2.17	2.81
	U251	1.79	0.62	0.85	2.28	2.19	3.18	1.85	1.67
Melanoma	LOX IMVI	1.53	1.23	1.36	1.69	1.66	1.90	1.70	1.65
	MALME-3M	2.12	2.54	2.31	3.05	2.72	3.14	1.67	1.68
	M14	2.37	1.57	2.03	2.36	2.95	☯	2.41	2.20
	MDA-MB-435	2.31	1.30	2.71	2.03	2.41	3.12	2.19	2.16
	SK-MEL-2	2.31	1.49	2.56	4.08	☯	☯	3.99	4.93
	SK-MEL-28	3.21	1.36	1.94	4.02	☯	☯	2.74	2.91
	SK-MEL-5	2.13	1.66	1.52	3.87	☯	☯	4.70	3.13
	UACC-257	4.12	4.43	☯	☯	☯	☯	4.70	☯
	UACC-62	1.72	1.37	2.28	2.86	☯	☯	☯	☯
Ovarian Cancer	IGROV1	1.82	2.22	1.93	4.71	3.69	☯	2.81	2.75
	OVCAR-3	2.09	1.61	1.35	1.85	1.58	3.14	1.88	1.96
	OVCAR-4	3.08	2.84	☯	4.49	3.67	☯	2.94	2.69
	OVCAR-5	2.47	1.54	2.78	2.78	☯	☯	3.05	3.53
	OVCAR-8	2.58	2.13	2.58	3.32	3.65	4.67	3.08	2.73
	NCI/ADR-RES	2.14	1.87	1.82	2.15	2.73	☯	3.12	2.41
	SK-OV-3	3.67	2.41	4.00	☯	3.56	1.30	2.25	3.75
Renal Cancer	786-0	3.22	0.36	0.84	1.92	2.15	4.37	1.87	2.00
	A498	4.90	1.20	2.01	2.03	4.88	☯	2.81	2.08
	ACHN	2.39	1.09	1.33	3.16	3.47	3.59	3.17	2.41
	CAKI-1	2.48	1.18	1.79	3.38	2.88	3.32	2.60	2.50
	RXF 393	2.17	0.15	0.23	1.89	3.34	4.57	2.71	2.33
	SN 12C	1.60	1.67	2.21	1.77	2.20	2.46	1.94	1.58
	TK-10	NT	NT	NT	NT	1.58	NT	4.14	4.33
	UO-31	1.83	0.93	1.65	2.08	2.47	☯	2.10	1.81
Prostate Cancer	PC-3	1.72	1.10	1.48	3.25	2.76	3.23	2.76	2.56
	DU-145	2.20	1.40	1.76	2.24	2.19	2.71	2.11	1.75
Breast Cancer	MCF7	1.92	0.59	1.21	1.84	2.98	2.87	2.64	2.22
	MDA-MB-231	1.96	1.46	1.78	2.05	2.21	2.25	1.92	1.48
	HS 578T	1.75	0.85	1.21	1.93	3.01	2.01	1.76	3.19
	BT-549	2.96	1.27	3.95	2.53	3.23	4.78	3.76	2.19
	T-47D	2.61	2.80	4.68	3.57	2.10	3.66	2.50	3.85
	MDA-MB-468	2.43	1.84	1.68	3.03	3.14	☯	2.79	2.26

“NT” Not tested. “☯” indicates GI₅₀ equal to or more than 5 μM. Red bolded numbers indicate activity less than 1 μM.

The results showed that most compounds had significant inhibitory effects, with GI_{50} values ranging from 0.15 μ M to 5 μ M. Only a few cell lines showed reduced sensitivity, with GI_{50} values not exceeding 15 μ M. Among the tested compounds, **8c** (a 4-hydroxyphenyl derivative) and **8e** (a 4-hydroxy-3-methoxyphenyl derivative) demonstrated remarkable potency, particularly against leukemia, followed by CNS, renal, and breast cancer cell lines.

Compound **8c** displayed outstanding activity with GI_{50} values ranging from 0.15 to 4.43 μ M among the tested NCI panel, including 15 cell lines with GI_{50} below 1.00 μ M. Interestingly, it showed sub-micromolar inhibitory activity towards all leukemia, all CNS (except SF-268 and SNB-19), renal (786-0, RXF 393, and UO-31), and breast cancer cell lines (MCF7 and HS 578T). Regarding compound **8e**, it displayed remarkable activity with GI_{50} values ranging from 0.23 to 4.68 μ M (with 10 cancer cell lines with GI_{50} below 1.00 μ M). Compound **8e** showed sub-micromolar inhibitory activity towards all leukemia (except K-562 and MOLT-4), all CNS (except SF-268 and SNB-19), and renal cancer cell lines (786-0 and RXF 393) (Table 2).

Additionally, an average activity metric for all cell lines is provided by the mean graph midpoint (MG-MID). The compounds **8b**, **8c**, **8e**, **8f**, **10b**, **10c**, **10e**, and **10f** showed MID (average sensitivity of all cell lines in μ M) values of 2.43, 1.33, 1.93, 3.04, 3.37, 4.23, 2.65, and 2.49 μ M, respectively (Table 3).

Table 3. Selectivity ratio of the compounds **8b**, **8c**, **8e**, **8f**, **10b**, **10c**, **10e** and **10f** towards NCI nine tumor subpanels.

Code	Parameter	Panel									GI_{50} -MID	MID ^a
		Leukemia	Lung Cancer (NSC)	Colon Cancer	CNS Cancer	Melanoma	Ovarian Cancer	Renal Cancer	Prostate Cancer	Breast Cancer		
8b	MID ^b	2.90	2.24	2.93	2.03	2.42	2.55	2.65	1.96	2.27	21.95	2.43
	Selectivity ^c	0.83	1.08	0.82	1.19	1.00	0.95	0.91	1.23	1.07		
8c	MID ^b	0.54	1.60	1.25	1.00	1.88	2.08	0.94	1.25	1.46	12.00	1.33
	Selectivity ^c	2.46	0.83	1.06	1.33	0.70	0.63	1.33	1.06	0.91		
8e	MID ^b	0.97	2.05	2.42	1.18	2.49	2.80	1.43	1.62	2.41	17.37	1.93
	Selectivity ^c	1.98	0.94	0.79	1.63	0.77	0.68	1.34	1.19	0.80		
8f	MID ^b	3.52	2.67	3.67	2.58	3.82	3.63	2.31	2.74	2.49	27.43	3.04
	Selectivity ^c	0.86	1.13	0.82	1.17	0.79	0.83	1.31	1.10	1.22		
10b	MID ^b	3.70	3.42	2.18	3.25	6.16	3.57	2.87	2.47	2.77	30.39	3.37
	Selectivity ^c	0.91	0.98	1.54	1.03	0.54	0.94	1.17	1.36	1.21		
10c	MID ^b	3.19	3.54	2.85	5.12	6.60	4.59	5.81	2.97	3.43	38.10	4.23
	Selectivity ^c	1.32	1.19	1.48	0.82	0.64	0.92	0.72	1.42	1.23		
10e	MID ^b	2.68	2.72	1.85	2.64	3.60	2.73	2.66	2.43	2.56	23.87	2.65
	Selectivity ^c	0.98	0.97	1.43	1.00	0.73	0.97	0.99	1.09	1.03		
10f	MID ^b	2.36	2.51	2.01	2.39	3.32	2.83	2.38	2.15	2.53	22.48	2.49
	Selectivity ^c	1.05	0.99	1.23	1.04	0.75	0.87	1.04	1.15	0.98		

^a MID is the average sensitivity of all cell lines in μ M; ^b MID is the average sensitivity of all cell lines in a particular subpanel in μ M; ^c Selectivity is the ratio of average sensitivity of all cell lines to the average sensitivity of all cell lines in a particular subpanel.

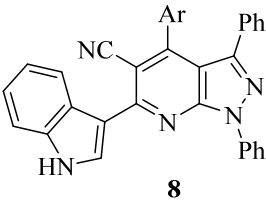
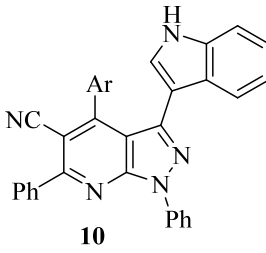
The ratio of the entire panel mean graph midpoint (MID) to the individual subpanel MID was used to calculate the selectivity of these substances. The subpanel MID shows the average sensitivity of cell lines within a particular tumor subpanel, whereas the whole panel MID shows the average sensitivity of all cell lines to the test agent. Compounds with selectivity ratios below this cutoff are regarded as nonselective, whilst those with ratios above 2 may suggest a preference for specific tumor types. With selectivity ratios ranging from 0.54 to 2.46, the drugs in this investigation showed broad-spectrum anticancer efficacy across the nine tumor subpanels examined. With an average MID of 1.33 μ M, compound **8c** demonstrated the highest potency overall and the most effective and selective treatment of leukemia (MID = 0.54 μ M, selectivity = 2.46). Likewise, compound **8e** had significant

potency and selectivity against leukemia, attaining a MID of 0.97 μM and a selectivity ratio of 1.98, with an average MID of 1.93 μM .

2.4. Antiproliferative Activity Against Leukemia Cells

Following the NCI 60 screening, the synthesized derivatives (**8b**, **8c**, **8e**, **8f**, **10b**, **10c**, **10e**, and **10f**) were further evaluated for their antiproliferative activity against leukemia cancer cell lines, including chronic myeloid leukemia (K562) and acute monocytic leukemia (MV4-11), as shown in Table 4.

Table 4. Antiproliferative activity of pyrazolo [3,4-*b*]pyridines **8b**, **8c**, **8e**, **8f**, **10b**, **10c**, **10e** and **10f** against leukemia MV4-11 and K562 cell lines.

 8				 10			
Code	Ar	GI ₅₀ (μM) ^a $\pm$ SD		Code	Ar	GI ₅₀ (μM) ^a $\pm$ SD	
		MV4-11	K562			MV4-11	K562
8b	3-OH-Ph	3.55 $\pm$ 0.37	6.52 $\pm$ 2.24	10b	3-OH-Ph	8.88 $\pm$ 0.04	3.32 $\pm$ 0.11
8c	4-OH-Ph	0.72 $\pm$ 0.27	0.73 $\pm$ 0.01	10c	4-OH-Ph	8.03 $\pm$ 1.41	2.50 $\pm$ 0.30
8e	3-OH-4-OCH ₃ -Ph	>10	>10	10e	3-OH-4-OCH ₃ -Ph	9.03 $\pm$ 0.20	5.13 $\pm$ 0.12
8f	4-OH-3-OCH ₃ -Ph	3.70 $\pm$ 0.04	3.17 $\pm$ 0.03	10f	4-OH-3-OCH ₃ -Ph	8.73 $\pm$ 0.40	4.00 $\pm$ 0.21
	Cisplatin	0.75 $\pm$ 0.21	4.57 $\pm$ 1.40		Quizartinib	0.003 $\pm$ 2.24	>10

^a Assayed in triplicate.

With the exception of compound **8e**, the chosen pyrazolo [3,4-*b*]pyridine derivatives efficiently suppressed the proliferation of MV4-11 cells, with GI₅₀ values ranging from 0.72 to 9.03 μM . With a GI₅₀ value of 0.72 μM , pyrazolo [3,4-*b*]pyridine **8c** exhibited the strongest sub-micromolar cytotoxic effect. Furthermore, with GI₅₀ values of 3.55 and 3.70 μM , respectively, compounds **8b** and **8f** demonstrated low single-digit micromolar cytotoxicity against the MV4-11 cell line. The remaining compounds (**10b**, **10c**, **10e**, and **10f**), on the other hand, demonstrated substantial single-digit micromolar cytotoxic activity, as indicated by Table 4, with GI₅₀ values ranging from 8.03 to 9.03 μM .

The pyrazolo [3,4-*b*]pyridine derivatives showed GI₅₀ values ranging from 0.72 to 6.52 μM for the K562 leukemia cell line. Compound **8c** once more exhibited exceptional sub-micromolar cytotoxicity (GI₅₀ = 0.72 μM). With GI₅₀ values ranging from 2.50 to 6.52 μM , compounds **8b**, **8f**, **10b**, **10c**, **10e**, and **10f** demonstrated single-digit micromolar cytotoxic action. However, as Table 4 summarizes, compound **8e** exhibited noticeably less activity, with a GI₅₀ value exceeding 10 μM .

Overall, SAR analysis indicates that subtle structural modifications have a marked influence on biological activity, notably enhancing potency against leukemia cancer cell lines. Among the tested compounds, **8c** exhibited the highest potency and was therefore selected for further experiments to elucidate the molecular mechanism of action of the presented derivatives.

2.5. Immunodetection and Cell Cycle Analysis of MV4-11

Based on the promising antiproliferative activity observed in the initial screening and SAR analysis, further experiments were conducted to characterize the molecular mecha-

nisms underlying the cytotoxic effects of the most potent compound **8c**. Asynchronously growing MV4-11 cells were incubated with increasing concentrations of **8c** (1.25, 2.5, 5.0, and 10 μ M) for 24 h and subsequently analyzed by immunodetection (Figure 5) and cell cycle flow cytometry (Figure 6).

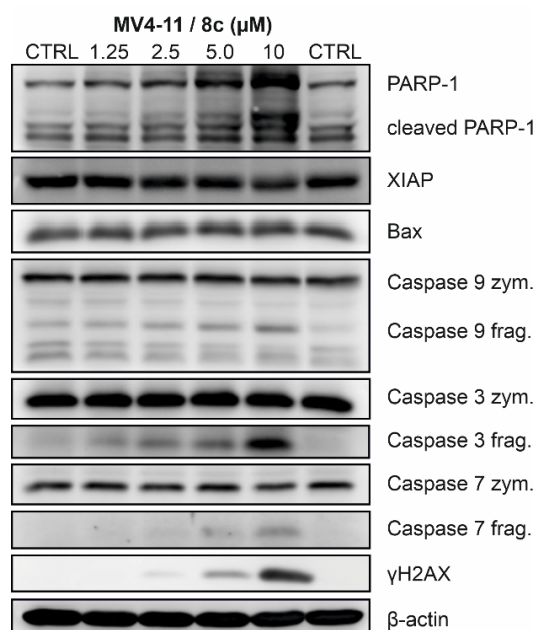


Figure 5. Effect of **8c** on the induction of cell death and DNA damage in MV4-11 cells treated for 24 h with indicated concentrations (μ M). Representative results from three independent experiments are presented; quantitative data are provided in the Supplementary Materials Information.

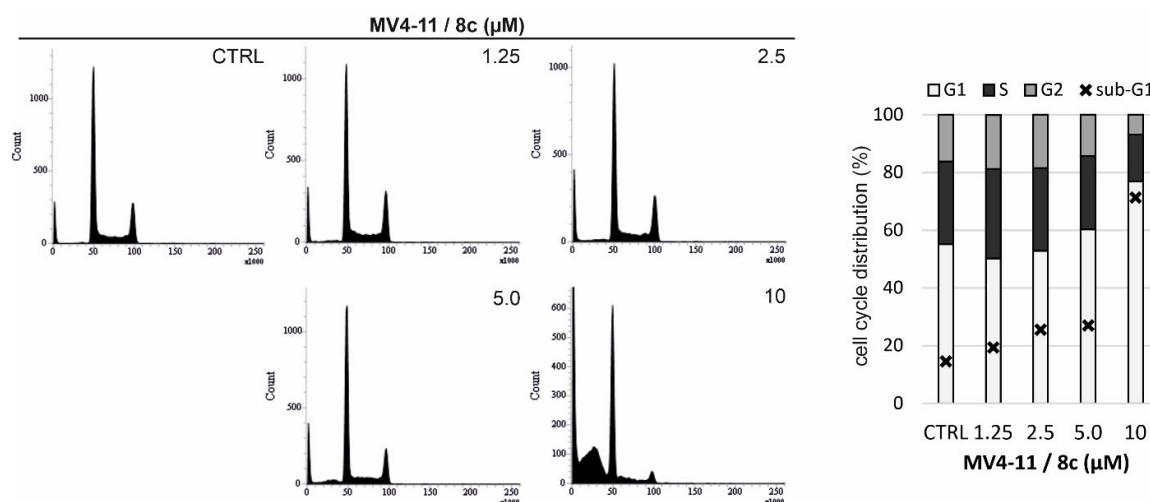


Figure 6. The cell cycle phases (%) in MV4-11 cells treated with the indicated concentrations (μ M) of compound **8c** for 24 h.

Treatment with **8c** efficiently induced the cleavage of initiator caspase-9, which was accompanied by cleavage of executioner caspases –3 and –7. Once activated, they cleave a variety of cellular substrates, including poly(ADP-ribose) polymerase 1 (PARP-1), thereby promoting commitment to cell death [66]. The presence of the cleaved 89 kDa fragment of PARP-1 clearly indicates a concentration-dependent progression of cell death in MV4-11 cells following **8c** treatment. This was further associated with a slight increase in the proapoptotic protein Bax and, conversely, a decrease in the antiapoptotic protein XIAP. In addition, **8c** induced phosphorylation of histone H2AX at serine 139 (γ -H2AX),

indicative of DNA damage, which is likely the trigger responsible for the initial activation of caspases [67,68] (Figure 5).

To further support these findings, flow cytometry analysis of cell cycle distribution was performed in MV4-11 cells treated with **8c** for 24 h, using propidium iodide staining. Treatment with **8c** resulted in a dose-dependent decrease in the S-phase cell population, accompanied by a reduction in the number of cells progressing to the G₂/M phase and a significant increase in the sub-G₁ population. These changes are indicative of replication stress during the S-phase and corroborate the strong induction of cell death previously observed by immunoblotting (Figure 6).

2.6. Topoisomerase Relaxation Assay

Finally, we sought to investigate the underlying mechanism responsible for the replication stress induced by compound **8c**, leading to cell death. Replication stress is often associated with inhibiting TOPs, enzymes essential for maintaining DNA topology during replication. Notably, several established TOP inhibitors, such as camptothecin (a TOPI inhibitor) and etoposide (a TOPII inhibitor), possess planar aromatic structures that facilitate DNA intercalation [44]. Given the planar aromatic character of the synthesized compound series, we hypothesized that **8c** might interfere with the activity of TOPI or TOPII.

To test this hypothesis, we performed topoisomerase relaxation assays to evaluate the inhibitory potential of **8c** on the ability of TOPI and TOPII α to relax supercoiled plasmid DNA. Reaction products were separated by agarose gel electrophoresis and visualized using GelRed nucleic acid stain. Camptothecin (CPT) and etoposide were employed as positive controls for TOPI and TOPII α inhibition, respectively. **8c** did not inhibit TOPI-mediated DNA relaxation; however, it impaired the relaxation activity of TOPII α in a concentration-dependent manner (Figure 7).

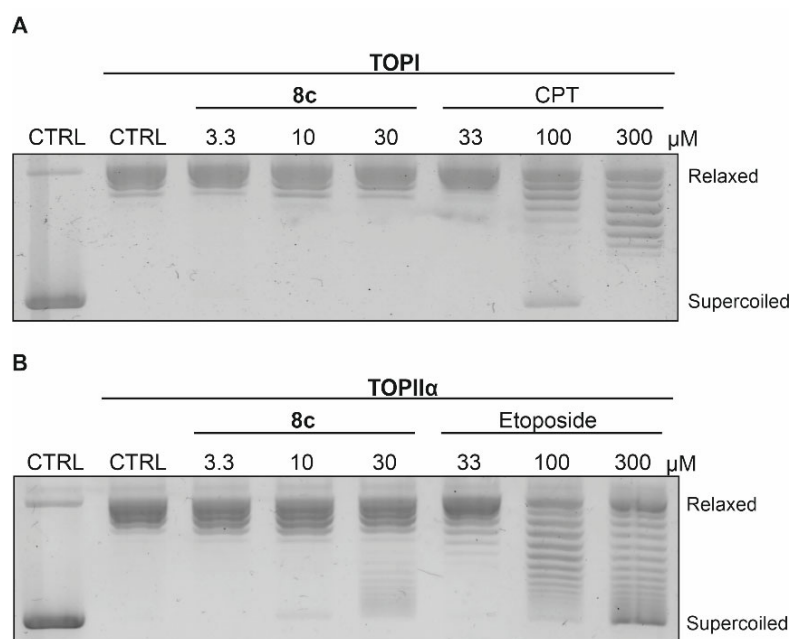


Figure 7. Effect of **8c** on the ability of (A) TOPI and (B) TOPII α to relax supercoiled plasmid DNA at the indicated concentrations using camptothecin (CPT) and etoposide as references.

2.7. Kinase Assays

The newly prepared pyrazolo [3,4-*b*]pyridines (**8b**, **8c**, **8e**, **8f**, **10b**, **10c**, **10e**, and **10f**) underwent further evaluation against a focused kinase panel (Abl, FLT3-ITD, and PDGFR) to assess off-target effects and confirm TOPII α selectivity. At concentrations up to 10 μ M,

3. Materials and Methods

3.1. Chemistry

3.1.1. General

Commercial suppliers provided all of the materials, which were used exactly as supplied. Silica gel plates (Merck KGaA, Darmstadt, Germany) were subjected to TLC in order to track the purity and development of the reaction. Uncorrected melting points were measured using a Stuart SMP30 apparatus (Cole-Parmer Ltd., Stone, Staffordshire, UK), and standard equipment was used for elemental analysis using a Vario EL III elemental analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany), FTIR using a PerkinElmer Spectrum Two FTIR spectrometer (Waltham, MA, USA), and NMR (^1H , ^{13}C , and DEPT-135) were obtained on a Bruker Avance III HD 400 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). A Bruker MicroTOF spectrometer was used to record high-resolution mass spectra (Bruker Daltonics, Billerica, MA, USA). As stated in the literature, starting ingredients **2** [54], **5** [55], and **9** [55] were synthesized. A ZORBAX Eclipse Plus C18 column (4.6×150 mm, $5 \mu\text{m}$; Agilent Technologies, Santa Clara, CA, USA) was used for HPLC analysis, which verified that the produced chemicals were more than 95% pure.

3.1.2. General Procedure for Synthesis of Pyrazolo [3,4-*b*]Pyridines (**8a–g**, **10a–g**, and **12**)

TLC was used to track the reaction progress of a combination of different aldehydes (**3a–g** and **11**) (0.0015 mole), equimolar quantities of 3-oxo-3-arylpropanenitriles (**2** and **4**) (0.0015 mole), and 3-substituted-1-phenyl-1*H*-pyrazol-5-amines (**5** and **9**) (0.0015 mole) that were refluxed in 20 mL of absolute ethanol for 12 h. The reaction mixture was finished and then left to cool to room temperature. Filtration, air drying, and recrystallization of the resultant precipitate from ethanol produced the required very pure pyrazolo [3,4-*b*]pyridine derivatives (**8a–g**, **10a–g**, and **12**).

3.1.3. 6-(1*H*-Indol-3-yl)-1,3,4-Triphenyl-1*H*-Pyrazolo [3,4-*b*]Pyridine-5-Carbonitrile (**8a**)

Yield (85%) as a white powder, with Mp: 245°C . HPLC: R_T 7.098 min (purity: 96.00%). ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ (ppm): 11.68 (d, $J = 2.9$ Hz, 1H, NH), 7.81–7.78 (m, 3H, Arom-H), 7.63–7.57 (m, 4H, Arom-H), 7.49–7.39 (m, 3H, Arom-H), 7.33–7.29 (m, 6H, Arom-H), 7.20–7.16 (m, 2H, Arom-H), 7.13 (tt, $J = 7.2, 2.3$ Hz, 2H, Arom-H). ^{13}C NMR (101 MHz, $\text{DMSO-}d_6$) δ (ppm): 149.7, 145.6, 145.3, 139.1, 138.2, 136.3, 134.5, 130.7, 129.8, 129.3, 129.1, 128.9, 128.3, 127.4, 127.2, 125.7, 125.2, 123.7, 122.4, 122.2, 121.4, 120.0, 111.8, 108.1, 99.4, 85.7. HRMS (ESI): m/z : $[\text{M} + \text{H}]^+$ calcd. 488.1870 and found 488.1862. Anal. Calcd. (Found) For $\text{C}_{33}\text{H}_{21}\text{N}_5$: C, 81.29 (81.15); H, 4.34 (4.35); N, 14.36 (14.29)%.

3.1.4. 4-(3-Hydroxyphenyl)-6-(1*H*-Indol-3-yl)-1,3-Diphenyl-1*H*-Pyrazolo [3,4-*b*]Pyridine-5-Carbonitrile (**8b**)

Yield (83%) as a white powder, with Mp: 232°C . HPLC: R_T 5.770 min (purity: 99.40%). ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ (ppm): 11.68 (d, $J = 2.9$ Hz, 1H, NH), 9.80 (s, 1H, OH), 7.81–7.78 (m, 3H, Arom-H), 7.72–7.54 (m, 7H, Arom-H), 7.21–7.07 (m, 5H, Arom-H), 6.83–6.74 (m, 2H, Arom-H), 6.71 (t, $J = 2.1$ Hz, 1H, Arom-H), 6.59 (dd, $J = 8.1, 2.4$ Hz, 1H, Arom-H). ^{13}C NMR (101 MHz, $\text{DMSO-}d_6$) δ (ppm): 158.5, 150.5, 144.8, 143.8, 139.3, 136.0, 134.1, 130.8, 129.7, 128.6, 128.1, 128.0, 127.3, 124.0, 121.5, 119.0, 115.2, 114.6, 112.9, 102.1. HRMS (ESI): m/z : $[\text{M} + \text{H}]^+$ calcd. 504.1819 and found 504.1810. Anal. Calcd. (Found) For $\text{C}_{33}\text{H}_{21}\text{N}_5\text{O}$: C, 78.71 (78.87); H, 4.20 (4.18); N, 13.91 (13.86)%.

3.1.5. 4-(4-Hydroxyphenyl)-6-(1H-Indol-3-yl)-1,3-Diphenyl-1H-Pyrazolo [3,4-b]Pyridine-5-Carbonitrile (8c)

Yield (89%) as a white powder, with Mp: 295 °C. HPLC: R_T 5.165 min (purity: 98.53%). ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 11.92 (s, 1H, NH), 9.85 (s, 1H, OH), 8.53–8.44 (m, 2H, Arom-H), 8.39–8.29 (m, 2H, Arom-H), 7.69–7.61 (m, 2H, Arom-H), 7.57 (dd, *J* = 8.3, 3.3 Hz, 1H, Arom-H), 7.49–7.43 (m, 1H, Arom-H), 7.31–7.25 (m, 2H, Arom-H), 7.23–7.11 (m, 7H, Arom-H), 6.71–6.56 (m, 2H, Arom-H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ (ppm): 159.2, 156.3, 153.7, 151.0, 147.5, 138.9, 136.9, 132.1, 131.5, 130.1, 129.7, 129.4, 128.5, 128.0, 127.4, 126.4, 124.8, 123.1, 122.3, 122.0, 121.4, 119.7, 115.2, 113.3, 112.7, 110.8, 100.3. ¹³C NMR-DEPT-135 (126 MHz, DMSO-*d*₆) δ: 131.5 (↑), 130.1 (↑), 129.7 (↑), 129.4 (↑), 128.5 (↑), 128.0 (↑), 127.4 (↑), 123.1 (↑), 122.3 (↑), 122.0 (↑), 121.4 (↑), 115.2 (↑), 112.7 (↑). HRMS (ESI): *m/z*: [M + H]⁺ calcd. 504.1819 and found 504.1809. Anal. Calcd. (Found) For C₃₃H₂₁N₅O: C, 78.71 (78.59); H, 4.20 (4.23); N, 13.91 (13.97)%.

3.1.6. 4-(2-Hydroxy-3-Methoxyphenyl)-6-(1H-Indol-3-yl)-1,3-Diphenyl-1H-Pyrazolo [3,4-b]Pyridine-5-Carbonitrile (8d)

Yield (85%) as a white powder, with Mp: 271 °C. HPLC: R_T 7.888 min (purity: 99.41%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 11.33 (s, 1H, NH), 9.41 (s, 1H, OH), 8.45–6.76 (m, 18H, Arom-H), 3.88 (s, 3H, OCH₃). ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm): 160.3, 153.2, 150.0, 149.7, 147.1, 143.0, 139.1, 138.3, 136.2, 130.5, 129.9, 129.8, 128.9, 127.8, 127.5, 126.9, 126.2, 122.4, 121.7, 120.5, 118.3, 116.9, 112.5, 112.0, 106.4, 102.5, 56.2. Anal. Calcd. (Found) For C₃₄H₂₃N₅O₂: C, 76.53 (76.38); H, 4.34 (4.30); N, 13.13 (13.09)%.

3.1.7. 4-(3-Hydroxy-4-Methoxyphenyl)-6-(1H-Indol-3-yl)-1,3-Diphenyl-1H-Pyrazolo [3,4-b]Pyridine-5-Carbonitrile (8e)

Yield (91%) as a white powder, with Mp: 266 °C. HPLC: R_T 7.413 min (purity: 96.44%). ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 11.92 (s, 1H, NH), 9.13 (s, 1H, OH), 8.48 (d, *J* = 7.9 Hz, 2H, Arom-H), 8.36–8.33 (m, 2H, Arom-H), 7.67–7.63 (m, 2H, Arom-H), 7.57 (d, *J* = 8.0 Hz, 1H, Arom-H), 7.48–7.45 (m, 1H, Arom-H), 7.28 (ddt, *J* = 8.1, 5.6, 1.9 Hz, 2H, Arom-H), 7.22 (td, *J* = 7.5, 1.2 Hz, 1H, Arom-H), 7.18–7.11 (m, 4H, Arom-H), 6.82 (d, *J* = 2.1 Hz, 1H, Arom-H), 6.78 (d, *J* = 8.3 Hz, 1H, Arom-H), 6.73 (dd, *J* = 8.2, 2.1 Hz, 1H, Arom-H), 3.77 (s, 3H, OCH₃). ¹³C NMR (126 MHz, DMSO-*d*₆) δ (ppm): 156.2, 153.4, 150.9, 149.3, 147.4, 146.8, 138.9, 136.9, 132.1, 130.1, 129.7, 129.2, 128.6, 127.9, 127.4, 126.9, 126.4, 123.1, 122.4, 122.0, 121.4, 119.5, 117.0, 113.3, 112.7, 112.2, 110.8, 100.3, 56.3. ¹³C NMR-DEPT-135 (126 MHz, DMSO-*d*₆) δ: 130.1 (↑), 129.7 (↑), 129.2 (↑), 128.6 (↑), 127.9 (↑), 127.4 (↑), 123.1 (↑), 122.4 (↑), 122.0 (↑), 121.4 (↑), 117.0 (↑), 112.7 (↑), 112.2 (↑), 56.3 (↑). HRMS (ESI): *m/z*: [M + H]⁺ calcd. 534.1925 and found 534.1922. Anal. Calcd. (Found) For C₃₄H₂₃N₅O₂: C, 76.53 (76.65); H, 4.34 (4.32); N, 13.13 (13.16)%.

3.1.8. 4-(4-Hydroxy-3-Methoxyphenyl)-6-(1H-Indol-3-yl)-1,3-Diphenyl-1H-Pyrazolo [3,4-b]Pyridine-5-Carbonitrile (8f)

Yield (87%) as a white powder, with Mp: 239 °C. HPLC: R_T 7.355 min (purity: 98.55%). ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 11.92 (d, *J* = 2.9 Hz, 1H, NH), 9.40 (s, 1H, OH), 8.49 (d, *J* = 2.7 Hz, 1H, Arom-H), 8.47 (d, *J* = 7.9 Hz, 1H, Arom-H), 8.36–8.31 (m, 2H, Arom-H), 7.68–7.63 (m, 2H, Arom-H), 7.57 (d, *J* = 8.0 Hz, 1H, Arom-H), 7.49–7.45 (m, 1H, Arom-H), 7.31–7.25 (m, 2H, Arom-H), 7.22 (td, *J* = 7.4, 1.2 Hz, 1H, Arom-H), 7.17 (d, *J* = 4.4 Hz, 4H, Arom-H), 7.05 (dd, *J* = 8.1, 2.1 Hz, 1H, Arom-H), 6.82 (d, *J* = 8.1 Hz, 1H, Arom-H), 6.74 (d, *J* = 2.0 Hz, 1H, Arom-H), 3.34 (s, 3H, OCH₃). ¹³C NMR (126 MHz, DMSO-*d*₆) δ (ppm): 156.4, 153.5, 151.0, 148.6, 147.5, 147.4, 138.9, 136.9, 132.4, 130.2, 129.7, 129.4, 128.6, 127.9, 127.4, 126.4, 125.0, 123.1, 123.0, 122.4, 122.0, 121.4, 119.8, 115.5, 114.9, 113.3, 112.7, 110.7, 100.2, 55.7. ¹³C NMR-DEPT-135 (126 MHz, DMSO-*d*₆) δ: 130.2 (↑), 129.7 (↑), 129.4 (↑), 128.6 (↑), 127.9 (↑),

127.4 (↑), 123.1 (↑), 123.0 (↑), 122.4 (↑), 122.0 (↑), 121.4 (↑), 115.5 (↑), 114.9 (↑), 112.7 (↑), 55.7 (↑). HRMS (ESI): m/z : $[M + H]^+$ calcd. 534.1925 and found 534.1912. Anal. Calcd. (Found) For $C_{34}H_{23}N_5O_2$: C, 76.53 (76.67); H, 4.34 (4.38); N, 13.13 (13.18)%.

3.1.9. 4-(3,4-Dimethoxyphenyl)-6-(1H-Indol-3-yl)-1,3-Diphenyl-1H-Pyrazolo [3,4-b]Pyridine-5-Carbonitrile (**8g**)

Yield (88%) as a white powder, with Mp: 228 °C. HPLC: R_T 10.632 min (purity: 99.55%). 1H NMR (500 MHz, $DMSO-d_6$) δ (ppm): 12.26 (s, 1H, NH), 8.44 (s, 2H, Arom-H), 8.22–8.18 (m, 4H, Arom-H), 7.81 (d, J = 2.2 Hz, 2H, Arom-H), 7.73 (dd, J = 8.5, 2.1 Hz, 2H, Arom-H), 7.56 (d, J = 7.1 Hz, 2H), 7.28 (td, J = 7.6, 1.5 Hz, 4H, Arom-H), 7.19 (d, J = 8.5 Hz, 2H, Arom-H), 3.88 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3). ^{13}C NMR (126 MHz, $DMSO-d_6$) δ (ppm): 181.9, 153.1, 152.9, 149.1, 137.1, 135.8, 126.7, 126.3, 125.4, 123.9, 122.8, 121.9, 119.1, 114.2, 113.3, 112.9, 112.3, 108.4, 56.3, 56.0. ^{13}C NMR-DEPT-135 (126 MHz, $DMSO-d_6$) δ : 152.9 (↑), 135.8 (↑), 126.3 (↑), 123.9 (↑), 122.8 (↑), 121.9 (↑), 113.3 (↑), 112.9 (↑), 112.3 (↑), 56.3 (↑), 56.0 (↑). HRMS (ESI): m/z : $[M + H]^+$ calcd. 548.2081 and found 548.2080. Anal. Calcd. (Found) For $C_{35}H_{25}N_5O_2$: C, 76.77 (76.91); H, 4.60 (4.57); N, 12.79 (12.89)%.

3.1.10. 3-(1H-Indol-3-yl)-1,4,6-Triphenyl-1H-Pyrazolo [3,4-b]Pyridine-5-Carbonitrile (**10a**)

Yield (86%) as a white powder, with Mp: 280 °C. HPLC: R_T 7.001 min (purity: 98.27%). 1H NMR (500 MHz, $DMSO-d_6$) δ (ppm): 11.22–11.19 (m, 1H, NH), 8.38 (d, J = 8.0 Hz, 2H, Arom-H), 8.20 (d, J = 7.7 Hz, 1H, Arom-H), 8.02 (dd, J = 6.6, 2.9 Hz, 2H, Arom-H), 7.68–7.61 (m, 6H, Arom-H), 7.59 (s, 1H, Arom-H), 7.56–7.53 (m, 2H, Arom-H), 7.48 (t, J = 7.6 Hz, 2H, Arom-H), 7.42 (t, J = 7.4 Hz, 1H, Arom-H), 7.35 (d, J = 7.8 Hz, 1H, Arom-H), 7.17–7.12 (m, 2H, Arom-H). ^{13}C NMR (126 MHz, $DMSO-d_6$) δ (ppm): 160.4, 153.1, 150.0, 142.8, 139.0, 138.2, 136.1, 135.0, 130.6, 130.5, 129.9, 129.9, 129.9, 129.0, 128.9, 127.3, 127.1, 126.2, 122.4, 121.8, 121.4, 120.5, 118.1, 112.4, 112.0, 106.3, 102.4. ^{13}C NMR-DEPT-135 (126 MHz, $DMSO-d_6$) δ : 130.6 (↑), 130.5 (↑), 129.9 (↑), 129.9 (↑), 129.9 (↑), 129.0 (↑), 128.9 (↑), 127.3 (↑), 127.1 (↑), 122.4 (↑), 121.8 (↑), 121.4 (↑), 120.5 (↑), 112.0 (↑). HRMS (ESI): m/z : $[M + H]^+$ calcd. 488.1870 and found 488.1868. Anal. Calcd. (Found) For $C_{33}H_{21}N_5$: C, 81.29 (81.42); H, 4.34 (4.37); N, 14.36 (14.41)%.

3.1.11. 4-(3-Hydroxyphenyl)-3-(1H-Indol-3-yl)-1,6-Diphenyl-1H-Pyrazolo [3,4-b]Pyridine-5-Carbonitrile (**10b**)

Yield (83%) as a white powder, with Mp: 247 °C. HPLC: R_T 5.736 min (purity: 99.79%). 1H NMR (500 MHz, $DMSO-d_6$) δ (ppm): 11.32 (d, J = 2.9 Hz, 1H, NH), 9.88 (s, 1H, OH), 8.40 (d, J = 8.0 Hz, 2H, Arom-H), 8.03–8.01 (m, 2H, Arom-H), 7.68–7.62 (m, 6H, Arom-H), 7.39–7.32 (m, 3H, Arom-H), 7.20–7.13 (m, 3H, Arom-H), 7.04–7.02 (m, 1H, Arom-H), 6.98 (d, J = 7.6 Hz, 1H, Arom-H), 6.96 (d, J = 2.1 Hz, 1H, Arom-H). HRMS (ESI): m/z : $[M + H]^+$ calcd. 504.1819 and found 504.1824. Anal. Calcd. (Found) For $C_{33}H_{21}N_5O$: C, 78.71 (78.90); H, 4.20 (4.19); N, 13.91 (13.94)%.

3.1.12. 4-(4-Hydroxyphenyl)-3-(1H-Indol-3-yl)-1,6-Diphenyl-1H-Pyrazolo [3,4-b]Pyridine-5-Carbonitrile (**10c**)

Yield (85%) as a white powder, with Mp: 259 °C. HPLC: R_T 5.054 min (purity: 99.32%). 1H NMR (500 MHz, $DMSO-d_6$) δ (ppm): 11.33 (d, J = 2.8 Hz, 1H, NH), 10.07 (s, 1H, OH), 8.38 (d, J = 8.1 Hz, 2H, Arom-H), 8.24–8.22 (m, 1H, Arom-H), 8.02–8.00 (m, 2H, Arom-H), 7.65–7.61 (m, 5H, Arom-H), 7.42 (s, 1H, Arom-H), 7.37 (dd, J = 8.6, 6.9 Hz, 3H, Arom-H), 7.20–7.09 (m, 3H, Arom-H), 6.86–6.83 (m, 2H, Arom-H). HRMS (ESI): m/z : $[M + H]^+$ calcd. 504.1819 and found 504.1817. Anal. Calcd. (Found) For $C_{33}H_{21}N_5O$: C, 78.71 (78.86); H, 4.20 (4.16); N, 13.91 (13.95)%.

3.1.13. 4-(2-Hydroxy-3-Methoxyphenyl)-3-(1H-Indol-3-yl)-1,6-Diphenyl-1H-Pyrazolo [3,4-b]Pyridine-5-Carbonitrile (**10d**)

Yield (90%) as a white powder, with Mp: 277 °C. HPLC: R_T 7.845 min (purity: 99.81%). ^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ (ppm): 11.67 (s, 1H, NH), 9.21 (s, 1H, OH), 8.27 (d, $J = 7.6$ Hz, 1H, Arom-H), 7.88 (d, $J = 2.5$ Hz, 1H, Arom-H), 7.81–7.71 (m, 3H, Arom-H), 7.60 (t, $J = 7.9$ Hz, 3H, Arom-H), 7.51–7.42 (m, 3H, Arom-H), 7.32–7.30 (m, 1H, Arom-H), 7.21–7.14 (m, 5H, Arom-H), 6.95 (s, 1H, Arom-H), 3.82 (s, 3H, OCH_3). ^{13}C NMR (101 MHz, $\text{DMSO-}d_6$) δ (ppm): 163.3, 150.1, 148.9, 148.8, 148.4, 139.4, 137.0, 129.6, 127.6, 125.4, 124.6, 124.5, 123.4, 122.1, 121.4, 120.2, 120.1, 119.7, 116.3, 112.2, 109.3, 91.9, 56.3. HRMS (ESI): m/z : $[\text{M} + \text{H}]^+$ calcd. 534.1925 and found 534.1918. Anal. Calcd. (Found) For $\text{C}_{34}\text{H}_{23}\text{N}_5\text{O}_2$: C, 76.53 (76.66); H, 4.34 (4.32); N, 13.13 (13.16)%.

3.1.14. 4-(3-Hydroxy-4-Methoxyphenyl)-3-(1H-Indol-3-yl)-1,6-Diphenyl-1H-Pyrazolo [3,4-b]Pyridine-5-Carbonitrile (**10e**)

Yield (93%) as a white powder, with Mp: 249 °C. HPLC: R_T 7.380 min (purity: 99.95%). ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ (ppm): 11.35 (d, $J = 2.9$ Hz, 1H, NH), 9.45 (s, 1H, OH), 8.39 (d, $J = 8.0$ Hz, 2H, Arom-H), 8.33–8.30 (m, 1H, Arom-H), 8.03–7.99 (m, 2H, Arom-H), 7.67–7.62 (m, 5H, Arom-H), 7.55–7.35 (m, 3H, Arom-H), 7.17 (tt, $J = 7.1, 5.4$ Hz, 2H, Arom-H), 7.05 (d, $J = 8.3$ Hz, 1H, Arom-H), 6.98 (d, $J = 2.2$ Hz, 1H, Arom-H), 6.95 (dd, $J = 8.2, 2.2$ Hz, 1H, Arom-H), 3.89 (s, 3H, OCH_3). ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) δ (ppm): 160.4, 153.3, 150.0, 149.7, 147.1, 143.0, 139.1, 138.3, 136.2, 130.6, 129.9, 129.9, 129.6, 129.0, 128.6, 127.8, 127.5, 127.0, 126.2, 122.4, 121.8, 121.7, 121.2, 120.6, 118.3, 116.9, 112.5, 112.4, 112.0, 106.4, 102.6, 56.2. ^{13}C NMR-DEPT-135 (126 MHz, $\text{DMSO-}d_6$) δ : 130.6 ($\uparrow$), 129.9 ($\uparrow$), 129.9 ($\uparrow$), 129.0 ($\uparrow$), 127.8 ($\uparrow$), 127.0 ($\uparrow$), 122.4 ($\uparrow$), 121.8 ($\uparrow$), 121.7 ($\uparrow$), 121.2 ($\uparrow$), 120.6 ($\uparrow$), 116.9 ($\uparrow$), 112.5 ($\uparrow$), 112.0 ($\uparrow$), 56.2 (OCH_3 $\uparrow$). HRMS (ESI): m/z : $[\text{M} + \text{H}]^+$ calcd. 534.1925 and found 534.1929. Anal. Calcd. (Found) For $\text{C}_{34}\text{H}_{23}\text{N}_5\text{O}_2$: C, 76.53 (76.38); H, 4.34 (4.28); N, 13.13 (13.08)%.

3.1.15. 4-(4-Hydroxy-3-Methoxyphenyl)-3-(1H-Indol-3-yl)-1,6-Diphenyl-1H-Pyrazolo [3,4-b]Pyridine-5-Carbonitrile (**10f**)

Yield (88%) as a white powder, with Mp: 276 °C. HPLC: R_T 7.283 min (purity: 99.42%). ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ (ppm): 11.34 (d, $J = 2.8$ Hz, 1H, NH), 9.61 (s, 1H, OH), 8.38 (d, $J = 8.3$ Hz, 2H, Arom-H), 8.17 (d, $J = 7.8$ Hz, 1H, Arom-H), 8.04–7.99 (m, 2H, Arom-H), 7.67–7.59 (m, 5H, Arom-H), 7.45–7.36 (m, 2H, Arom-H), 7.15 (dt, $J = 21.7, 7.2$ Hz, 2H, Arom-H), 7.05 (dt, $J = 8.1, 1.8$ Hz, 1H, Arom-H), 7.01 (d, $J = 1.7$ Hz, 1H, Arom-H), 6.88 (dd, $J = 8.0, 1.4$ Hz, 1H, Arom-H), 6.16 (t, $J = 2.0$ Hz, 1H, Arom-H), 3.38 (s, 3H, OCH_3). ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) δ (ppm): 160.6, 153.4, 150.1, 148.9, 147.7, 142.9, 139.0, 138.3, 136.1, 130.6, 130.0, 129.8, 128.9, 127.7, 127.0, 126.2, 125.1, 123.2, 122.3, 121.8, 121.2, 120.5, 118.6, 115.7, 114.9, 112.4, 111.9, 106.5, 102.5, 55.9. ^{13}C NMR-DEPT-135 (126 MHz, $\text{DMSO-}d_6$) δ : 130.6 ($\uparrow$), 130.0 ($\uparrow$), 129.8 ($\uparrow$), 128.9 ($\uparrow$), 127.7 ($\uparrow$), 127.0 ($\uparrow$), 123.3 ($\uparrow$), 122.3 ($\uparrow$), 121.8 ($\uparrow$), 121.2 ($\uparrow$), 120.5 ($\uparrow$), 115.7 ($\uparrow$), 114.9 ($\uparrow$), 111.9 ($\uparrow$), 55.9 (OCH_3 $\uparrow$). HRMS (ESI): m/z : $[\text{M} + \text{H}]^+$ calcd. 534.1925 and found 534.1920. Anal. Calcd. (Found) For $\text{C}_{34}\text{H}_{23}\text{N}_5\text{O}_2$: C, 76.53 (76.37); H, 4.34 (4.33); N, 13.13 (13.11)%.

3.1.16. 4-(3,4-Dimethoxyphenyl)-3-(1H-Indol-3-yl)-1,6-Diphenyl-1H-Pyrazolo [3,4-b]Pyridine-5-Carbonitrile (**10g**)

Yield (91%) as a white powder, with Mp: 284 °C. HPLC: R_T 9.795 min (purity: 99.32%). ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ (ppm): 11.30 (d, $J = 2.8$ Hz, 1H, NH), 8.38 (d, $J = 8.0$ Hz, 2H, Arom-H), 8.12 (d, $J = 7.9$ Hz, 1H, Arom-H), 8.05–8.00 (m, 2H, Arom-H), 7.69–7.61 (m, 5H, Arom-H), 7.41 (dd, $J = 20.8, 7.7$ Hz, 2H, Arom-H), 7.19–7.01 (m, 5H, Arom-H), 6.12 (d, $J = 2.7$ Hz, 1H, Arom-H), 3.85 (s, 3H, OCH_3), 3.36 (s, 3H, OCH_3). ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) δ (ppm): 160.6, 153.1, 150.7, 150.1, 148.7, 142.9, 139.0, 138.3, 136.1, 130.6, 130.0,

129.9, 129.0, 127.6, 127.1, 126.6, 126.2, 122.9, 122.3, 121.8, 121.1, 120.4, 118.5, 114.3, 112.5, 112.0, 111.9, 106.4, 102.4, 56.2, 55.7. ^{13}C NMR-DEPT-135 (126 MHz, $\text{DMSO}-d_6$) δ : 130.6 (↑), 130.0 (↑), 129.9 (↑), 129.8 (↑), 129.0 (↑), 127.6 (↑), 127.1 (↑), 122.9 (↑), 122.3 (↑), 121.8 (↑), 121.1 (↑), 120.4 (↑), 114.3 (↑), 112.0 (↑), 111.9 (↑), 56.2 (OCH_3 ↑), 55.7 (OCH_3 ↑). HRMS (ESI): m/z : $[\text{M} + \text{H}]^+$ calcd. 548.2081 and found 548.2074 Anal. Calcd. (Found) For $\text{C}_{35}\text{H}_{25}\text{N}_5\text{O}_2$: C, 76.77 (76.59); H, 4.60 (4.62); N, 12.79 (12.85)%.

3.1.17. 4-(1H-Indol-3-yl)-1,3,6-Triphenyl-1H-Pyrazolo [3,4-b]Pyridine-5-Carbonitrile (12)

Yield (89%) as a white powder, with Mp: 257 °C. HPLC: R_T 7.166 min (purity: 99.25%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ (ppm): 11.62 (d, J = 2.8 Hz, 1H, NH), 8.35–8.29 (m, 2H, Arom-H), 8.09–8.03 (m, 2H, Arom-H), 7.66–7.61 (m, 5H, Arom-H), 7.46–7.39 (m, 4H, Arom-H), 7.18–7.09 (m, 4H, Arom-H), 7.02–6.94 (m, 3H, Arom-H). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ (ppm): 161.4, 150.7, 147.7, 147.6, 138.8, 138.4, 136.2, 132.2, 130.6, 130.0, 129.8, 129.0, 128.9, 128.7, 127.7, 127.4, 126.2, 122.4, 122.3, 120.4, 120.0, 118.9, 112.7, 112.2, 109.0, 102.9. ^{13}C NMR-DEPT-135 (126 MHz, $\text{DMSO}-d_6$) δ : 130.6 (↑), 130.0 (↑), 129.8 (↑), 129.0 (↑), 128.9 (↑), 128.9 (↑), 128.7 (↑), 127.7 (↑), 127.4 (↑), 122.4 (↑), 122.3 (↑), 120.4 (↑), 120.0 (↑), 112.2 (↑). HRMS (ESI): m/z : $[\text{M} + \text{H}]^+$ calcd. 488.18697 and found 488.18698. Anal. Calcd. (Found) For $\text{C}_{33}\text{H}_{21}\text{N}_5$: C, 81.29 (81.44); H, 4.34 (4.32); N, 14.36 (14.41)%.

3.2. Biological Evaluation

3.2.1. In Vitro Antitumor Screening Against 60 Cancer Cell Lines

In accordance with the NCI's usual technique, the synthetic compounds' in vitro anticancer activity was assessed against a panel of 60 human tumor cell lines from nine different tissue types (Figures S67–S105). To evaluate each compound's potency and cytotoxic profile, key dose–response parameters, including GI_{50} , TGI, and LC_{50} , were computed [69–77], and detailed data are provided in the Supplementary Information.

3.2.2. Cell Lines

Recognized cell banks provided the MV4-11 and K562 cancer cell lines, which were then cultivated at 37 °C in a humidified CO_2 incubator under standard conditions in RPMI-1640 or DMEM medium supplemented with fetal bovine serum and antibiotics, and detailed data are provided in the Supplementary Materials Information.

3.2.3. Cell Viability Assay

In order to calculate GI_{50} values from dose–response curves using fluorescence measurements, cells were seeded in 96-well plates and treated with different doses of the test substances for 72 h. This was followed by a viability assessment based on resazurin, and detailed data are provided in the Supplementary Materials Information.

3.2.4. Flow Cytometry

The test substance was applied at different concentrations to asynchronously developing cells, which were then harvested after 24 h, fixed in ice-cold 70% ethanol, and incubated on ice for 30 min. Cell cycle distribution was examined by flow cytometry with a 488 nm laser after PBS washing and propidium iodide staining, and ModFit LT software (version 5.0.9) was used to quantify the results, and detailed data are provided in the Supplementary Materials Information.

3.2.5. Immunoblotting

RIPA buffer was used to generate cell lysates, and proteins were separated using SDS-PAGE before being transferred to nitrocellulose membranes. Following blocking, membranes were incubated with certain primary antibodies overnight at 4 °C. Peroxidase-

conjugated secondary antibodies were then added, and SuperSignal West Pico reagents and a LAS-4000 CCD camera were used for detection, and detailed data are provided in the Supplementary Materials Information.

3.2.6. Topoisomerase Relaxation Assay

Supercoiled pBR322 plasmid and the corresponding assay buffers were used for the Topoisomerase I and II α assays. The assays were then incubated for 30 min at 37 °C at 350 rpm. Complete experimental details are included in the Supplementary Materials Information. Reactions were halted, products were separated using 5% agarose gel electrophoresis, and they were then seen using GelRed staining and a FLA-7000 digital image analyzer.

3.2.7. Molecular Docking

The RCSB-PDB site was used to obtain the coordinates of TOPII (PDB: 3QX3 [78]). The three-dimensional structures of **8c** and the cocrystal ligand were prepared and optimized using Marvin Sketch [79] as part of the docking investigation. AutoDock Vina [80] was used to carry out docking operations. The active site's coordinates (x, y, z) were 32.9/95.4/50.8 with a size 24.5/20.2/17.5. The 2D schematic presentation and 3D visualization were created using the Discovery Studio 2021 client [81].

4. Conclusions

The synthesized pyrazolo [3,4-*b*]pyridine derivatives demonstrated antiproliferative activity, particularly against leukemia-derived cancer cell lines, with **8c** emerging as the most potent candidate. Detailed mechanistic studies revealed that **8c** induces DNA damage leading to cell death in MV4-11 cells, as evidenced by caspase activation, PARP-1 cleavage, and modulation of pro- and antiapoptotic proteins. Additionally, **8c** caused S-phase cell cycle perturbations indicative of replication stress and confirmed strong induction of cell death. Topoisomerase relaxation assays identified TOPII α as a potential molecular target, with **8c** inhibiting its activity in a concentration-dependent manner. These findings highlight **8c** as a promising lead compound for the further development of anticancer agents targeting TOPII α -associated pathways.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ph18111770/s1>, Figures S1–S37. NMR spectra of compounds **8a-g**, **10a-g** and **12**, Figures S38–S51. HRMS spectra of compounds **8a-c**, **8e-g**, **10a-g** and **12**, Figures S52–S66. HPLC spectrum of compounds **8a-g**, **10a-g** and **12**: title, Figures S67–S105. NCI charts of compounds **8a-g**, **10a-g** and **12**; Table S1. Kinases assays.

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