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Research paper

2,4-Diaryl-pyrimido[1,2-*a*]benzimidazole derivatives as novel anticancer agents endowed with potent anti-leukemia activity: Synthesis, biological evaluation and kinase profiling

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ABSTRACT

Acute myeloid leukemia (AML) stands as one of the most aggressive type of human cancer that can develop rapidly and thus requires immediate management. In the current study, the development of novel derivatives of pyrimido[1,2-*a*]benzimidazole (**5a-p**) as potential anti-AML agents is reported. The prepared compounds **5a-p** were inspected for their *in vitro* anti-tumor activity at NCI-DTP and subsequently **5h** was selected for full panel five-dose screening to assess its TGI, LC_{50} and GI_{50} values. Compound **5h** showed effective anti-tumor activity at low micromolar concentration on all tested human cancer cell lines with GI_{50} range from 0.35 to 9.43 μM with superior sub-micromolar activity towards leukemia. Furthermore, pyrimido[1,2-*a*]benzimidazoles **5e-l** were tested on a panel ofhuman acute leukemia cell lines, namely HL60, MOLM-13, MV4-11, CCRF-CEM and THP-1, where **5e-h** reached single-digit micromolar GI_{50} values for all the tested cell lines. All prepared compounds were first tested for inhibitory action against the leukemia-associated mutant FLT3-ITD, as well as against ABL, CDK2, and GSK3 kinases, in order to identify the kinase target for the herein described pyrimido[1,2-*a*]benzimidazoles. However, the examined molecules disclosed non-significant activity against these kinases. Thereafter, a kinase profiling on a panel of 338 human kinases was then used to discover the potential target. Interestingly, pyrimido [1,2-*a*]benzimidazoles **5e** and **5h** significantly inhibited BMX kinase. Further investigation for the effect on cell cycle of HL60 and MV4-11 cells and caspase 3/7 activity was also performed. In addition, the changes in selected proteins (PARP-1, Mcl-1, pH3-Ser10) associated with cell death and viability were analyzed in HL60 and MV4-11 cells by immunoblotting.

1. Introduction

Acute myeloid leukemia (AML) is a resistant and aggressive kind of hematological cancer with unmet therapeutic needs, in which hematopoietic progenitor cells in the myeloid lineage lack the ability to differentiate and ignore normal proliferation regulators [1]. Consequently, non-functional hematopoietic cells amass, causing AML symptoms like dyspnea, anemia, bleeding and serious infections [2]. In 2020, the estimations of the American Cancer Society for the newly diagnosed AML cases and fatalities in the USA 19940 and 11180, respectively [3], with the overall five-year survival percentage for AML patients being less than 50%. AML patients' genome sequencing revealed that mutations in Fms-like tyrosine kinase 3 (FLT3) are a prevalent hallmark in the disease [2,4].

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Targeted therapies against AML have still not led to significant clinical benefits, due to drug resistance and molecular heterogeneity [5]. Several genes are frequently altered in AML cells; among these, activating internal tandem duplication (ITD) mutations in Fms-like tyrosine kinase 3 (FLT3-ITD) are detected in about a quarter of AML patients [6].

The non-receptor bone marrow tyrosine kinase on chromosome X (BMX) is a hypoxia-inducible gene that leads to therapeutic resistance in AML by activating pro-survival signaling pathways [7,8]. BMX controls a variety of cellular functions, such as cell growth, differentiation, motility, and apoptosis. It can be activated downstream of PI3K by PH domain–mediated membrane targeting and SRC-mediated phosphorylation of a kinase domain tyrosine. A number of proteins, including TNFR2, PAK1, TP53, PIM1, and STAT3, as well as BAK, have been shown to be directly or indirectly regulated by BMX. Furthermore, BMX has been discovered to be overexpressed in a variety of cancer types suggesting that increased levels of BMX are associated with cancer cell survival.

Considering the potential pathological role of BMX in several malignancies, diverse small molecule inhibitors have been developed [9–11]. For example, BMX–IN–1 (Fig. 1) disclosed potent BMX inhibition (IC_{50} = 25 nM), as well as excellent anticancer activity towards prostate cancer [12]. In addition, CHMFL-BMX-078 (Fig. 1) emerged as a highly potent irreversible BMX kinase inhibitor with an IC_{50} value of 11 nM [13]. In addition, it was also reported to overcome the resistance of melanoma to vemurafenib by suppressing the AKT signaling pathway [14].

Pyrimido[1,2-*a*]benzimidazole is a type of tricyclic fused ring between benzimidazole and pyrimidine with a total of three nitrogen atoms. Literature surveying disclosed that several pyrimido[1,2-*a*] benzimidazole derivatives were reported for diverse pharmacological activities, such as anti-inflammatory [15,16], anti-microbial [17,18], anti-viral [19–21], anti-neurodegenerative [22], anti-hypertensive [23, 24], CRF_1 receptor antagonist [25], and anti-cancer activities [26]. Specifically, benzimidazole derivative **I** [27] (Fig. 1) has been reported to exhibit high cytotoxicity against leukemia cells with GI_{50} values of 1.15–7.33 μM. Another report published by Gowda et al. [28] evaluated the anti-cancer activity of benzimidazole-5-carboxylic acid derivatives **II** (Fig. 1), which showed anti-leukemic activities in the range of 3–240 μM. Benzimidazolyl isoxazole-4-carboxamide derivatives **III** (Fig. 1) were reported to be selective FLT3 inhibitors with IC_{50} range of 0.495–13.4 μM [29]. Furthermore, the pyrazolo[1,5-*a*]pyrimidine bearing benzothiazole moiety **IV** showed potent anti-leukemic activity against CCRF-CEM cell line with an GI_{50} of 16.34 μM [30]. Owing to the promising anticancer activity profiles of these motifs, we proposed to combine the two benzimidazole and imidazo[1,2-*a*]pyrimidine pharmacophores into a single tricyclic entity affording the target 2,4-disubstituted pyrimido[1,2-*a*]benzimidazoles **5a-p** as potential anti-cancer molecules (Fig. 1).

All synthetized pyrimido[1,2-*a*]benzimidazoles **5a-p** were evaluated for their anti-cancer activity at the USA-NCI, then the IC_{50} values were determined against a panel of 5 human acute leukemia cell lines, namely HL60, MOLM-13, MV4-11, CCRF-CEM and THP-1. Furthermore, they were tested for inhibitory action against the leukemia-associated mutant FLT3-ITD, as well as against in-house ABL, CDK2, and GSK3 kinases, in order to identify their kinase target. Thereafter, the kinase profiling service at Reaction Biology Corporation, over a panel of 338 human kinases, was exploited to discover the potential target. Further investigation for the effect on cell cycle of HL60 and MV4-11 cells and caspase 3/7 activity was also performed. In addition, the changes of selected proteins (PARP-1, Mcl-1, pH3-Ser10) related to cell death and viability were analyzed in HL60 and MV4-11 cells by immunoblotting.

2. Results and discussion

2.1. Chemistry

Scheme 1 shows the route for preparing pyrimido[1,2-*a*]benzimidazole **5a-p** using the proposed metal-free cascade process. In the

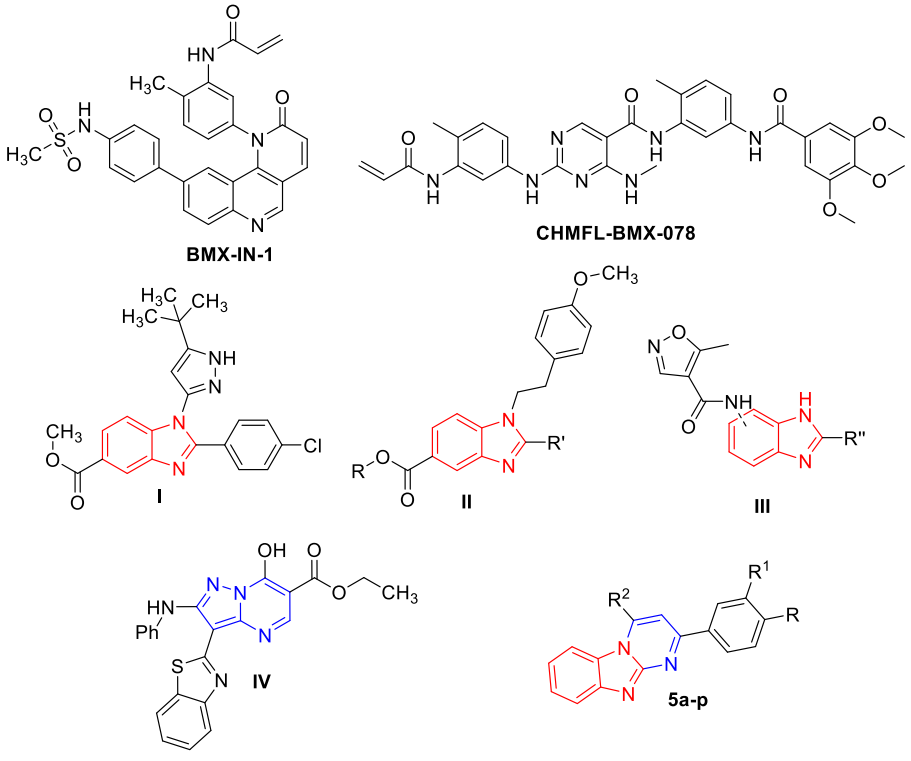
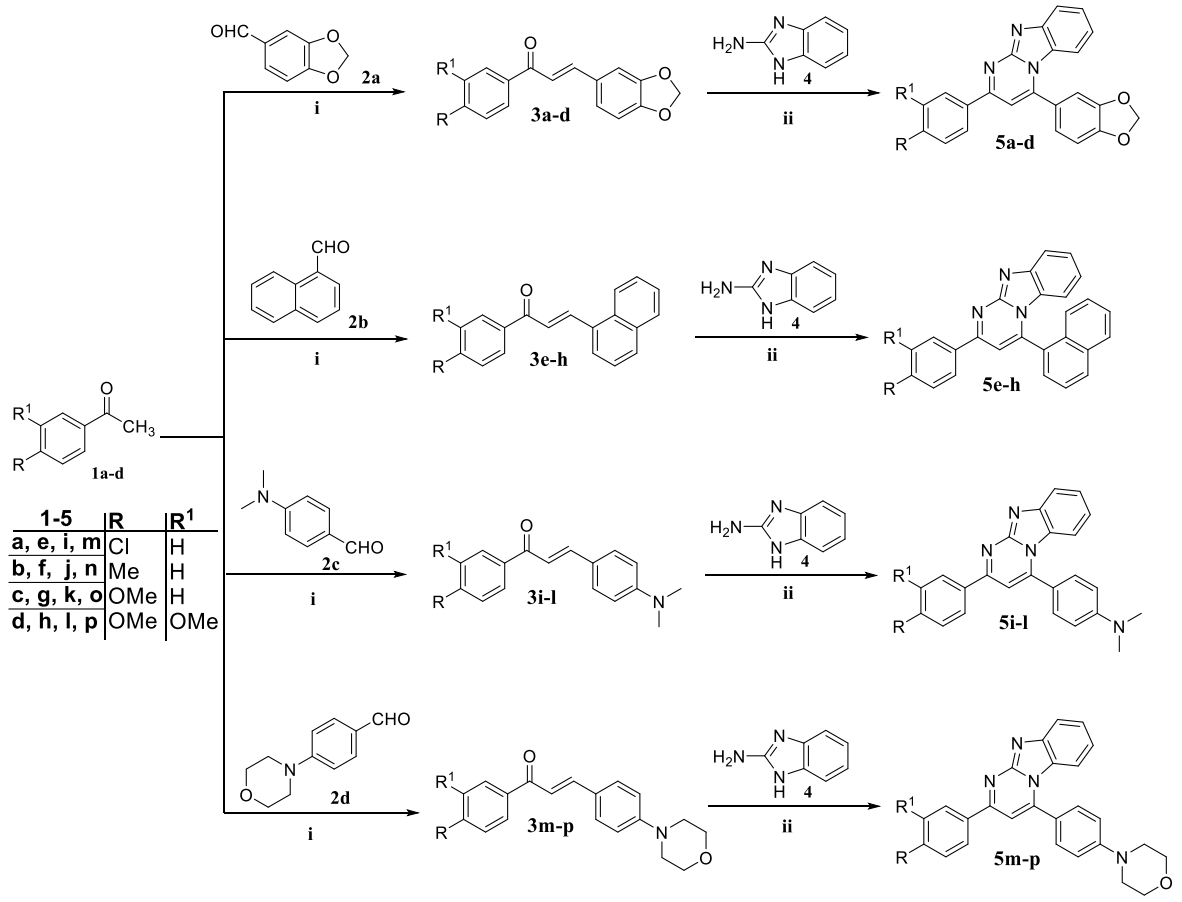


Fig. 1. Structure of BMX inhibitors (BMX–IN–1 and CHMFL-BMX-078), benzimidazole-based anti-leukemic agents (**I-IV**), and the proposed 2,4-disubstituted pyrimido[1,2-*a*]benzimidazole **5a-p** (R′ and R’ represent different substituents).



Scheme 1. Synthetic pathway of targeted pyrimido[1,2-*a*]benzimidazoles **5a-p**.
Reagents and conditions: i) KOH, CH_3OH, rt, 24 h; **ii**) NaOH, DMF, reflux, 24 h.

presence of a base, acetophenone derivatives **1a-d** and benzaldehyde derivatives **2a-d** undergo an aldol reaction, yielding the *α*,*β*-unsaturated carbonyl intermediates **3a-p**. When intermediates **3a-p** are condensed with 2-aminobenzimidazole **4**, and one water molecule is removed, an imine is formed which in turn is cyclized and auto-oxidized, resulting in the production of pyrimido[1,2-*a*]benzimidazoles **5a-p**.

The structure of sixteen pyrimido[1,2-*a*]benzimidazoles synthesized in this study **5a-p** was confirmed using ^{1}H NMR, ^{13}C NMR, ESI-MS and elemental analysis. Spectra can be found in the Supplementary Materials.

2.2. Biological evaluation

2.2.1. Screening on NCI cancer cells

Following National Cancer Institute (NCI) protocol, sixteen pyrimido [1,2-*a*]benzimidazoles **5a-p** have been evaluated for their possible *in vitro* anti-tumor effect on 58 human cancer cell lines representing melanoma, leukemia, prostate, CNS, breast, NSCLC, colon, ovarian and renal cancers at the NCI-USA [31].

2.2.2. Preliminary one dose screening

The antiproliferative action of pyrimido[1,2-*a*]benzimidazoles **5a-p** was initially evaluated using the NCI's default one-dose SRB assay at 10 μM. According to the SRB assay outcomes, the newly prepared pyrimido [1,2-*a*]benzimidazoles exerted weak to remarkable anticancer action against almost all of the evaluated cells.

Evaluating the attained average percentage inhibition of growth (GI %) values (Table 1) showed that naphthyl-bearing pyrimido[1,2-*a*] benzimidazole **5h** is the highly efficient antitumor agent among the herein prepared molecules (mean GI % = 65). The results of the NCI evaluation uncovered the efficacy of **5h** as anti-proliferative agent on 57 human cancer types, implying a broad-spectrum of action. Remarkably, **5h** displayed superior cytotoxic activity with GI % greater than 80% against leukemia (CCRF-CEM, MOLT-4), non-small cell lung (HOP-92, NCI–H226, NCI–H460), CNS (SF-295, SNB-19), melanoma (MALME-3M, SK-MEL-5, UACC-62), renal (786-0, ACHN, CAKI-1, SN 12C), prostate (DU-145), and breast cancer cell lines (MCF7, T-47D). In turn, **5h** was found to be lethal towards leukemia (HL-60, RPMI-8226), and non-small cell lung (HOP-62) cancer cell lines (GI % = 107, 118, and 101 respectively). Moreover, the naphthyl-bearing analogue **5e** (mean GI % = 42) exerted potent cytotoxic activity concerning all leukemia (except K-562 and RPMI-8226), non-small cell lung (A549, NCI–H460), colon (HCT-116), CNS (U251), melanoma (MALME-3M), renal (ACHN, CAKI-1, UO-31), and breast cancer cell lines (MCF7, T-47D) (Table 1).

In addition, the methylated analogues **5f** and **5j** displayed generally moderate antitumor activity with mean GI % = 41 and 40, respectively. The naphthyl-bearing analogue **5f** showed potent cytotoxic action towards all leukemia (excluding K-562 and RPMI-8226), breast (MCF7), melanoma (MALME-3M), renal (ACHN, CAKI-1, UO-31), and non-small cell lung cancer (A549, NCI–H460). In addition, compound **5j** exerted potent cytotoxic activities against all leukemia (K-562, MOLT-4, RPMI-8226), non-small cell lung (HOP-92, NCI–H522), colon (HCT-116), melanoma (UACC-62, SK-MEL-2), breast (T-47D), and renal cancer cell lines (A498). The results also revealed a lethal effect of **5j** against breast cancer (HS T578) and melanoma cell lines (LOX IMVI).

The methoxylated analogues **5g** and **5k** described more potent antitumor action, with an average GI of 55% and 47%, compared with their methylated analogues **5f** and **5j**. Compound **5g** displayed high cytotoxic

Table 1
Percentage growth inhibition of *in vitro* 58 human cancer cell lines treated at one dose of 10 μM for each compound **5a-p**.

Cancer type/cells	Compound[a]															
	5a	5b	5c	5d	5e	5f	5g	5h	5i	5j	5k	5l	5 m	5n	5o	5p
Leukemia																
MOLT-4	27	37	28	29	87	95	97	92	22	70	79	37	–	–	–	31
K-562	20	13	23	21	40	48	62	67	81	63	76	40	–	10	–	42
RPMI-8226	15	11	24	34	31	34	67	118	15	45	75	27	–	–	–	36
CCRF-CEM	10	16	16	32	74	62	90	96	–	61	86	40	–	–	–	48
HL-60(TB)	16	21	23	26	65	92	101	107	13	32	81	31	–	–	–	28
Non-Small Cell Lung Cancer																
NCI–H23	–	–	–	–	25	17	37	55	–	16	22	–	–	–	–	–
EKVX	–	17	14	–	26	21	25	30	–	42	43	–	–	–	–	11
NCI–H322 M	–	–	–	–	19	–	26	24	–	42	39	–	–	–	–	14
HOP-92	–	32	36	43	42	28	61	88	15	74	82	12	–	–	–	12
NCI–H226	12	17	13	–	38	42	50	80	–	–	17	–	–	–	–	–
A549/ATCC	12	14	20	10	75	60	70	75	–	42	32	–	–	–	–	–
NCI–H522	24	20	–	–	25	23	49	51	–	78	105	75	27	10	33	104
NCI–H460	–	13	–	10	78	78	86	96	–	38	46	–	–	–	–	13
HOP-62	–	10	–	–	51	32	74	101	15	43	61	14	–	–	–	17
Colon Cancer																
HT29	–	–	–	–	33	26	66	78	–	21	37	–	–	–	–	–
SW-620	–	17	–	–	48	54	59	70	10	43	49	12	–	–	–	16
HCT-116	–	14	11	14	74	56	62	68	–	60	84	27	–	–	–	–
HCT-15	10	15	13	10	48	52	57	72	–	19	23	–	–	–	–	–
COLO 205	–	–	–	–	24	40	25	34	–	34	45	–	–	–	–	–
KM 12	–	–	–	–	–	21	37	31	–	19	15	–	–	–	–	–
HCC-2998	–	–	–	–	–	–	11	27	–	–	40	–	–	–	–	–
CNS Cancer																
SNB-19	10	19	15	12	38	46	60	83	10	49	32	12	–	–	–	14
U251	–	–	10	–	65	51	66	79	–	–	–	–	–	–	–	16
SF-539	–	–	–	–	41	50	54	68	–	10	17	10	–	–	–	23
SNB-75	–	17	–	–	49	53	64	59	–	43	59	10	–	–	–	13
SF-295	–	–	11	–	55	59	79	97	–	11	23	16	–	–	–	–
SF-268	–	21	25	11	23	33	53	64	–	44	51	–	–	–	–	–
Melanoma																
MDA-MB-435	11	17	–	–	27	35	46	52	–	84	103	–	–	–	–	–
UACC-257	–	–	–	–	43	24	31	39	–	–	–	–	–	–	–	–
M14	–	–	–	–	49	38	47	63	11	64	51	–	–	–	–	–
SK-MEL-28	–	–	–	–	24	22	29	48	–	34	25	–	–	–	–	–
SK-MEL-2	17	–	–	11	–	–	20	–	–	34	42	–	–	–	–	–
MALME-3M	–	–	–	–	67	67	67	83	–	47	22	–	–	–	–	–
SK-MEL-5	55	38	45	75	55	59	69	82	–	16	33	10	–	–	–	21
UACC-62	27	27	17	–	53	56	33	80	–	22	70	16	–	–	–	–
LOX IMVI	24	–	16	–	44	43	54	69	11	137	96	45	–	–	–	44
Ovarian Cancer																
OVCAR-5	–	–	–	–	12	–	18	47	11	45	60	12	–	–	–	17
SK-OV-3	–	–	–	–	17	–	41	64	–	–	–	–	–	–	–	–
OVCAR-4	–	–	11	–	17	20	35	28	–	39	43	–	–	–	–	12
NCI/ADR-RES	–	13	–	–	32	32	45	52	–	18	44	–	–	–	–	–
OVCAR-8	–	19	10	–	57	41	57	65	–	–	45	12	–	–	–	11
OVCAR-3	–	10	–	–	–	20	38	33	–	43	40	–	–	–	–	12
IGROV1	–	10	13	–	43	40	51	47	–	13	22	–	–	–	–	–
Renal Cancer																
CAKI-1	34	41	40	34	72	76	78	82	31	40	38	45	–	12	–	49
UO-31	22	28	29	38	67	62	71	70	47	84	48	39	–	24	34	37
ACHN	11	13	15	–	72	75	80	91	29	58	64	48	–	18	–	60
TK-10	–	–	–	–	20	30	57	46	11	38	59	24	–	–	–	–
SN 12C	–	10	16	–	45	38	63	89	–	37	35	–	–	–	–	21
A498	56	46	42	48	49	47	58	61	–	50	36	–	–	–	–	–
786–0	13	–	13	18	52	52	63	89	17	19	57	24	–	–	–	15
Prostate Cancer																
DU-145	–	–	–	–	20	30	62	84	–	12	26	–	–	–	–	–
PC-3	20	22	32	–	47	39	64	69	16	52	71	26	–	–	–	26
Breast Cancer																
MDA-MB-468	31	36	10	–	23	29	47	36	–	16	17	–	–	–	–	–
T-47D	32	17	27	40	61	45	75	80	–	33	24	14	–	–	–	29
Hs 578T	–	–	–	–	37	43	45	53	–	101	49	–	–	–	–	–
BT-549	–	–	–	17	37	30	30	65	18	40	59	23	–	–	–	–
MCF7	21	28	25	16	80	77	80	84	35	93	97	27	–	–	–	17
MDA-MB-231	15	25	26	21	26	26	21	33	–	17	59	43	–	–	–	17
GI % Mean	10	13	11	–	42	41	55	65	–	40	47	13	–	–	–	15
Sensitive Cell lines	25	35	32	22	54	53	58	57	19	52	55	29	1	5	2	31

[a] Values higher than 10% are only displayed.

activity on all leukemia types, non-small cell lung (A549, HOP-62, NCI–H460, HOP-92), melanoma (MALME-3M, SK-MEL-5), and breast cancers (MCF7, T-47D), and all CNS (except SF-268 and SF-539), colon (HCT-116, HT29), renal (except A498 and TK-10), and prostate cancer cell lines. Similarly, compound **5k** exhibited potent anti-proliferative actions against all leukemia types, colon (HCT-116), ovarian (OVCAR-8), non-small cell lung (A549, NCI–H522), melanoma (M14 and SK-MEL-5), breast (T-47D), prostate (PC-3), and renal cancer cell lines (UO-31). In turn, compound **5g** exerted a lethal effect against leukemia cell line HL-60, whereas **5k** was lethal for melanoma MDA-MB-435, and non-small cell lung cancer cell line NCI–H522.

On the other hand, analogues **5d**, **5i**, **5m**, **5n** and **5o** did not display any significant cytotoxic activity on the assessed cancer cells as their average GI % was lower than ten. Compounds **5a** (mean GI = 10%), **5b** (13%), **5c** (11%), **5l** (13%), and **5p** (15%) displayed moderate sensitivity against few representative cancer cells following NCI standards (40% or less decrease in proliferation on any type of cancer cells) (Table 1).

It is worthy to emphasize that the type of substitution at C-4 of the pyrimido[1,2-*a*]benzimidazole scaffold is a crucial element for the anticancer activity of the molecules **5a-p**. Introduction of a 4-morpholinophenyl group led to compounds (**5m-o**), which exerted only negligible growth inhibition against the 58 tested cell lines, with average GI value less than 1%, except **5p,** which showed modest potency (GI < 15%). Grafting a benzodioxole led to compounds **5a-d**, which displayed weak or non-significant anti-proliferative actions (mean GI = 10, 13, 11 and 0%, respectively) (Table 1). Moreover, decoration of the pyrimido[1,2-*a*]benzimidazole motif with a *N*,*N*-dimethylamino phenyl substituent at C-4 enhanced antiproliferative activity, but only when a 4-methylphenyl or a 4-methoxyphenyl group is present in position 2 (**5j** and **5k** with GI of 40% and 47%, respectively). Finally, introduction of a 1-naphthyl moiety at C-4 afforded the most potent pyrimido[1,2-*a*]benzimidazoles in this work (**5e-h**), with average GI of 42, 41, 55 and 65%, respectively (Table 1). A comparison of 4-naphthylpyrimido[1,2-*a*]benzimidazoles bearing different substituents in position 2 showed that the strongest growth inhibition is obtained when a 3,4-dimethoxyphenyl group is present (**5h**, mean GI of 65%).

2.2.3. *In vitro five dose assay on full NCI panels*

The preliminary one dose assay findings showed that **5h** was the most effective anti-tumor agent in this study, with encouraging inhibitory action on an assortment of cancer types from various subpanels (Table 1). Pyrimido[1,2-*a*]benzimidazole **5h** has been selected for further biological examination at five-dose assay (0.01–100 μM)and GI_{50}, TGI, and LC_{50} values were attained for the examined cancer cell lines. TGI denotes the value of cytostatic effect, reflecting the level of growth inhibition by GI_{50} values. LC_{50} reflects the cytotoxic activity of examined hits.

As shown in Table 2, compound **5h** showed high anti-proliferative activity at low micromolar concentration on almost all evaluated human cancer types with GI_{50} values of 0.35–9.43 μM. Interestingly, **5h** exhibited superior sub-micromolar activity against leukemia (MOLT-4, HL-60, SR, CCRF-CEM), CNS (SF-295, SNB-75), colon (COLO 205, HCT-15, SW-620), melanoma (LOX IMVI, MALME-3M, SK-MEL-5), non-small cell lung (A549, HOP-62, NCI–H460), renal cancer (A498, ACHN, CAKI-1, UO-31), and breast cancer cell lines (MCF7) with GI_{50} value range between 0.35 and 0.97 μM. In addition, **5h** demonstrated high cytostatic action at low micromolar concentration (TGI values 1.75–9.24 μM) towards 17 cancer types among the herein evaluated cell lines, except ovarian and prostate cancer subpanels (Table 2). It demonstrated cytostatic action against non-small cell lung (A549), melanoma (MALME-3M, UACC-257), colon (HCT-116, HT29), ovarian cancer cell lines (SK-OV-3) with TGI ranging from 15.2 to 49.0 μM. Compound **5h** showed no cytostatic effect against the left-over cancer cells (TGI >100 μM).

Conversely, molecule **5h** appeared as a non-lethal agent, which has LC_{50} greater than 100 μM for most of the examined cancer cells,

Table 2
In vitro five-dose assay of compound **5h** (NSC: 830655) at NCI-USA.

Cancer type/cells	Compound 5h (NSC 830655)		
	GI_{50} (μM)	TGI (μM)	LC_{50} (μM)
Leukemia			
MOLT-4	**0.35**	100 <	100 <
RPMI-8226	1.34	**4.29**	100 <
K-562	1.32	100 <	100 <
SR	**0.36**	100 <	100 <
CCRF-CEM	**0.55**	100 <	100 <
HL-60(TB)	**0.41**	**3.29**	100 <
Non-Small Cell Lung Cancer			
HOP-92	1.65	**7.99**	100 <
NCI–H226	1.16	**6.70**	100 <
NCI–H522	2.75	100 <	100 <
NCI–H322 M	7.36	100 <	100 <
NCI–H460	**0.35**	**1.75**	**7.76**
NCI–H23	1.92	100 <	100 <
EKVX	2.79	100 <	100 <
HOP-62	**0.65**	**4.51**	100 <
A549/ATCC	**0.58**	15.2	100 <
Colon Cancer			
KM 12	9.43	100 <	100 <
SW-620	**0.78**	100 <	100 <
HT29	1.91	28.2	100 <
HCT-15	**0.92**	100 <	100 <
COLO 205	**0.62**	100 <	100 <
HCC-2998	1.62	**4.73**	100 <
HCT-116	1.66	49.0	100 <
CNS Cancer			
SNB-75	**0.86**	**4.60**	100 <
U251	1.16	100 <	100 <
SF-539	1.02	**7.67**	100 <
SNB-19	2.59	100 <	100 <
SF-295	**0.58**	**5.33**	100 <
SF-268	1.95	100 <	100 <
Melanoma			
MDA-MB-435	2.72	100 <	100 <
UACC-62	1.03	**7.20**	**65.3**
M14	2.08	100 <	100 <
UACC-257	2.14	40.3	100 <
SK-MEL-5	**0.96**	**2.84**	**8.23**
SK-MEL-28	2.88	100 <	100 <
LOX IMVI	**0.97**	**6.65**	**77.0**
MALME-3M	**0.88**	16.7	100 <
SK-MEL-2	2.57	**7.47**	**36.5**
Ovarian Cancer			
IGROV1	1.48	100 <	100 <
OVCAR-4	4.77	100 <	100 <
OVCAR-5	3.03	100 <	100 <
OVCAR-8	1.06	100 <	100 <
NCI/ADR-RES	2.07	100 <	100 <
SK-OV-3	2.55	44.9	100 <
Renal Cancer			
786-0	1.57	100 <	100 <
A498	**0.65**	NT	100 <
ACHN	**0.46**	**3.35**	100 <
CAKI-1	**0.55**	100 <	100 <
RXF 393	2.75	100 <	100 <
SN 12C	1.33	100 <	100 <
TK-10	2.47	100 <	100 <
UO-31	**0.67**	100 <	100 <
Prostate Cancer			
PC-3	2.50	100 <	100 <
DU-145	2.16	NT	100 <
Breast Cancer			
MCF7	**0.50**	100 <	100 <
MDA-MB-231	1.52	100 <	100 <
HS 578T	1.42	100 <	100 <
BT-549	2.27	**6.09**	100 <
T-47D	1.73	**9.24**	100 <
MDA-MB-468	2.70	100 <	100 <

[NT] Not tested.

excluding non-small cell lung (NCI–H460), and melanoma cell lines (LOX IMVI, SK-MEL-2, SK-MEL-5) (LC_{50} = 7.76, 77.0, 36.5, 8.23, and 65.3 μM, respectively (Table 2).

Regarding the sensitivity towards various tumor cell lines, pyrimido[1,2-*a*]benzimidazole **5h** owned potent growth inhibitory activity against the whole NCI panel, with a median GI_{50} against the full panel (MG-MID) of 1.81 μM, and effective sub-panel median GI_{50} (MG-MID) values between 0.72 and 2.49 μM. Leukemia sub-panel was the most susceptible cancer type to **5h** [GI_{50} (MG-MID) = 0.72 μM] (Table 3). Moreover, the ratio of the MG-MID of the full-panel to its individual sub-panels offers the selectivity index. Notably, **5h** showed the best selectivity index (2.51) for the leukemia cell sub-panel (Table 3).

2.2.4. *In vitro activity against leukemia cell lines*

As mentioned above, all prepared pyrimido[1,2-*a*]benzimidazoles **5a-p** were evaluated by the NCI-USA for their potential anti-cancer activities. The screening outputs demonstrated that C-4 substitution of pyrimido[1,2-*a*]benzimidazole scaffold with either 1-naphthyl or *N*,*N*-dimethylamino phenyl moiety is a crucial element for anti-tumor activities of the target compounds that resulted in the most potent molecules in this work (**5e-l**). In addition, leukemia was the most susceptible cancer subpanel to the synthesized pyrimido[1,2-*a*]benzimidazoles, as evidenced by the values of GI% (Table 1), as well as GI_{50} (MG-MID) for compound **5h** that equals 0.72 μM in the five-dose assay (Table 3).

Hence, compounds **5e-l** were tested toward a panel of 5 human acute leukemia cell lines; namely HL60, MOLM-13, MV4-11, CCRF-CEM and THP-1, and their GI_{50} values were showed in Table 4. In agreement with NCI results, the naphthyl-bearing derivatives **5e-h** exerted much pronounced anti-leukemic activity than dimethylamino phenyl-bearing members **5i-l**, that displayed no or only weak cytotoxicity across tested cell lines.

Regarding the anti-leukemic activity of **5e-h**, they reached single-digit micromolar GI_{50} values for all investigated acute leukemia cell lines. In accordance with previous results from NCI screen, **5h** showed to be the most potent compound in this series. In details, compound **5h** showed sub-micromolar to low single single-digit micromolar GI_{50} values of 0.8, 0.9, 1.2, 2.9 and 2.8 μM against MV4-11, MOLM-13, HL60, CCRF-CEM and THP-1, respectively (Table 4).

2.2.5. *Cell cycle effects*

Next, we examined the effect of pyrimido[1,2-*a*]benzimidazoles **5e-l** on the cell cycle of HL60 and MV4-11 cells, as they were one of the most sensitive cell lines to the tested compounds (Supplementary Figs. S1 and S2). Asynchronously growing cells were treated for 24 h, stained by propidium iodide and subsequently analyzed using flow cytometry.

Derivatives **5e-h** induced G2/M block of cell cycle with pronounced cytotoxic effect in HL60, whereas **5i-l** treatment was followed by G1 arrest, which was, on the other hand, strongest in MV4-11 cell line. Compound **5h**, as the most active, was selected for further experiments in an expanded time and concentration range. As shown in Fig. 2, both cell lines were blocked in the G2/M phase, with an obvious increase in cell death, especially in HL60 cells.

Table 3
Median GI_{50} values (μM) for compound **5h** on cancer cells.

Subpanel tumor cell line	Compound 5h (NSC 830655)	
	MG-MID	Selectivity index
Leukemia	0.72	2.51
Non-Small Cell Lung Cancer	2.13	0.85
Renal Cancer	1.31	1.38
CNS Cancer	1.36	1.33
Breast Cancer	1.69	1.07
Melanoma	1.80	1.00
Colon Cancer	2.42	0.75
Prostate Cancer	2.33	0.78
Ovarian Cancer	2.49	0.73
Full panel MG-MID	1.81	–

Table 4
Anti-proliferative activities of compounds **5e-l** on a panel of human acute leukemia cell lines (MOLM-13, MV4-11, CCRF-CEM, HL60 and THP-1).

Compounds	GI_{50} (μM)				
	MV4-11	MOLM-13	HL60	CCRF-CEM	THP-1
5a	>40	n.t.	>40	n.t.	n.t.
5b	>40	n.t.	>40	n.t.	n.t.
5c	27 ± 1.7	n.t.	>40	n.t.	n.t.
5d	9.1 ± 1.7	n.t.	>40	n.t.	n.t.
5e	2.1 ± 0.8	2.5 ± 0.0	1.7 ± 0.0	8.4 ± 2.1	9.4 ± 2.5
5f	3.5 ± 0.2	3.2 ± 0.1	2.4 ± 0.5	9.9 ± 2.2	12.7 ± 0.8
5g	3.0 ± 0.2	2.1 ± 0.0	2.7 ± 0.4	7.4 ± 1.5	4.2 ± 0.3
5h	0.8 ± 0.1	0.9 ± 0.1	1.2 ± 0.3	2.9 ± 0.4	2.8 ± 0.0
5i	>40	>20	>20	>20	>20
5j	34.9 ± 2.5	18.1 ± 0.1	>20	>20	>20
5k	34.2 ± 1.5	19.2 ± 0.3	>20	>20	>20
5l	11.6 ± 0.1	15.9 ± 3.4	>20	>20	>20

2.2.6. *Apoptosis and caspase 3/7 activity*

Thereafter, pyrimido[1,2-*a*]benzimidazole **5h** was analyzed for its apoptosis-inducing activity *via* analysing the level of caspase 3/7 activity in HL60 and MV4-11 cell lines after 8 and 24 h treatment with increasing concentrations of **5h**. In line with flow cytometry results, **5h** increased caspase activity in HL60 cell line. Caspase 3/7 activity was more than ten-times higher in HL60 cells treated with 5 μM of **5h** for 24 h, but no change was found in MV4-11 cells (Fig. 3).

2.2.7. *Levels of regulatory proteins*

Finally, changes in levels of proteins involved in regulation of proliferation and apoptosis were analyzed by immunoblotting (Fig. 4). HL60 and MV4-11 cells were treated for 8 and 24 h with increasing concentrations of **5h**. To distinguish whether cells were blocked in mitosis or not, we detected phosphorylation of Ser10 in the tails of histone H3, which is tightly correlated with chromatin condensation during mitosis and is usually considered a mitotic marker [32]. We did not observe any increase in H3 phosphorylation in both treated cell lines, which would be comparable with nocodazole treatment. On the contrary, we revealed that **5h** reduced levels of phosphorylated H3 on Ser10, which corresponds with the inability to condensate chromatin followed by G2 phase arrest and cell death.

Levels of Mcl-1 and PARP-1, which is cleaved during apoptosis by caspases 3/7 [33], were monitored as well. In particular, the cleaved 89 kDa PARP-1 fragment was detected after 24 h treatment with higher doses of **5h** in HL60 cell line. In parallel, decreased Mcl-1 level was observed as well. The cleavage of PARP-1 occurred to a lesser extent in both treatment periods of MV4-11 cells, but with no changes in Mcl-1.

2.2.8. *Kinase assay*

2.2.8.1. FLT3, ABL, CDK2 and GSK3β inhibitory activities. On account of its overexpression on the majority of AML cells as well as its significant role in the aggressive nature and increased relapse of this cancer, FLT3-ITD stands out as a promising target for management of AML [34–36]. Moreover, ABL, CDK2 and GSK3*β* kinases were reported to be overexpressed in leukemia cells.

In the current work, the synthesized benzimidazoles **5a-p** were examined further for inhibitory activity against mutant FLT3-ITD, as well as against ABL, CDK2 and GSK3*β* kinases, and the results are shown in Table S1 (Supplementary Materials).

The examined derivatives displayed weak FLT3-ITD inhibitory activity. Compounds **5b-d** exerted single-digit micromolar activity against FLT3-ITD (IC_{50} = 7.8, 6.0 and 2.6 μM, respectively). Also, compounds **5a** and **5f-h** displayed weak activity with IC_{50} values equal to 18, 19.4, 17.8

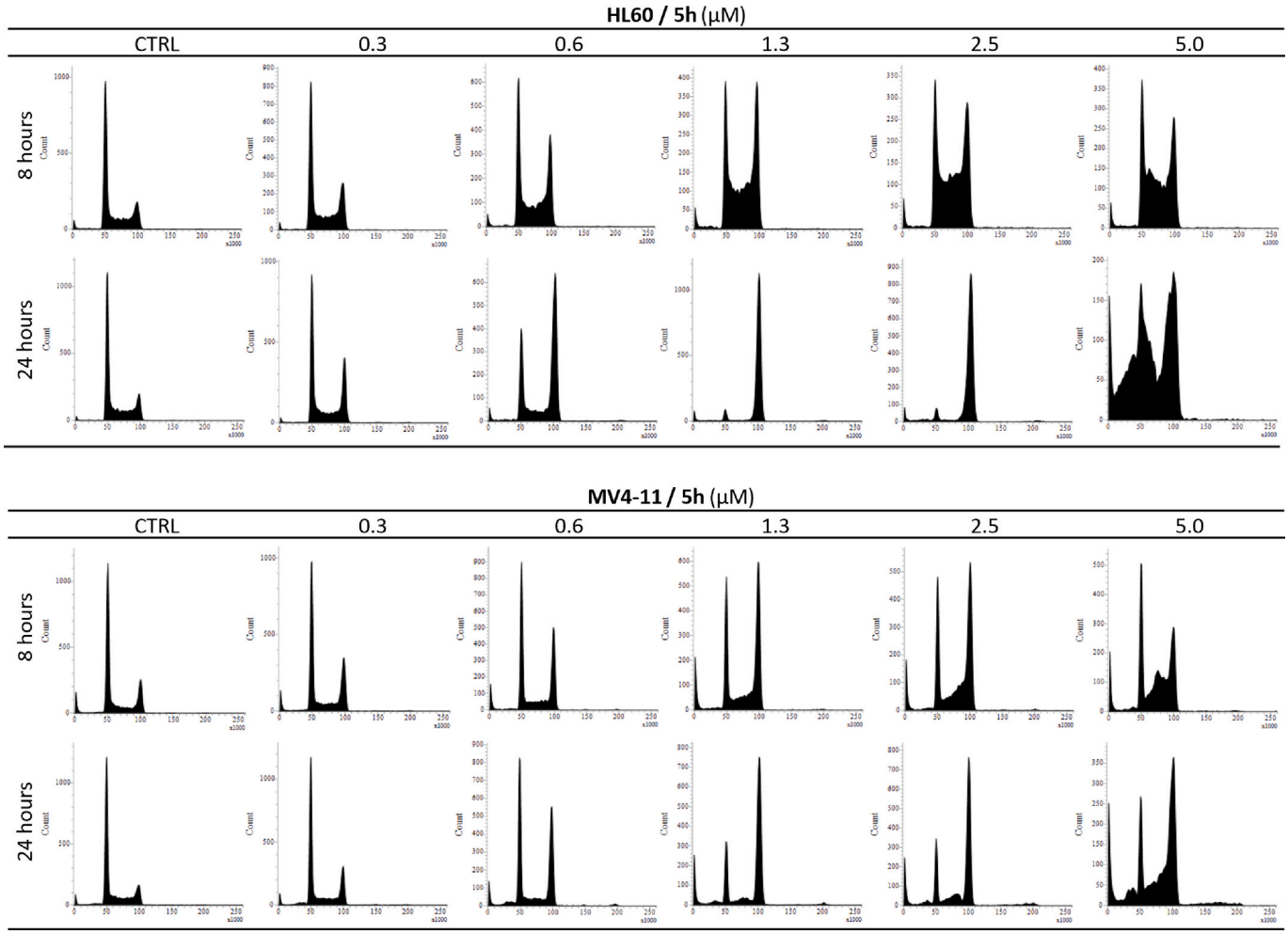


Fig. 2. Impact of **5h** on cell cycle in HL60 and MV4-11 cells treated for 8 and 24 h with the indicated doses.

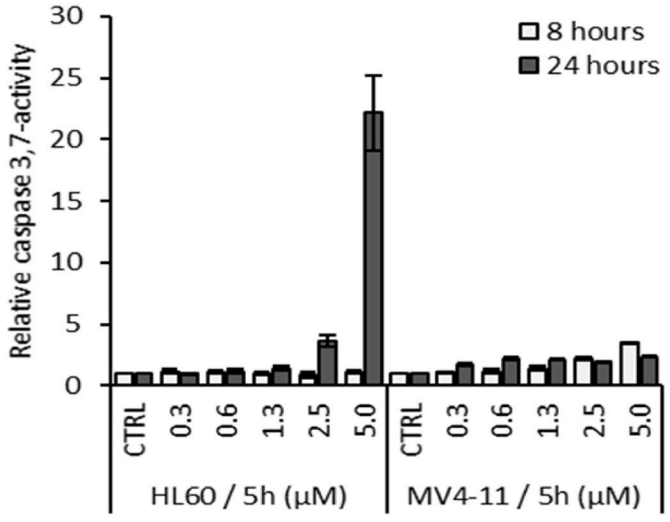


Fig. 3. Relative caspase 3/7 activity in HL60 and MV4-11 cells after 8 and 24 h treatment with **5h**.

and 13.3 μM, respectively. Moreover, no significant inhibitory activity was observed up to 20 μM for all compounds tested against the examined ABL, CDK2 and GSK3β kinases (Table S1, Supplementary Materials).

The most potent anti-leukemic agents **5e-h** in this study didn't exerted significant inhibitory activity against these kinases, we realized additional investigations to identify other potential targets for the pyrimido[1,2-*a*]benzimidazoles described here.

2.2.8.2. Kinase profiling at Reaction Biology Corporation. In another attempt to identify the kinase target for herein reported pyrimido[1,2-*a*] benzimidazoles, compound **5h** that displayed the best anticancer activity was tested at a single dose (10 μM) concentration over a panel of 338 human kinases at Reaction Biology Corporation. The screened kinases spanned seven families including AGC, CAMK, CK1, CMGC, STE, TK and TKL in addition to atypical kinases and other eukaryotic protein kinases that don't fit into the kinase's groups (Fig. 5) [37]. The results were expressed as inhibition percentage and listed in Table S2 (Supplementary Materials).

Compound **5h** showed a percentage of inhibition above 40% for about 26 kinases (Fig. 5), the majority of which belong to the TK family (22 kinases). Analyzing the results for the involved TK enzymes, the oncogenic BMX and TIE2 kinases were inhibited by 81% and 77%, respectively, whereas TEC kinase that is mainly involved in inflammatory diseases was 82% inhibited (Fig. 6).

Accordingly, the promising anti-proliferative activity of target pyrimido[1,2-*a*]benzimidazole derivatives could be attributed to the inhibition of BMX and/or TIE2. Thereafter, the inhibition percentages for compounds **5e-g** towards BMX and TIE2 kinases were determined (Table 5). Compounds **5e** and **5h** demonstrated significant inhibition of BMX kinase at rates of 93% and 81%, respectively, overcoming their inhibition of TIE2 kinase at rates of 82% and 74%, respectively

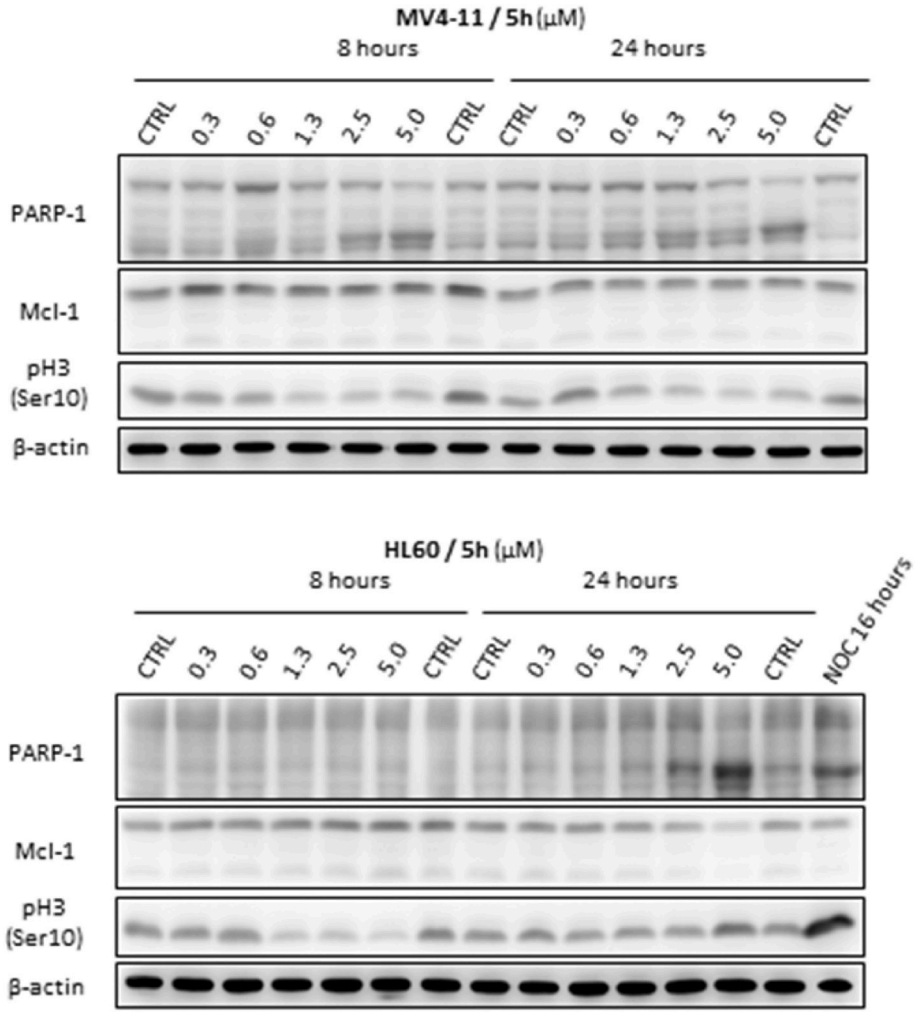


Fig. 4. Effect of **5h** on the phosphorylation of histone H3 on Ser10 and the induction of cell death in HL60 and MV4-11 cell lines upon 8 and 24 h treatment. β-Actin level was detected to verify equal protein loading.

(Table 5). Furthermore, compounds **5e** and **5h** displayed IC_{50} values equal 0.59 and 0.81 μM, respectively, against BMX kinase (Table 6).

3. Conclusion

In summary, a series of 2,4-disubstituted pyrimido[1,2-*a*]benzimidazole **5a-p** was prepared as potential anti-leukemic agents. The synthesized pyrimido[1,2-*a*]benzimidazoles were studied for their *in vitro* anti-tumor activity at NCI-DTP. SAR analysis revealed that insertion of a 1-naphthyl moiety at C-4 into the pyrimido[1,2-*a*]benzimidazole scaffold resulted in the most potent anti-proliferative pyrimido[1,2-*a*]benzimidazoles in this work (**5e-h**) with average GI of 42, 41, 55 and 65%, respectively. In particular, **5h** bearing a 3,4-dimethoxyphenyl group at C-2 showed the strongest and the broadest growth inhibition in the NCI assays. In addition, compounds bearing a *N*,*N*-dimethylamino phenyl substituent at C-4 and a 4-methylphenyl or 4-methoxyphenyl group in position 2 led to active compounds **5j** and **5k** (GI = 40% and 47%, respectively). Pyrimido[1,2-*a*]benzimidazoles **5a-l** were then tested toward a panel of 5 human leukemia cell lines; namely HL60, MOLM-13, MV4-11, CCRF-CEM and THP-1; where **5e-h** reached single-digit micromolar GI_{50} values for all the investigated cell lines. Compound **5h** showed sub-micromolar to low single-digit micromolar GI_{50} values of 0.8, 0.9, 1.2, 2.9 and 2.8 μM, respectively. Further investigation of their effect on cell cycle on HL60 and MV4-11 cells and caspase 3/7 activity was also performed. In addition, the changes of regulatory proteins (PARP-1, Mcl-1, pH3-Ser10) related to cell death and viability were analyzed in HL60 and MV4-11 cells by immunoblotting. In our effort to determine the potential kinase target for the pyrimido[1,2-*a*]benzimidazoles disclosed here, compounds **5a-p** were first tested for inhibitory action against the leukemia-associated mutant FLT3-ITD, as well as, against in-house ABL, CDK2, and GSK3 kinases. The examined molecules disclosed non-significant activity against these kinases. Thereafter, the kinase profiling service at Reaction Biology Corporation, over a panel of 338 human kinases, was exploited to discover the potential target. Superiorly, pyrimido[1,2-*a*]benzimidazoles **5e** and **5h** significantly inhibited BMX kinase by 93% and 81%, respectively. Ultimately, it should be noted that the aforementioned findings will be employed to optimize the pyrimido[1,2-*a*]benzimidazole scaffold and to delve more comprehensively into the kinase inhibitory mechanisms of these compounds.

4. Experimental section

4.1. Chemistry

^{1}H and ^{13}C NMR spectra were performed utilizing the Bruker spectrophotometer where ^{1}H NMR was at 400–500 MHz and ^{13}C NMR at 125 MHz, using TMS as an internal standard and chemical shifts were reported in ppm on the δ scale using deuterated chlorform. Coupling constant (J) values were estimated in Hertz (Hz). Splitting patterns are

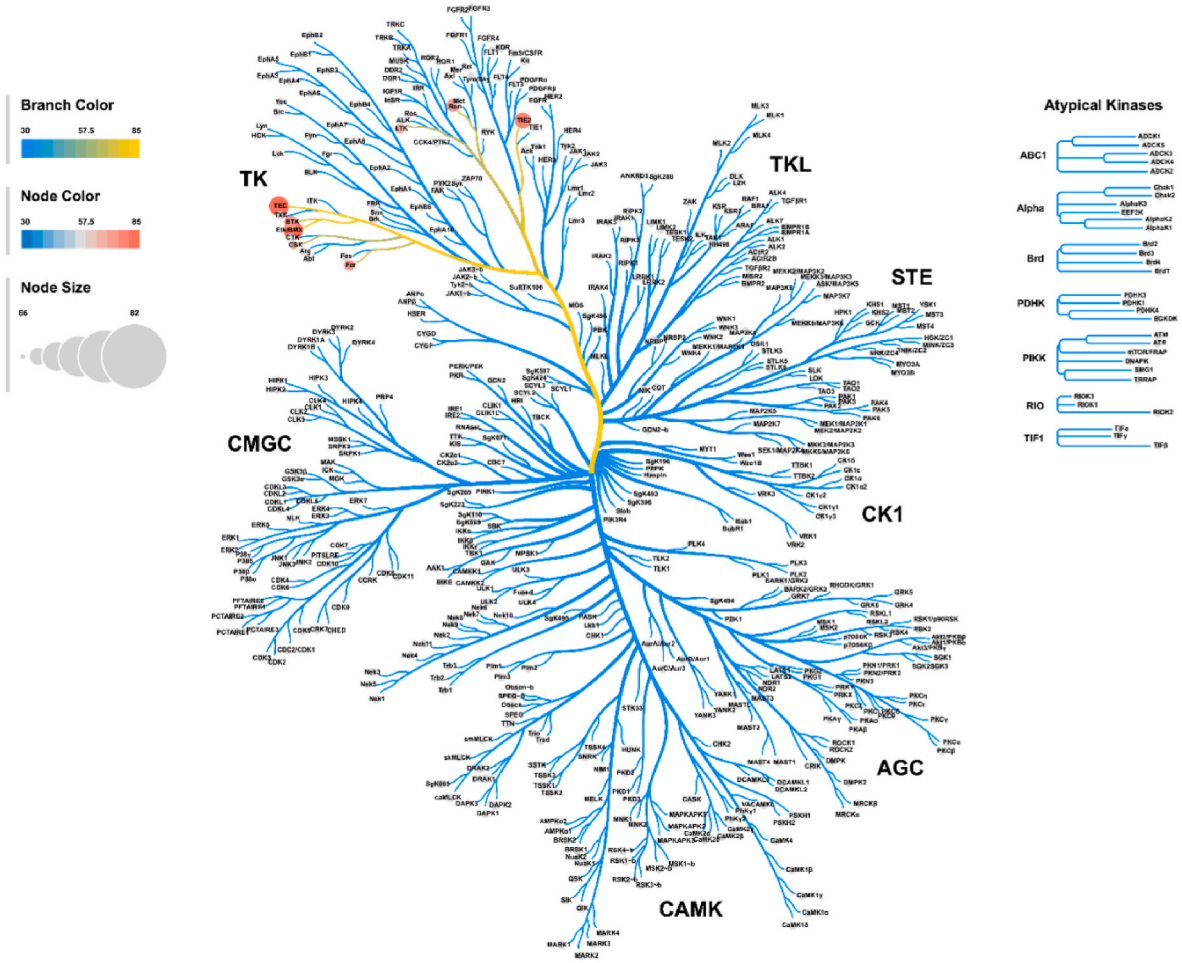


Fig. 5. Tree plot for Kinome-kinase profile of **5h** created by Coral.

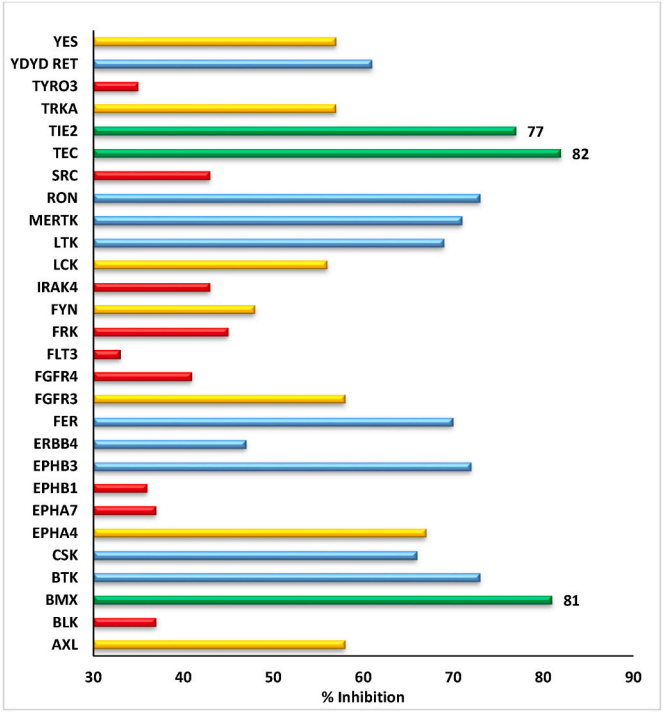


Fig. 6. Plot of the percentage inhibition of **5h** against TK family kinases.

Table 5
Percent inhibition of BMX and **TIE2** kinases activity exerted by pyrimido[1,2-*a*] benzimidazoles **5e-h** at a single dose of 10 μM.

Compound	BMX (% inhibition)	TIE2 (% inhibition)
5e	93%	82%
5f	16%[a]	27%[a]
5g	26%	16%
5h	81%	74%
Staurosporine	98%	98%

[a] Inhibition activities for **5f** were measured at 1 μM due to a solubility issue.

Table 6
Enzymatic inhibition activity of compounds **5e** and **5h** against BMX kinase.

Compound	IC_{50} (μM)
5e	0.59
5h	0.81

designated as follows: s, singlet; d, doublet, t, triplet; q, quartet; m, multiplet. The electrospray ionization (ESI) mass spectra were recorded using a Advion compact mass spectrometer (CMS) NY | USA. Micro-analysis was measured for C, H, N using PerkinElmer 2400 and was within ±0.4% of theoretical values. Melting points were measured using a Stuart SMP30 apparatus in open-glass capillaries.

4.1.1. General procedure for preparation of compounds **3a-p**

All chalcones **3a-p** were synthesized by stirring of different ketones **1a-d** (1 mmol) and different aldehydes **2a-d** (1 mmol) using methyl alcohol (20 mL) and 50% w/v KOH (5 mL), at room temperature for 24 h. Distilled water was added after total precipitation of the compounds, which were attained by filtration and later recrystallized in absolute ethanol to give compounds **3a-3d** [38–41], **3e-3h** [38,42], **3i-3l** [43–46] and **3m-3p** [47].

4.1.2. General procedure for preparation of compounds **5a-p**

A solution of 2-aminobenzimidazole **4** (0.16 g, 1.2 mmol) in DMF (10 mL) was mixed with the appropriate α,β-unsaturated ketones **3a-p** (1 mmol) in the presence of sodium hydroxide (0.05 g, 1.25 mmol). The mixture was then heated under reflux for 24 h. The mixture was poured into ice-water and the obtained residue was filtered, then dissolved in hot methanol and kept overnight. The obtained solid was washed with mixture of methanol/diethyl ether to yield compounds **5a-p (**Supplementary Materials).

4.2. Biological evaluation

The comprehensive procedures of biological assays of the target pyrimido[1,2-*a*]benzimidazoles (**5a-p**) were presented in the Supplementary Materials including; NCI-USA screening [31], cell viability assays [48], flow cytometry [49], immunoblotting [49], caspase 3/7-activity assay [48], FLT3-ITD [49], ABL [50], CDK2 [50,51] GSK3β assay [52], and protein kinases screening.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2023.115610.

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