

Article

3,5,7-Substituted pyrazolo[4,3-d]pyrimidine inhibitors of cyclin-dependent kinases and their evaluation in lymphoma models

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5 **Their Evaluation in Lymphoma Models**
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51 **KEYWORDS**
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53 cyclin-dependent kinase, lymphoma, pyrazolo[4,3-*d*]pyrimidine, venetoclax
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ABSTRACT

Cyclin-dependent kinases are therapeutic targets frequently deregulated in various cancers. By convenient alkylation of the 5-sulfanyl group, we synthesized 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidines with various substitutions at position 5 with potent antiproliferative activity in non-Hodgkin lymphoma cell lines. The most potent derivative **4.35** also displayed activities across more than sixty cancer cell lines. The kinase profiling confirmed high selectivity of **4.35** toward CDKs 2, 5 and 9, and the cocrystal with CDK2/cyclin A2 revealed its binding in the active site. Cultured lymphoma cell lines treated with **4.35** showed dephosphorylation of CDK substrates, cleavage of PARP-1, downregulation of XIAP and Mcl-1 and activation of caspases, which collectively confirmed ongoing apoptosis. Moreover, **4.35** demonstrated significant activity in various cell line xenograft and patient-derived xenograft mouse models in vivo both as a monotherapy and as a combination therapy with the BCL2-targeting venetoclax. These findings support further studies of combinatorial treatment based on CDK inhibitors.

INTRODUCTION

Non-Hodgkin lymphomas (NHLs) are the most frequent hematologic malignancies in the Western hemisphere, comprising approximately 30% of all hematologic cancers. Based on the behavior of the cancer, NHLs can be classified as indolent, aggressive and highly aggressive lymphomas. The latter two NHL types include diffuse large B-cell lymphoma, Burkitt's lymphoma, mantle cell lymphoma and peripheral T-cell lymphomas. The deregulation of cell cycle machinery together with the disruption of programmed cell death are hallmarks of aggressive NHL. These hallmarks include mutated or overexpressed oncogenes (e.g., MYC, BCL6, MYD88, cyclin D1, BCL2, CDK4/6) or the deletion of tumor suppressors (e.g., TP53, TP63, RB1, CDKN2A, ATM). Double- and triple-hit lymphomas characterized by the concurrent genetic aberrations of MYC, BCL-2 and/or BCL6 represent a subgroup of NHL with an extremely dismal prognosis.¹

Some of the recurrently overexpressed or mutated genes encode drug targets, such as BCL-2 protein, Bruton's tyrosine kinase or phosphoinositide 3-kinase, which can be modulated by the small molecule inhibitors venetoclax, ibrutinib or idelalisib, respectively.²⁻⁴ Another emerging pharmacological approach targets cyclin-dependent kinases (CDK), which were first discovered for their role in regulating the cell cycle.⁵ Depending on their selectivity, CDK inhibitors can interfere either with cell cycle progression by blocking RB protein phosphorylation (governed by CDKs 2/4/6) or with global transcription by inactivating RNA polymerase II (governed by CDKs 7/8/9/12). However, many inhibitors have broad specificity and simultaneously obstruct both of these processes,⁶⁻⁸ providing the advantage of overcoming possible compensatory functions among the CDK family.⁹

CDK inhibitors, including clinical candidate drugs such as flavopiridol, roscovitine, dinaciclib and the recently approved drugs palbociclib and abemaciclib, have been increasingly evaluated in various lymphomas.¹⁰⁻¹⁴ In NHL, similar to other cancers, the

1
2
3 pharmacological inhibition of transcriptional CDKs leads to the downregulation of short-lived
4 mRNAs related to cell survival, such as those of MCL-1, XIAP, MYC and cyclin D1. The
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6 downregulation of cyclin D1 and MCL-1 protein levels was observed in mantle cell
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8 lymphomas and in aggressive MYC-driven B-cell lymphoma treated with roscovitine and
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10 dinaciclib, respectively.^{11,12} Despite these compounds inhibit multiple CDKs, a widely
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12 accepted mechanism of action involves CDK9, which was independently supported by
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14 findings obtained with the highly CDK9-selective drug atuvaciclib.¹⁵ Importantly, the loss of
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16 MCL-1 as a consequence of CDK9 or CDK7 inhibition by these drugs has been found to
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18 strongly enhance apoptosis induction in NHL by small-molecule antagonists of BCL-2.¹⁶⁻²¹
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24 The pan-selective purine CDK inhibitor roscovitine, which was discovered in our
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26 laboratory, was among the first CDK inhibitors that entered clinical trials.^{22,23} The further
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28 exploration of roscovitine has been mainly oriented toward either modifications in its
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30 substitutable positions²⁴⁻²⁶ or the redistribution of nitrogen atoms of the purine scaffold; the
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32 latter approach culminated in nanomolar inhibitors based on pyrazolo[4,3-*d*]pyrimidines and
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34 pyrazolo[1,5-*a*][1,3,5]triazine.²⁷⁻³⁰
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38 The objective of this work was to synthesize novel potent pyrazolo[4,3-*d*]pyrimidine
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40 CDK inhibitors, which proved to be more potent than related purines,²⁸⁻³⁰ and explore their
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42 activity toward lymphoma models. The heterocyclic core was substituted with groups that
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44 conferred high activity in purines, identified during our previous structure-activity studies^{24,26};
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46 i.e. isopropyl moiety at position 3, benzylamines at position 7 and alkyl- or cycloalkyl-
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48 amines at position 5.²⁹⁻³⁰ The compounds were prepared using more convenient alkylation of
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50 5-sulfanyl derivatives, providing substantially higher yields over the 5-amino-substituted
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52 pyrazolo[4,3-*d*]pyrimidines described earlier.^{30,31} In line with our expectations, the newly
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54 prepared derivatives displayed nanomolar potency against CDKs and cancer cell lines. Our
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56 previous study demonstrated that roscovitine strongly sensitizes leukemia and lymphoma cells
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to TRAIL-induced apoptosis by altering the levels of BCL-2 proteins in the mitochondria³² and, together with other observations,¹¹ this led us to explore anticancer activity of new pyrazolo[4,3-*d*]pyrimidines against several lymphoma models. The compounds proved to potently kill various lymphoma cell lines and also displayed significant *in vivo* activity both in monotherapy and in a combination with venetoclax, which corresponds to the abovementioned findings.

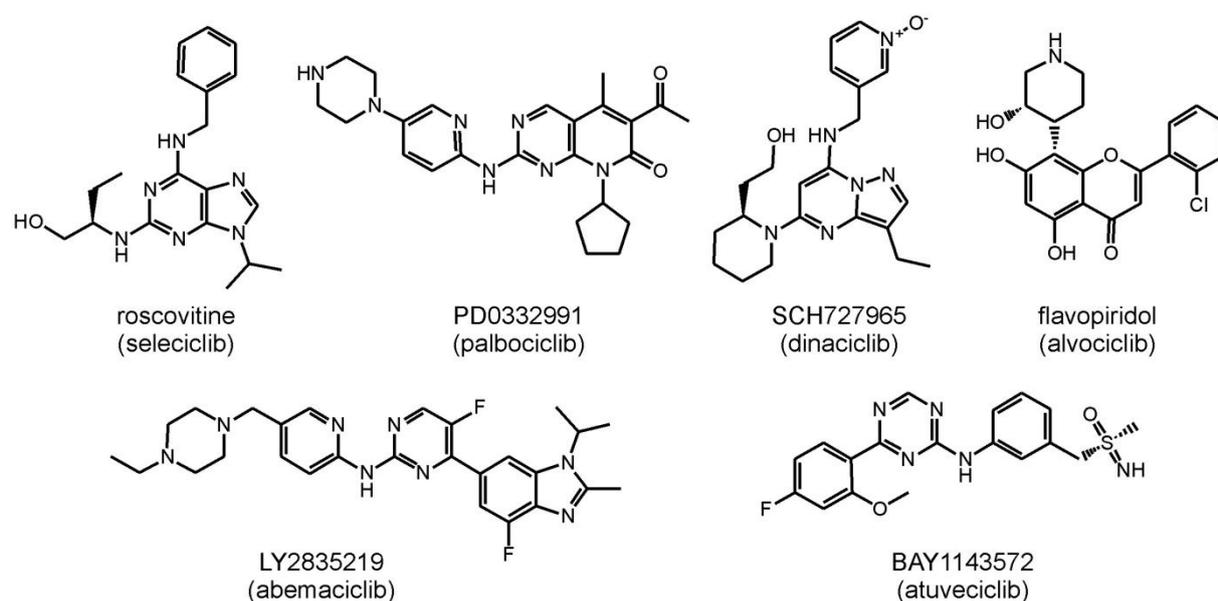


Figure 1. Some cyclin-dependent kinase inhibitors with activity in lymphoma.

Results and Discussion

Design and Synthesis

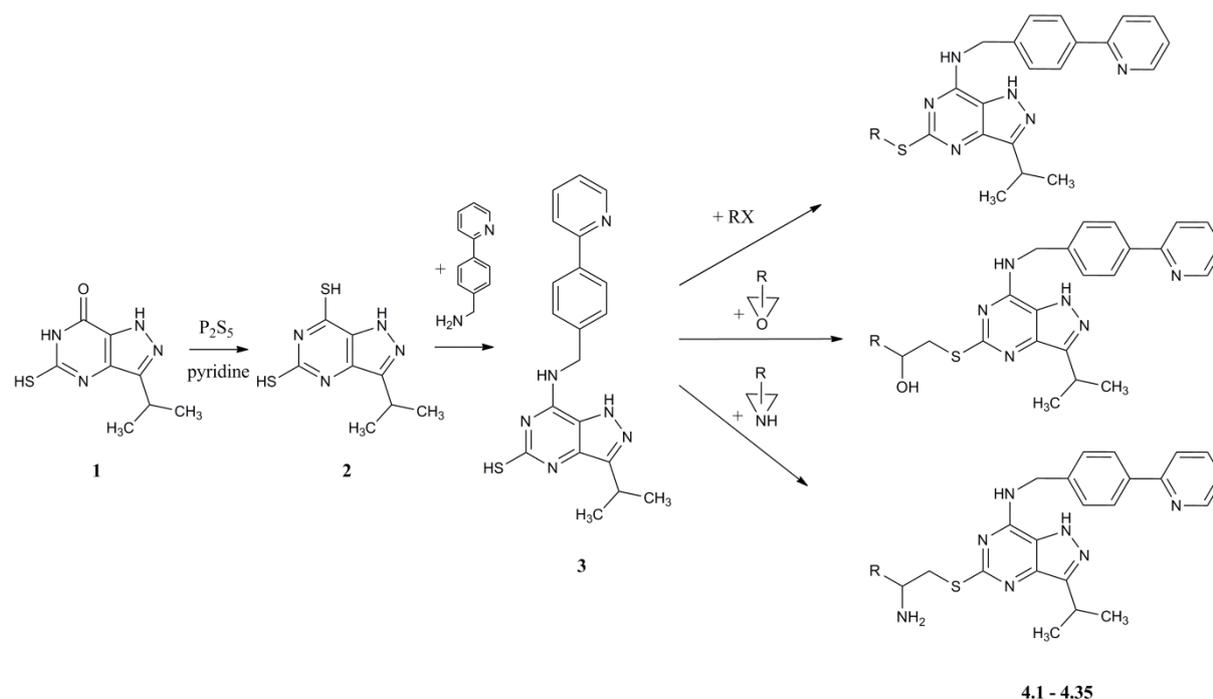
We recently described 5-alkylamino-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2*H*)-pyrazolo[4,3-*d*]pyrimidines that share some of the same substitutions as the purine CDK inhibitor roscovitine but that showed substantially higher potency against CDKs and cancer cell lines than did roscovitine.^{28,30} Due to the laborious synthesis of these derivatives, we sought to improve the synthetic accessibility of these compounds while retaining or even improving their biochemical potency. We focused on the substituted position 5 on the

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3 pyrazolo[4,3-*d*]pyrimidine scaffold and report here a new series of 3,5,7-trisubstituted
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5 pyrazolo[4,3-*d*]pyrimidines in which the alkylamino group at the 5-position of the heterocycle
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7 is replaced by a 5-alkylthio group. To verify the effect of this modification, we employed
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9 molecular modeling. We applied the computational procedure delineated in our previous
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11 work, in which we identified the pyrazolo[4,3-*d*]pyrimidine core as the most favorable central
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13 heterocycle of the purine bioisosteres of CDK2 inhibitors.³³ Importantly, we have shown that
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15 the contributions of the central heterocyclic cores and the individual substituents, quantified
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17 and evaluated in relation to conformations of the optimized protein-inhibitor complexes, are
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19 not simply additive. Depending on the scaffold, the same substituents can be associated with
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21 different interaction “free” energies $\Delta G'_{\text{int}}$ (in kcal mol⁻¹). Our preliminary calculations
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23 suggested that the replacement of the alkylamino group at the 5-position with the 5-alkylthio
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25 group can increase the binding affinity to CDK2 (selected data are available in
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27 **Supplementary Information**).

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33 The new compounds have been prepared from 3-isopropyl-5-sulfanyl-1(2*H*)-
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35 pyrazolo[4,3-*d*]pyrimidin-7-ol **1** (synthesized according to a described procedure²⁸), which
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37 was first converted to 5,7-dithiol **2** and subsequently substituted at the heterocycle position 7
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39 with 4-(2-pyridyl)benzylamine. The final compounds **4** were prepared by the alkylation of the
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41 reactive 5-sulfanyl functional group of **3** by various agents (**Scheme 1**); in the final step, we
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43 successfully prepared 24 alkyl halogens (yielding compounds **4.2 - 4.4**, **4.6 - 4.11**, **4.17**, **4.18**,
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45 **4.21 - 4.23**, **4.25**, **4.26**, **4.28**, **4.30 - 4.33**, **4.35**), 8 epoxides (yielding compounds **4.1**, **4.5**, **4.12**,
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47 **4.16**, **4.19**, **4.29**, **4.33**, **4.34**) and aziridines (yielding compounds **4.13**, **4.24** and **4.35**).

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51 The reactivity of the sulfanyl functional group enables the use of different classes of
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53 alkylating agents, eventually leading to the same compounds with similar yields (**4.33** and
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55 **4.35** were prepared not only by using alkyl halogens but also by using suitable epoxide or
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57 aziridine, respectively). This new synthetic approach overcomes the less convenient
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nucleophilic aromatic substitution (S_NAr , which requires difficult reaction conditions, makes undesired by-reactions and subsequently by-products) described in our previous article examining 5-alkylamino-1(2)H-pyrazolo[4,3-*d*]pyrimidine derivatives.³⁰



Scheme 1. General procedure for the synthesis of novel 5-alkylthio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-*d*]pyrimidines.

Structure-Activity Relationships

The implementation of the most suitable substitution at the 5 position of the pyrazolo[4,3-*d*]pyrimidine core was mostly inspired by previous SAR studies on trisubstituted pyrazolo[4,3-*d*]pyrimidine CDK inhibitors,^{29,30} related purines^{26,34-37} and pyrazolo[1,5-*a*]pyrimidines.³⁸ The compounds we prepared included inhibitors with significant activity against CDK2/E; all compounds exhibited an IC_{50} below 200 nM, and approximately 50% of inhibitors blocked the activity of the simple 5-methylthio derivative **4.14** ($IC_{50} = 48$ nM). The substituent in position 5 is mostly in contact with the flexible parts of the enzyme surface or

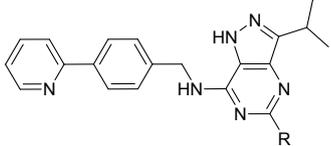
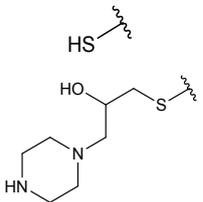
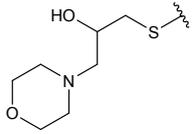
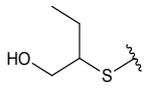
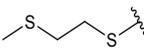
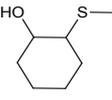
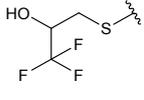
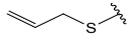
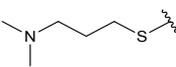
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3 with the solvent (water molecules) and therefore can be more variable, whereas the side
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5 chains at positions 3 and 7 do not allow such variability. Despite low differences in the CDK2
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7 inhibition measured with the studied compounds, we found some relationships that are
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9 associated with the size/length of the substituent at position 5 or its polarity. Compound **3**,
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11 which contains an acidic sulfanyl functional group in position 5, was the weakest in the series,
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13 with nearly three-fold lower potency than an analogous 3,7-disubstituted compound described
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15 earlier.³⁰ The methylation of the 5-sulfanyl functional group results in short lipophilic
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17 substituents in **4.14**, increasing the potency more than ten-fold.
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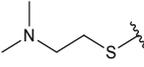
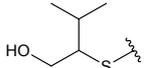
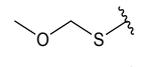
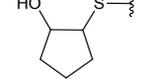
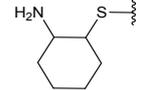
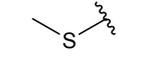
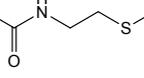
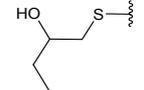
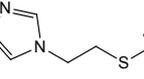
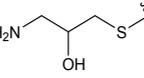
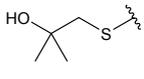
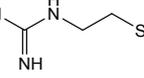
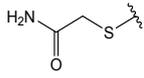
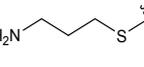
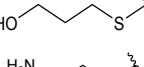
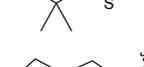
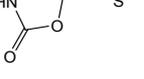
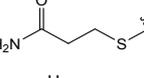
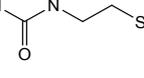
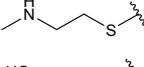
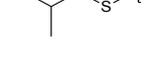
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21 The weakest CDK2 inhibition (IC_{50} between 100 - 200 nM) was observed for
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23 compounds bearing rigid piperazine and morpholine moieties (**4.2** and **4.1**, respectively).
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25 Additional groups with weak activity against CDK2 included compounds with less polar (**4.4**,
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27 **4.7**, **4.11**, **4.14**) or cycloalkyl (**4.5**, **4.12**, **4.13**) substituents at position 5 or those with tertiary
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29 amino groups (**4.8**, **4.9**) (IC_{50} = 48 - 138 nM). In contrast, the most potent inhibitors of the
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31 series (IC_{50} = 2 - 39 nM) bear at least one polar group (mainly a hydroxy or primary amino
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33 group) placed either on a short linear (**4.35**, **4.33**, **4.22**, **4.23**, **4.28**) or on a small branched
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35 chain (**4.34**, **4.32**, **4.30**, **4.29**, **4.27**, **4.26**). Further modification of 2-aminoethylthio in the
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37 most active compound **4.35** led to the impairment of CDK2 inhibition; this effect was
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39 observed when the terminal amino group was modified by methylation, acetylation and
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41 conversion to urea or guanidine (**4.28**, **4.15**, **4.9**, **4.27** and **4.20**, respectively) and is probably
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43 due to steric hindrance due to possible hydrogen bonding, increased basicity of the amine
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45 (**4.28** and **4.20**), or a combination of both. Other attempts to modify the primary amine in **4.35**
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47 diminished its activity, such as its extension with aminomethyl (**4.30**) or its cyclization (**4.13** a
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49 **4.17**). A similar scenario is also evident for the modified hydroxyethylthio derivatives, among
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51 which only compound **4.34** reached the same potency as the analog **4.33**; all other compounds
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53 (**4.32**, **4.29**, **4.19**, **4.18**, **4.16**, **4.12**, **4.11**, **4.5**, **4.3**, **4.2**, **4.1**, **4.31**) have worse CDK2 inhibition.
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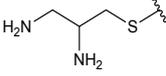
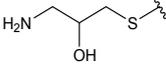
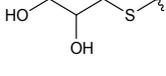
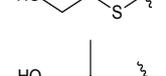
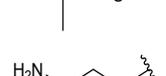
Recent experiments have shown that pharmacological CDK inhibition is an effective and rational treatment option for aggressive MYC-driven lymphomas.¹¹ We therefore screened all prepared compounds against the non-Hodgkin lymphoma cell lines HT, OCI-LY2 and MINO.

The antiproliferative activity of the prepared compounds was consistent with enhanced CDK2 inhibition (**Table 1**). The most potent compounds, **4.28**, **4.29**, **4.31**, **4.32**, **4.33**, **4.34** and **4.35**, had notably improved potency compared with the related purine CR8, with an average GI₅₀ value 5-fold higher than that of CR8.

Table 1. CDK2/E inhibitory and cellular activities of novel pyrazolo[4,3-*d*]pyrimidines.

Compound	R - substitution	IC ₅₀ (μM)*			GI ₅₀ (μM)*	
		CDK2/E	HT	OCI-LY2	MINO	
3		0.558	2.760	3.240	2.175	
4.1		0.171	0.332	0.435	0.767	
4.2		0.156	0.200	0.200	0.243	
4.3		0.15	0.255	0.181	0.119	
4.4		0.138	0.975	0.668	0.773	
4.5		0.134	0.636	0.680	0.530	
4.6		0.132	0.690	0.585	0.641	
4.7		0.100	1.005	0.858	0.923	
4.8		0.099	0.105	0.109	0.076	

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4	4.9		0.077	0.054	0.061	0.093
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6	4.10		0.077	0.980	1.001	1.307
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8	4.11		0.059	0.340	0.285	0.243
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11	4.12		0.052	0.567	0.373	0.450
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14	4.13		0.052	0.139	0.136	0.157
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17	4.14		0.048	0.783	1.207	1.050
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20	4.15		0.041	0.071	0.054	0.042
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23	4.16		0.039	0.273	0.280	0.427
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26	4.17		0.032	0.155	0.145	0.135
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29	4.18		0.031	0.055	0.055	0.064
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32	4.19		0.030	0.507	0.610	0.477
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35	4.20		0.030	0.39	0.812	0.39
36						
37						
38	4.21		0.027	0.192	0.163	0.197
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41	4.22		0.022	0.120	0.151	0.156
42						
43	4.23		0.021	0.109	0.102	0.089
44						
45	4.24		0.020	0.085	0.100	0.064
46						
47	4.25		0.019	0.080	0.053	0.069
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50	4.26		0.019	0.120	0.084	0.081
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52						
53	4.27		0.018	0.240	0.198	0.136
54						
55						
56	4.28		0.013	0.056	0.092	0.040
57						
58	4.29		0.012	0.062	0.152	0.081
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4	4.30		0.012	0.480	0.780
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6	4.31		0.011	0.063	0.099
7					
8					
9	4.32		0.010	0.021	0.022
10					
11	4.33		0.003	0.048	0.050
12					
13					
14	4.34		0.003	0.190	0.141
15					
16	4.35		0.002	0.022	0.028
17					
18					
19	CR8		0.062	0.263	0.307
20	dinaciclib		0.002	0.019	0.011
21	ibrutinib		>25	15.9	18.9
22					10.7

* measured at least in triplicate

Crystal Structure of the Complex of CDK2/cyclin A2 with Pyrazolo[4,3-*d*]pyrimidine

4.35

To obtain structural insight into the interaction of our inhibitors with the enzyme, we determined the crystal structure of the active Thr160-phosphorylated CDK2 with a fragment of cyclin A2 (residues 175-432) in complex with compound **4.35** at 2.15 Å resolution (PDB code: 6GVA). For data collection and refinement statistics, see **Supplementary Table 2**. The asymmetric unit contains one CDK2/cyclin A2 heterodimer. The residues 39-40 and C-terminal residues 291-298 of CDK2 could not be modeled into the electron density map due to disorder.

The inhibitor binds to the active site located in a narrow cleft between the N- and C-terminal domains of CDK2 in the standard binding mode typical for pyrazolo[4,3-*d*]pyrimidine-based inhibitors (see **Figure 2**). Specifically, the pyrazolo[4,3-*d*]pyrimidine core is sandwiched between the hydrophobic side-chains of Ile10 and Leu134 and interacts *via* three conserved hydrogen bonds with the hinge region residues (7-amino N-H \cdots O:Leu83, N2-H \cdots O:Glu81, and a weak N1 \cdots HN:Leu83 bond). The R³ isopropyl group forms

hydrophobic interactions with the gatekeeper residue Phe80. The terminal amino group of the R⁵ moiety forms a hydrogen bond with the carbonyl oxygen of Gln131 from the phosphate-ribose pocket. The proximal phenyl ring of the R⁷ moiety is sandwiched between Ile10 and the backbone of His84, close to the specificity surface. The terminal pyridine ring sticks out of the binding cleft, roughly in plane with the proximal phenyl ring. Moreover, N4 from the pyrazolo[4,3-*d*]pyrimidine core of the inhibitor interacts through a chain of three water molecules (W414, W435, and W436) with the side chains of Lys33 and Glu51 and with the main chain of Asp145. Another chain of three water molecules (W409, W426, and W471) provides additional hydrogen bonding of the amino group on the R⁵ moiety to the main chain of Glu12 and to the side chains of Asp86 and Lys89 at the edge of the binding cleft.

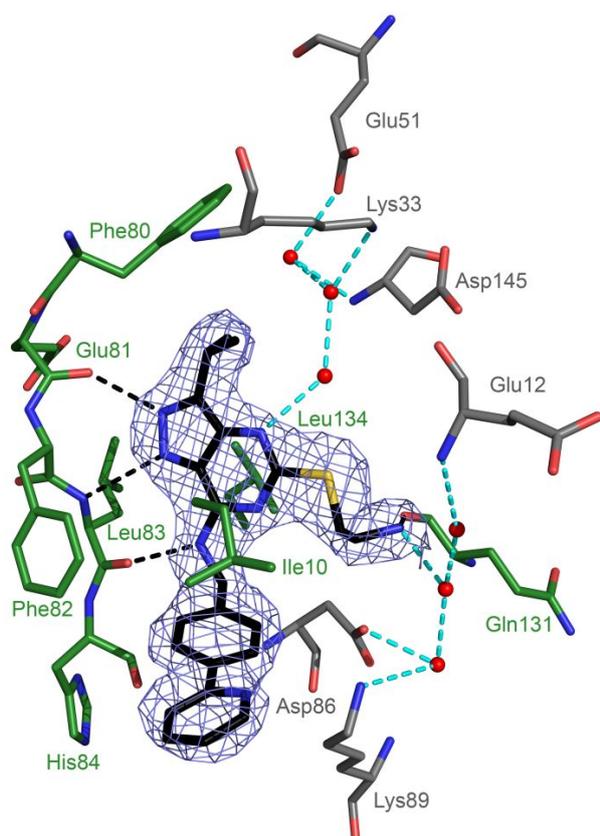


Figure 2. Inhibitor 4.35 bound to the active site of CDK2 (PDB: 6GVA). CDK2 residues interacting with the inhibitor directly (black dashed lines) or through water molecules (cyan dashed lines and red spheres) are shown in green or gray stick representations, respectively. A refined $2|F_o|-|F_c|$ electron density map for the inhibitor contoured at 1σ is shown.

Kinase Selectivity

As the most potent compound, **4.35** was then assayed on a panel of related CDKs. **4.35** had low nanomolar activity not only against CDK2 but also against CDK5 and CDK9 (**Table 2**). The compound displayed at least 10-fold increased potency toward most of the assayed CDKs compared to the related purine CR8, with the highest increase (43-fold) toward CDK4/cyclin D1.

Table 2. CDK selectivity profile of pyrazolo[4,3-*d*]pyrimidine **4.35** and purine CR8 (assayed as a control).

Kinase	IC ₅₀ (μM)*		
	4.35	CR8	Dinaciclib
CDK1/B1	0.090 ± 0.012	0.787 ± 0.100	0.072 ± 0.024
CDK2/E1	0.002 ± 0.001	0.062 ± 0.026	0.002 ± 0.001
CDK2/A2	0.006 ± 0.003	0.144 ± 0.002	0.003 ± 0.001
CDK4/D1	0.603 ± 0.318	26.09	0.115 ± 0.018
CDK5/p25	0.015 ± 0.003	0.225 ± 0.013	0.067 ± 0.032
CDK7/H/MAT	0.124 ± 0.007	1.769 ± 0.013	0.170 ± 0.008
CDK9/T1	0.025 ± 0.002	0.272 ± 0.038	0.015 ± 0.006

* measured at least in triplicate

Next, preliminary selectivity profiling of **4.35** was performed on a panel of 50 additional protein kinases, a representative sampling of the human kinome. The compound was assayed at a single concentration of 1 μM. As shown in **Supplementary Table 3**, significant inhibition was observed for CK1δ and CHK2; the residual activity of these kinases decreased to 11 and 18%, respectively. We then determined the IC₅₀ values for CK1δ and CHK2 (0.045 μM and 0.525 μM, respectively). Weak inhibition of PAK4, CAMK1 or RSK1 kinases was

also observed; these kinases are known to be sensitive to related CDK inhibitors with pyrazolo[4,3-*d*]pyrimidine or purine scaffolds.^{29, 39–41} These results confirmed the reasonable selectivity of **4.35**.

Antiproliferative Effects

We next assessed the anticancer activity of **4.35** against additional lymphoma cell lines as well as other human cancer cell lines (Table 3). **4.35** displayed a broad range of activity across all assayed cell lines, with the average concentration to reduce cell proliferation by 50% (GI₅₀ value) of 21.6 nM. The most sensitive cell lines were the diffuse large B-cell lymphomas OCI-LY3, RIVA and HBL-1. The clinical candidate dinaciclib, assayed in parallel, displayed similar potency.

Table 3: Effect of pyrazolo[4,3-*d*]pyrimidine **4.35** on various human cancer cell lines.

Cancer type	cell line	IC ₅₀ (μM)*	
		4.35	Dinaciclib
lymphoma (DLBCL-ABC)	RIVA	0.011	0.031
	OCI-LY3	0.007	n.d.
	U2932	0.030	0.011
	HBL-1	0.010	0.013
	TMD8	0.012	0.017
lymphoma (DLBCL-GCB)	OCI-LY2	0.023	0.011
	OCI-LY7	0.014	0.002
	SU-DHL10	0.017	n.d.
	SU-DHL4	0.015	0.010
	HT	0.019	0.019
lymphoma (MCL)	HBL-2	0.043	0.013
	JEKO-1	0.014	0.014

	MAVER-1	0.013	0.017
	MINO	0.016	0.011
	UPF1H	0.025	0.013
	UPF7U	0.012	0.019
	Z138	0.013	n.d.
lymphoma (BL)	Ramos	0.044	0.015
	Raji	0.038	0.025
leukemia (CML)	K562	0.036	0.011
leukemia (ALL)	CEM	0.033	0.007
leukemia (AML)	THP-1	0.019	0.007
multiple myeloma	RPMI8226	0.015	0.009
breast	MCF-7	0.016	0.006
cervix	HeLa	0.017	0.013
colon	HCT-116	0.022	0.007
melanoma	G361	0.036	0.016

* measured at least in triplicate; DLBCL–ABC, Diffuse large B–cell lymphoma, subtype – activated B–cell; DLBCL–GCB, Diffuse large B–cell lymphoma, subtype – germinal center B–cell; MCL – mantle cell lymphoma; BL – Burkitt lymphoma; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; AML, acute monocytic leukemia

To further assess the anticancer selectivity of **4.35**, we also screened the NCI60 cell line panel. Notably, **4.35** robustly inhibited proliferation across the entire cancer cell line panel with a mean GI_{50} of 23 nM (**Supplementary Table 4**). In general, **4.35** was effective against leukemia, melanoma and breast cancer cells but showed decreased activity in ovarian and renal cells.

Mechanism of Action in Cells

Compound **4.35** emerged as the most potent CDK inhibitor in this series, and because of its superior proapoptotic activity in several lymphoma cell lines (**Supplementary Figures S6 and S7**), we sought to characterize its cellular effects. We therefore performed immunoblotting analyses of MINO cells exposed to **4.35** both at different doses and for different lengths of time. The treated cells contained clear markers of ongoing apoptotic cell death, including the appearance of the PARP-1 fragment (89 kDa), diminution of full-length PARP-1, activation of caspases 3 and 7 and reduction in the levels of the anti-apoptotic proteins Mcl-1 and XIAP (**Figure 3A, B**). On the other hand, the anti-apoptotic proteins BCL-2 and BCL-XL did not show any change, probably due to their high stability. Clear markers of apoptosis were also detected in UPF1H and MAVER-1 cells (**Supplementary Figure S8**). These results are consistent with the known mechanisms of the pro-apoptotic action of other CDK inhibitors.^{11, 12, 24, 30}

The kinetics of CDK inhibition by **4.35** was studied in MINO cells over the period of 24 h (**Figure 3C**). The dose of 12.5 nM was selected because it induced a strong response within 24 h in previous experiments (**Figure 3A, B**), and it was anticipated that CDK inhibition would be evident at earlier time points. Importantly, a gradual decrease in the phosphorylation of the C-terminus of RNA polymerase II (at serines 2 and 5) and retinoblastoma protein (at serines 608 and 780) in a time-dependent manner was observed, suggesting that at least CDK2, CDK7 and CDK9 were effectively inhibited.

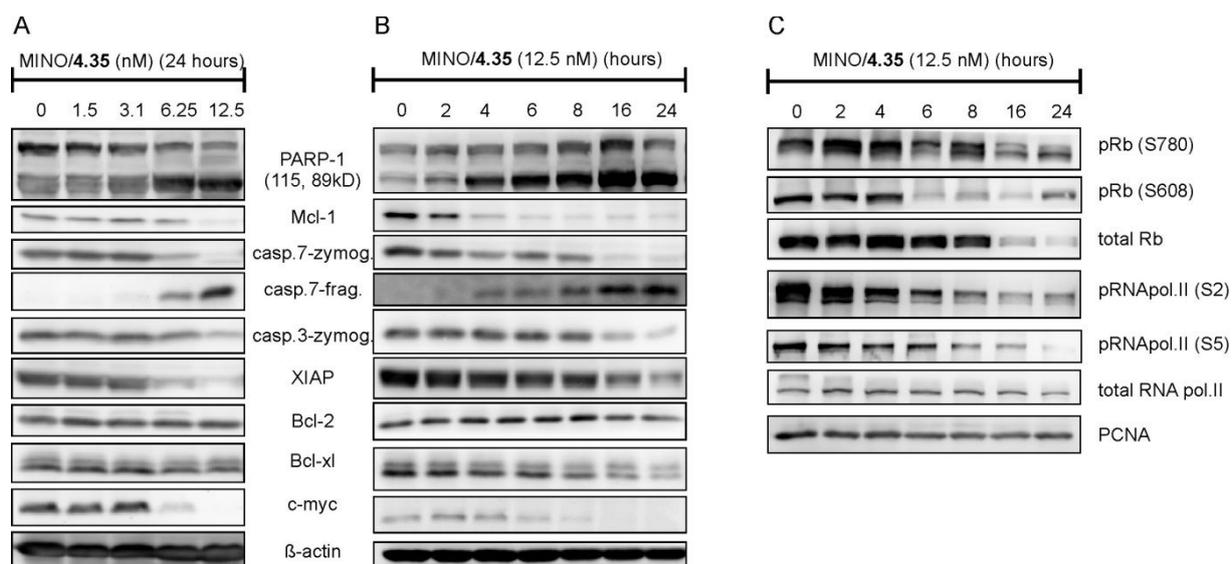


Figure 3. Immunoblotting analysis of expression of CDK substrates and apoptosis-related proteins in MINO lymphoma cells treated with **4.35** in a dose (A) and time-dependent (B, C) manner.

In Vivo Activity: Monotherapy

The in vivo efficacy of **4.35** was then assessed in murine models of aggressive lymphomas based on subcutaneous xenotransplantation of three established lymphoma cell lines (MINO, MAVER-1 and HBL-2) and one patient-derived lymphoma xenograft (PDX - VFN-M1) in immunodeficient mice. Due to relatively rapid pharmacokinetics and limited oral availability (for details see **Supplementary Information**), compound **4.35** was administered daily by i.v. injections. Treatment with **4.35** was associated with a significant reduction in the growth of all three xenografted lymphoma cell lines compared to controls (**Figure 4**). Importantly, anti-lymphoma activity was also clearly achieved in the PDX mouse model VFN-M1, which represents a surrogate model for primary lymphoma cells from patients (**Figure 4**).

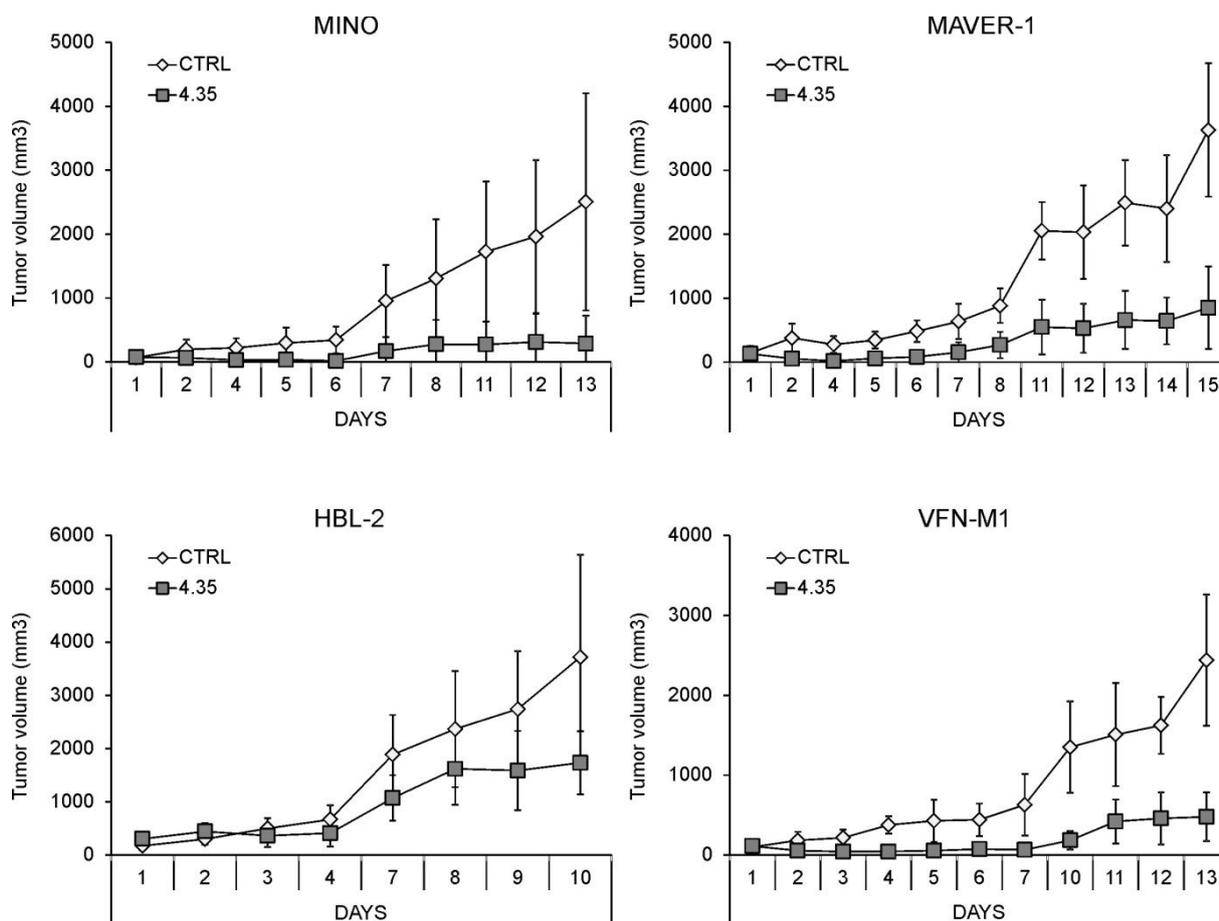


Figure 4. Determination of tumor volume of vehicle- or **4.35**-treated (10 mg/kg) mice after i.v. dosing in MINO (n = 10), MAVER-1 (n = 12), Hbl-2 (n = 8) and VFN-M1 patient-derived xenograft (PDX, n = 10).

Ex vivo analysis of selected pharmacodynamic targets of **4.35** in subcutaneously grown lymphoma tumors 24 h after a single i.v. administration of **4.35** confirmed the induction of apoptosis and the downregulation of MCL-1 and XIAP proteins but had no impact on the expression of BCL-2 (**Figure 5, Supplementary Figure S10**). Notably, the changes were also evident in the PDX model VFN-M1, a reliable surrogate for the primary cells of patients (**Figure 5C**). In addition, reduced RB and RNA polymerase II phosphorylation at several phosphorylation sites was also observed (**Supplementary Figure S11**).

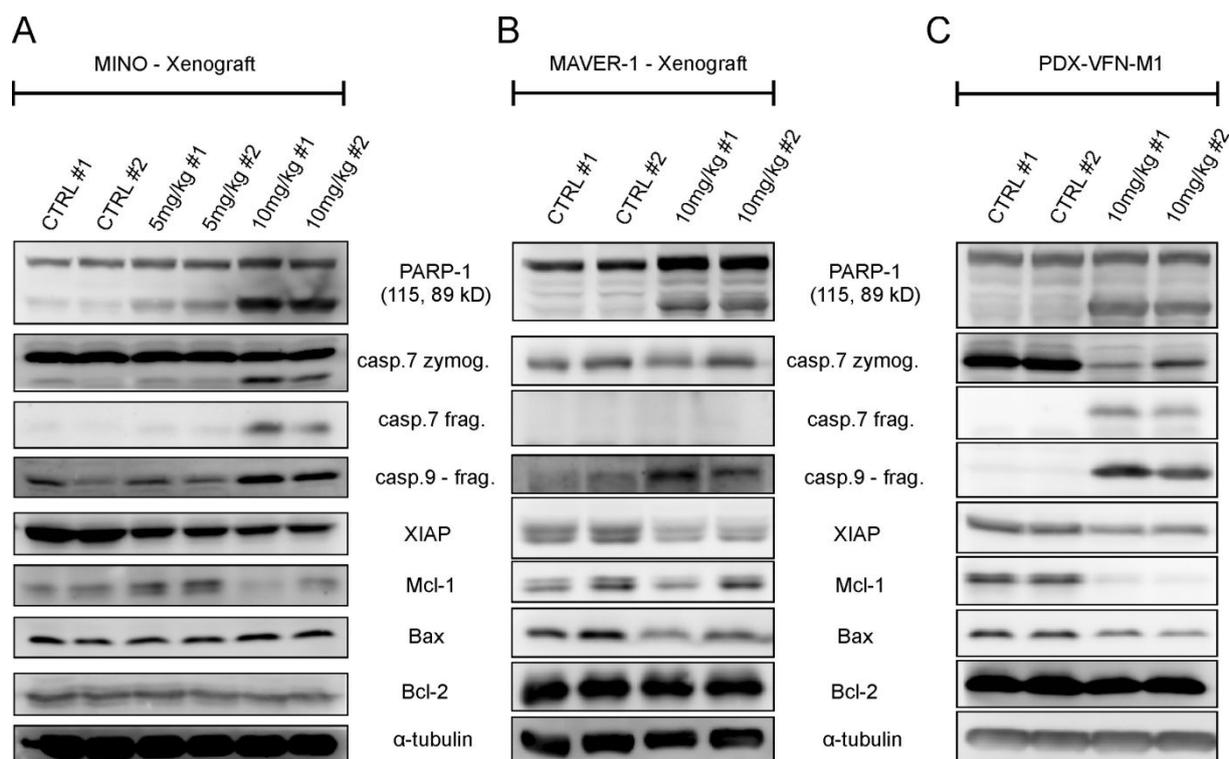


Figure 5. Inhibitory effect of pyrazolo[4,3-*d*]pyrimidine **4.35** on the growth of lymphoma xenografts derived from the MINO (A) and MAVER-1 (B) cell lines and from patient-derived VFN-M1 cells (C). Tumor-bearing mice were intravenously injected with **4.35**. After 24 h, the mice were euthanized, and the tumors were removed and investigated for the expression of apoptosis markers. Each lane represents a different animal.

In Vivo Activity: A Combination with Venetoclax

We and others have previously demonstrated that the concurrent inhibition of BCL-2 and MCL-1 results in marked cytotoxic synergy in lymphoma models.^{42,43} A loss of the antiapoptotic protein MCL-1 observed in the cell line and xenograft lymphoma models treated with **4.35** (Figures 3 and 5) suggests that the depletion may confer the lymphoma cell dependency on other BCL2 family members. Therefore, we investigated the response of a MAVER-1 xenograft to the combination **4.35** and venetoclax (ABT199), an inhibitor of BCL-2 family proteins. The individual treatment of both compounds, **4.35** and venetoclax, significantly inhibited tumor growth. Importantly, the inhibitory effect was more pronounced

when the mice were treated with a combination of these compounds, and the tumors did not start to expand over the observed time period (**Figure 6**). The results are in line with recent observations made with the CDK inhibitor dinaciclib, which clearly sensitized MYC-BCL-2 double-hit lymphomas to the BCL-2 inhibitor venetoclax.^{17, 19}

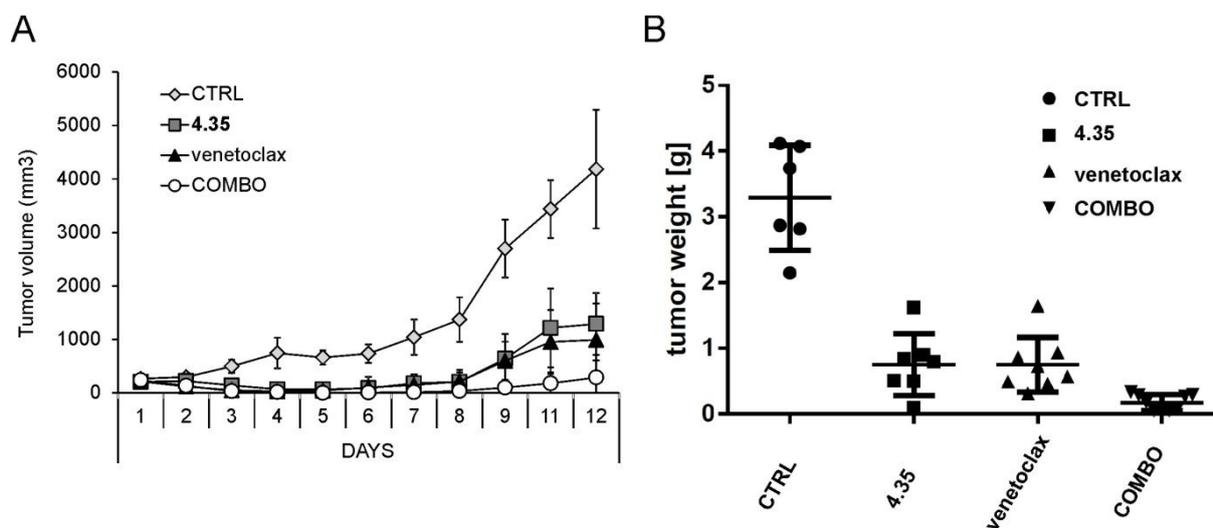


Figure 6. A combination of **4.35** and venetoclax in MAVER-1 xenografts. (A) Mean calculated tumor volumes (\pm SD) of MAVER-1 s.c. tumors from the initiation of therapy (day 1). MAVER-1 cells were xenografted to NSG mice and treated with vehicle (control, $n = 6$), **4.35** (9 mg/kg, $n = 8$), venetoclax (20 mg/kg, $n = 8$) or **4.35** + venetoclax combination ($n = 10$). (B) Evaluation of the mean calculated tumor weight (\pm SD) of MAVER-1 s.c. tumors from the abovementioned therapies (= day 13).

Conclusion

Relatively slow progression of pan-CDK inhibitors in clinical trials, which is generally attributed to their complicated mechanisms of action, narrow therapeutic windows and the lack of robust patient selection criteria, collectively emphasize the necessity to develop and explore new compounds with altered kinase profile. Herein, we describe the synthesis and biological activity of new pyrazolo[4,3-*d*]pyrimidines that potentially inhibited cyclin-

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3 dependent kinase 2 as demonstrated by biochemical assays and crystallographic analysis. Our
4 findings confirmed the in vitro and in vivo sensitivity of aggressive non-Hodgkin lymphomas
5 to CDK inhibitors and provide rationale for their future clinical evaluation. In addition, the
6 combination of CDK inhibitors selected based on biological mechanism (targeting
7 predominantly MCL-1) and the BCL-2 targeting agent venetoclax was proven to exert
8 synergistic effects exhibit synthetic lethality in vivo and should be considered for
9 combinatorial treatment approaches in patients.
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21 **Experimental Section**

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23 NMR spectra were recorded on a JEOL ECA-500 spectrometer operating at the frequencies of 500.16
24 MHz (^1H) and 125.76 MHz (^{13}C). ^1H NMR and ^{13}C NMR chemical shifts were referenced to the solvent
25 signals; for ^1H , $\delta(\text{residual CHCl}_3) = 7.25$ ppm, $\delta(\text{residual DMSO} - d_6) = 2.50$ ppm, $\delta(\text{residual CD}_3\text{OD}) =$
26 3.31 ppm; ^{13}C : $\delta(\text{CDCl}_3) = 77.23$ ppm, $\delta(\text{DMSO}-d_6) = 39.52$ ppm, and $\delta(\text{CD}_3\text{OD}) = 49.15$ ppm. Chemical
27 shifts are provided in the δ scale [ppm] and the coupling constants are provided in Hz.
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35 Melting points were determined on a Kofler block and are uncorrected. Reagents were of
36 analytical grade from standard commercial sources or were synthesized according to the referenced
37 procedure. Thin layer chromatography (TLC) was performed using aluminum sheets with silica gel
38 F254 from Merck. Spots were visualized under UV light (254 nm). ESI or APCI mass spectra were
39 determined using a Waters Micromass ZMD mass spectrometer (sample solution in MeOH, direct
40 inlet, coin voltage was in the range of 10–30 V, trace amounts of HCOOH or NH_4OH was used to
41 influence ionization). Column chromatography was performed using Merck silica gel Kieselgel 60
42 (230–400 mesh). The purity of all synthesized compounds was determined by HPLC–PDA (200–500
43 nm) and gave satisfactory results (> 95 %). Specific optical rotation was measured on a polarimeter
44 polAAR 3001 (wave length: 589.0 nm, tube length: 50 mm, at 23 °C). All compounds gave satisfactory
45 elemental analyses ($\pm 0.4\%$).
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3 **3-Isopropyl-5-sulfanyl-1(2)H-pyrazolo[4,3-d]pyrimidine-7-ol (1)**. This compound was prepared
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5 according to literature.²⁸
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10 **3-Isopropyl-1(2)H-pyrazolo[4,3-d]pyrimidine-5,7-dithiol (2)**. 3-Isopropyl-5-sulfanyl-1(2)H-
11 pyrazolo[4,3-d]pyrimidin-7-ol (**1**) (74 g, 0,35 mol) was added to a solution of phosphorus pentasulfide
12 (93 g, 0.42 mol) in pyridine (550 mL) at 75 °C. The reaction mixture under nitrogen was refluxed for 3
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14 h. Then, the mixture was concentrated by the distillation of pyridine at atmospheric pressure to a
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16 syrupy consistency. Water (demineralized, approx. 400 mL) was added dropwise at 65 – 75 °C. The
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18 resulting foamy mixture was heated at 95 °C for 1.5 h, and then the mixture was cooled to r.t. and
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20 the solid product was filtered off and washed with water and EtOH (200 mL). The product was dried
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22 under vacuum at 75 °C for a total of 61.7 g and a 76% yield. The analytical sample was crystallized
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24 from THF (m.p. 283–293 °C; UV (MeOH, nm): 274 λ_{\max} , 320 λ_{\max} , 372 sh). MS APCI + m/z 227.1
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26 (M+H)⁺, APCI and ES – m/z 225.1 (M-H)⁻. ¹H (500 MHz; DMSO-*d*₆): 1.20 (d, *J* = 7.03 Hz, 6H, -CH(CH₃)₂);
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28 3.30 (bs, 1H); 13.33 (bs, 1H); 13.45 (bs, 1H); 13.84 (bs, 1H). ¹³C (125 MHz; DMSO-*d*₆): 21.9; 24.8;
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30 121.5; 133.0; 143.4; 170.1; 175.1.
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39 **3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol (3)**. 4-(2-
40 pyridyl)benzylamine (**2**) (26.2 g; 0.14 mol) was dissolved in 2-methoxyethanole (150 mL) at 50 °C, and
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42 *3-isopropyl-1(2)H-pyrazolo[4,3-d]pyrimidin-5,7-dithiol (2)* (23 g, 0.10 mol) was added under nitrogen
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44 gas. The reaction mixture was then refluxed for 8 h. The product crystallized after slow cooling at r.t.
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46 and then at 5–10 °C. The product was filtered off, washed with 2-methoxyethanole and then with
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48 THF and dried under vacuum at 75 °C. The product (30 g) was obtained as nearly white fine-grain
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50 crystals with a 78% yield. Titration in AcOH with 0.1 N HClO₄ gave 100% of the product. Analytical
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52 samples were crystallized from DMSO/H₂O and dried under vacuum at 75 °C (m.p. 263–272 °C; UV
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54 (MeOH, nm): 255 λ_{\max} , 289 λ_{\max} , 336 λ_{\max}). MS ESI + 377.1 (M+H)⁺, ESI – 375.1 (M-H)⁻. ¹H (500 MHz;
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56 DMSO-*d*₆): 1.24 (d, *J* = 7.03 Hz, 6H); 3.32 (bs, 1H); 3.45 (bs, 1H); 4.80 (bs, 2H); 7.31–7.33 (m, 1H); 7.46
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(bd, $J = 6.42$ Hz, 2H); 7.83–7.86 (m, 1H); 7.91–7.93 (m, 1H); 8.04 (bd, $J = 7.34$ Hz, 2H); 8.64 (bd, $J = 3.97$ Hz, 1H). ^{13}C (125 MHz; DMSO- d_6): 21.8; 42.5; 54.8; 120.0, 122.4, 126.4, 127.8, 137.0, 149.4, 155.7.

5-[3-(1-Piperazinyl)-2-hydroxy-1-propyl]thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine (4.1). The precursor of the synthesis, 3-(4-Boc-piperazin-1-yl)-1,2-epoxypropane, was prepared by equimolar reaction of epichlorohydrin with N-Boc-piperazine in acetonitrile in the presence of K_2CO_3 (2 equivalents); the reaction mixture was stirred at 10 °C for 2 days. The crude product (used in subsequent synthesis) was isolated by filtration and then dried under vacuum at a temperature below 45 °C.

The mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (380 mg, 1 mmol), tetramethylammonium hydroxide (0.2 mL 25% solution in MeOH), trimethylamine (0.2 mL, 2.2 mmol) and 3-(4-Boc-piperazin-1-yl)-1,2-epoxypropane (0.45 g, 1.8 mmol) in MeOH (5 mL) was stirred at room temperature for 2 days. The crude Boc product was isolated by precipitation after adding water. The precipitate was dissolved in 3 mL TFA and was left 2 h at room temperature. The solution was evaporated under vacuum, and the product was purified stepwise (5%, 8% and 10%) with MeOH in CHCl_3 with a trace amount of aqueous NH_4OH by column chromatography. Chromatography provided (after evaporation under vacuum) an amorphous colorless glass foam (0.25 g, 48% yield). ESI + 519.1 ($\text{M}+\text{H}^+$), ESI – 517.1 ($\text{M}-\text{H}^-$). ^1H (500 MHz; DMSO- d_6): 1.36 (d, $J = 7.03$ Hz, 6H, $-\text{CH}-\text{CH}_3$); 1.37 (d, $J = 7.03$ Hz, 6H, $-\text{CH}-\text{CH}_3$); 2.33–2.42 (m, 6H, $\text{N}-\text{CH}_2-\text{CH}-\text{OH} + 2 \times -\text{CH}_2-$); 2.77–2.79 (m, 4H, $2 \times -\text{CH}_2-$); 3.01 (dd, $J = 13.45$ Hz, $J = 7.03$ Hz, 1H, $-\text{S}-\text{CH}_\alpha\text{H}_\beta-$); 3.27 (sept., $J = 7.03$ Hz, 1H, $-\text{CH}(\text{CH}_3)_2$); 3.35 (dd, $J = 13.60$ Hz, $J = 4.58$ Hz, 1H, $-\text{S}-\text{CH}_\alpha\text{H}_\beta-$); 3.90 (m, 1H, $-\text{CH}_2-\text{CH}-\text{OH}$); 4.78 (bs, 2H, $-\text{NH}-\text{CH}_2-$); 7.33 (qd, $J = 7.41$ Hz, $J = 4.89$ Hz, $J = 1.22$ Hz, 1H, H_{Ar}); 7.49 (d, $J = 8.25$ Hz, 2H, H_{Ar}); 7.86 (td, $J = 7.95$ Hz, $J = 1.83$ Hz, 1H, H_{Ar}); 7.93 (dt, $J = 8.25$ Hz, $J = 1.1$ Hz, 1H, H_{Ar}); 8.06 (d, $J = 8.25$ Hz, 2H, H_{Ar}); 8.34 (bs, 1H, $-\text{NH}-\text{CH}_2-$); 8.65 (qd, $J = 4.86$ Hz, $J = 1.83$ Hz, $J = 0.92$ Hz, 1H,

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3 H_{Ar}); 12.80 (bs, 1H, -NH-). ¹³C (125 MHz; DMSO-*d*₆): 21.6; 21.7; 25.7; 36.0; 42.9; 44.7; 53.1; 63.9; 67.2;
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5 120.0; 122.4; 126.5; 127.9; 137.1; 137.5; 138.3; 139.8; 149.4; 150.1; 155.7; 157.9; 161.2.
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10 **5[(3-(4-Morpholinyl)-2-hydroxy-1-propyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-**

11 **pyrazolo[4,3-d]pyrimidine (4.2).** 1-Chloro-3-morpholinopropan-2-ol (0.40 g, 2.2 mmol; prepared
12 according to the literature⁴⁴) was added to a mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-
13 1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (0.38 g, 1 mmol) and K₂CO₃ (0.17 g) in MeOH (10 mL), and
14 the mixture was stirred at 45 °C for 24 h. The crude product was precipitated by adding water and
15 then purified stepwise (3%, 4% and 5%) with MeOH in CHCl₃ by column chromatography.
16 Chromatography provided (after evaporation under vacuum) an amorphous colorless glass foam
17 (0.31 g, 60% yield). MS ESI + 520.1 (M+H)⁺ 15%, 542.1 (M+Na)⁺ 100%, ESI – 518.1 (M-H)⁻ 30%, 554.2
18 (M+Cl)⁻ 100%. ¹H (500 MHz; CDCl₃): 1.19–1.21 (m, 6H, -CH(CH₃)₂); 2.31–2.35 (m, 3H, -CH-CH_αH_β-N- + -
19 N-(CH_αH_β)₂-); 2.42–2.46 (m, 2H, -N-(CH_αH_β)₂-); 2.53 (dd, *J* = 12.53 Hz, *J* = 8.56 Hz, 1H, -CH-CH_αH_β-N);
20 3.11–3.19 (m, 2H, -CH(CH₃)₂ + S-CH_αH_β-CH-); 3.27 (d, *J* = 12.53 Hz, 1H, S-CH_αH_β-CH-); 3.50 (bs, 4H, O-
21 (CH₂)₂-); 4.09–4.10 (m, 1H, -CH₂-CH-CH₂-); 4.65 (bs, 2H, -NH-CH₂-); 7.17 (dd, *J* = 7.18 Hz, *J* = 5.20 Hz,
22 1H, H_{Ar}); 7.23 (d, *J* = 7.95 Hz, 2H, H_{Ar}); 7.42 (bs, 1H, -NH-); 7.59 (d, *J* = 7.95 Hz, 1H, H_{Ar}); 7.69 (td, *J* =
23 7.79 Hz, *J* = 1.83 Hz, 1H, H_{Ar}); 7.73 (d, *J* = 7.95 Hz, 2H, H_{Ar}); 8.58 (d, *J* = 4.58 Hz, 1H, H_{Ar}). ¹³C (125 MHz;
24 CDCl₃): 21.5; 21.6; 26.1; 36.3; 44.2; 53.8; 63.9; 66.5; 68.5; 120.7; 122.2; 127.0; 128.1; 137.0; 138.3;
25 138.6; 149.4; 156.8; 162.9.
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48 **5-(1-Hydroxy-2-butyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

49 **d]pyrimidine (4.3).** The precursor of the synthesis, 2-bromo-1-butanol, was obtained by introducing
50 diborane gas into a THF solution of 2-bromobutanoic acid at -10 °C for 5 h, then at 0 °C for 5 h, and
51 finally at 25 °C for 2 h. The excess diborane was consumed by adding 50% aq. HCOOH, and the
52 mixture was then concentrated by evaporation (15 torr, 25 °C). The remaining solution was
53 neutralized by aq. NaHCO₃, and the product was extracted by EtOAc. The combined organic phase
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3 was dried (Na_2SO_4) and evaporated (15 torr, 25 °C). The crude product ($\rho = 1.38 \text{ g/mL}$) was directly
4
5 used in the subsequent alkylation reaction.
6

7
8 The mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-
9
10 thiol **3** (0.225 g, 0.66 mmol), DMF (5 mL), K_2CO_3 (0.2 g) and 2-bromo-1-butanol (120 μL , 1.0 mmol)
11
12 was stirred at 50 °C for 8 h. Subsequently, the reaction mixture was evaporated at a temperature
13
14 below 50 °C, and the residue was partitioned between EtOAc and H_2O . The combined organic phase
15
16 was dried (MgSO_4) and evaporated under vacuum. The product was finally purified stepwise (2%, 3%
17
18 and 4%) with MeOH in CHCl_3 by column chromatography and was crystallized from absolute Et_2O
19
20 (0.07 g, 22% yield, m.p. 126–129 °C). MS ESI + 449.1 (M+H)⁺, ESI – 447.1 (M-H)⁻. NMR: ¹H (500 MHz,
21
22 $\text{DMSO-}d_6$) δ 0.96 (t, $J = 7.4 \text{ Hz}$, 3H, - CH_3); 1.35 – 1.39 (m, 6H, - $\text{CH}(\text{CH}_3)_2$); 1.55 – 1.63 (m, 1H, - $\text{CH}_\alpha\text{H}_\beta$ -
23
24 CH_3); 1.81 – 1.88 (m, 1H, - $\text{CH}_\alpha\text{H}_\beta$ - CH_3); 3.24 (m, $J = 6.9 \text{ Hz}$, 1H, - $\text{CH}(\text{CH}_3)_2$); 3.50 – 3.54 (m, 1H, -S-CH-);
25
26 3.70 – 3.75 (m, 2H, - CH_2 -OH); 4.77 (t, $J = 4.9 \text{ Hz}$, 2H, NH- CH_2 -); 4.87 (bs, 1H, -OH), 7.32 – 7.35 (m, 1H,
27
28 H_{Ar}); 7.45 – 7.55 (m, 2H, H_{Ar}); 7.85 – 7.95 (m, 2H, H_{Ar}); 8.00 – 8.09 (m, 3H, H_{Ar} + -NH); 8.65 – 8.66 (m,
29
30 1H, H_{Ar}); 12.16 (s, 1H, -NH). ¹³C (125 MHz, $\text{DMSO-}d_6$) δ 11.3, 21.5, 21.6, 21.7, 23.6, 24.9, 26.3, 40.4,
31
32 43.1, 48.5, 63.1, 120.1, 120.4, 122.3, 122.5, 126.3, 126.6, 127.7, 128.0, 130.4, 137.1, 137.6, 138.6,
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34 139.6, 139.9, 140.6, 148.6, 148.7, 149.4, 153.7, 155.6, 155.8, 161.1.
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41 **5-(2-Methylthio-1-ethyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

42 **d]pyrimidine (4.4).** To a solution of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-
43
44 d]pyrimidin-5-thiol **3** (0.38 g, 1.0 mmol) in DMF (6 mL) K_2CO_3 (0.2 g) and 2-chloroethyl methyl sulfide
45
46 (101 μL , 1 mmol) were added, and the mixture was stirred at room temperature for 16 h. The
47
48 reaction mixture was evaporated at a temperature below 50 °C, and the residue was partitioned
49
50 between CHCl_3 and H_2O . The combined organic phase was dried with sodium sulfate and evaporated
51
52 under vacuum. The product was purified stepwise (2%, 3% and 4%) with MeOH in CHCl_3 by column
53
54 chromatography, and the product was crystallized from abs. Et_2O (0.35 g, 74% yield, m.p. 140 – 141
55
56 °C). APCI + 474.3 (M+H)⁺, ESI – 472.2 (M-H)⁻. ¹H (500 MHz; CDCl_3): 1.33 (d, $J = 7.03 \text{ Hz}$, 6H, - $\text{CH}(\text{CH}_3)_2$);
57
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3 2.12 (s, 3H, -S-CH₃); 2.80–2.83 (m, 2H, H₃C-S-CH₂-); 3.26–3.30 (m, 3H, -S-CH₂- + -CH(CH₃)₂); 4.62 (bs,
4 2H, -NH-CH₂-); 6.66 (bs, 1H, -NH-CH₂-); 7.15–7.22 (m, 3H, H_{Ar}); 7.61 (d, *J* = 8.25 Hz, 1H, H_{Ar}); 7.70–7.74
5
6 (m, 3H, H_{Ar}); 8.59 (d, *J* = 4.89 Hz, 1H, H_{Ar}); 12.13 (bs, 1H, -NH-). ¹³C (125 MHz; CDCl₃): 15.2; 21.6; 26.2;
7
8 30.6; 34.0; 44.0; 121.0; 122.4; 127.0; 127.9; 137.2; 138.1; 138.7; 149.3; 157.0; 161.7.
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14 **5-(2-Hydroxycyclohexyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

15 **d]pyrimidine (4.5).** The 1,2-epoxycyclohexane (0.12 mL, 1.15 mmol) in MeOH (2 mL) was added to a
16
17 stirred mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3**
18
19 (0.38 g, 1 mmol) and tetramethylammonium hydroxide (catalytic amount, 80 mg) in MeOH (6 mL),
20
21 and the mixture was stirred at 40 °C for 7 h. The reaction mixture was evaporated at a temperature
22
23 below 50 °C, and the residue was partitioned between 2-methyltetrahydrofuran and H₂O. The
24
25 combined organic phase was dried with magnesium sulfate and evaporated under vacuum. The
26
27 product was purified stepwise (2%, 3% and 4%) with MeOH in CHCl₃ by column chromatography.
28
29 Chromatography provided (after evaporation under vacuum) an amorphous colorless glass foam
30
31 (0.22 g, 46% yield). MS ESI + 475.1 (M+H)⁺, ESI – 473.1 (M-H)⁻. ¹H (500 MHz; CDCl₃): 1.18 (d, *J* = 7.03
32
33 Hz, 3H, -CH₃); 1.20–1.22 (m, 2H, -CH_αH_β-CH₂-CH-S + -CH_αH_β-CH₂-CH-OH); 1.24 (d, *J* = 7.03 Hz, 3H, -
34
35 CH₃); 1.33–1.40 (m, 2H, -CH_αH_β-CH-S- + -CH_αH_β-CH-OH); 1.68–1.70 (m, 2H, -CH_αH_β-CH₂-CH-S- + -CH_αH_β-
36
37 CH₂-CH-OH); 2.08–2.17 (m, 2H, -CH_αH_β-CH-S- + -CH_αH_β-CH-OH); 3.19 (sept., *J* = 7.03 Hz, 1H, -
38
39 CH(CH₃)₂); 3.45 (ddd, *J* = 13.21 Hz, *J* = 10.09 Hz, *J* = 3.97 Hz, 1H, -S-CH-); 3.54 (td, *J* = 10.70 Hz, *J* = 4.26
40
41 Hz, 1H, -CH-OH); 4.65 (bs, 2H, -NH-CH₂-); 7.17–7.21 (m, 3H, H_{Ar}); 7.32 (bs, 1H, -NH-CH₂-); 7.58 (d, *J* =
42
43 7.95 Hz, 1H, H_{Ar}); 7.68–7.72 (m, 3H, H_{Ar}); 8.58 (d, *J* = 4.89 Hz, 1H, H_{Ar}). ¹³C (125 MHz; CDCl₃): 21.3;
44
45 21.7; 24.0; 26.0; 26.2; 30.2; 31.6; 36.5; 44.0; 51.2; 77.4; 120.8; 121.1; 127.0; 127.9; 137.0; 138.2;
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47 138.6; 149.3; 157.0; 163.6.
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55 **5-(3,3,3-Trifluoro-2-hydroxy-1-propyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-**

56
57 **pyrazolo[4,3-d]pyrimidine (4.6).** 2-Bromo-1,1,1-trifluoro-2-propanol (0.11 mL, 1.05 mmol) was
58
59
60

1
 2
 3 added dropwise to a mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-
 4 d]pyrimidine-5-thiol **3** (0.38 g, 1.0 mmol) in DMF (6 mL) and K₂CO₃ (0.12 g), and the reaction mixture
 5
 6
 7 was stirred at room temperature for 4 h. The reaction mixture was evaporated at a temperature
 8
 9 below 50 °C, and the residue was partitioned between CHCl₃ and H₂O. The product was purified
 10
 11 stepwise (2%, 3% and 4%) with MeOH in CHCl₃ by column chromatography. The product was
 12
 13 crystallized from DCM (0.15 g, 31% yield, m.p. 174–177 °C). MS ESI + 489.1 (M+H)⁺, ESI – 487.1 (M-H)⁻.
 14
 15 . NMR: Two tautomers: 2:1 (integration showed in relative values) ¹H (500 MHz; DMSO-*d*₆): 1.35 (d, *J*
 16
 17 = 7.03 Hz, 6H, -CH(CH₃)₂ form I); 1.38 (d, *J* = 7.03 Hz, 6H, -CH(CH₃)₂ form II); 2.83–2.92 (m, 1H, -S-
 18
 19 CH_αH_β- form I and II); 3.21–3.27 (m, H, -CH(CH₃)₂, form I and II); 3.62 (d, *J* = 13.75 Hz, 1H, -S-CH_αH_β,
 20
 21 form II); 3.68 (dd, *J* = 13.75 Hz, *J* = 2.14 Hz, 1H, -S-CH_αH_β, form I); 4.23 (bs, 1H, S-CH₂-CH-form I and II);
 22
 23 4.72 (d, *J* = 5.81 Hz, 2H, NH-CH₂-, form II); 4.78 (d, *J* = 5.50 Hz, 2H, NH-CH₂-, form I); 6.59 (d, *J* = 6.72
 24
 25 Hz, 1H, CH-OH, form I and II); 7.32–7.35 (m, 1H, H_{Ar}, form I and II); 7.45 (d, *J* = 7.95 Hz, 2H, H_{Ar}, form
 26
 27 II); 7.50 (d, *J* = 8.25 Hz, 2H, H_{Ar}, form I); 7.82–7.87 (m, 1H, H_{Ar}, form I and II); 7.90–7.95 (m, 1H, H_{Ar},
 28
 29 form I and II); 8.01 (d, *J* = 7.95 Hz, 2H, H_{Ar}, form II); 8.08–8.10 (m, 3H, H_{Ar} + -NH-CH₂-, form I); 8.64–
 30
 31 8.66 (m, 1H, H_{Ar}, form I and II); 8.79 (bt, *J* = 5.81 Hz, 1H, -NH-CH₂-, form II); 12.22 (bs, 1H, -NH-, form I);
 32
 33 13.80 (bs, 1H, -NH-, form II). ¹³C (125 MHz; DMSO-*d*₆): mixture of two forms 21.3; 21.4; 21.5; 25.0;
 34
 35 26.3; 26.4; 31.2; 42.4; 43.1; 67.9 (q, -CF₃, form I); 68.0 (q, -CF₃, form II); 120.0; 120.1 120.5; 122.3;
 36
 37 122.5; 124.2; 126.3; 126.5; 126.6; 127.6; 128.0; 130.5; 134.8; 137.10; 137.15; 137.2 137.7; 138.9;
 38
 39 139.3; 139.7; 140.3; 148.8; 149.43; 149.48; 154.0; 155.6; 155.8; 160.0.

5-(Prop-2-en-1-yl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine

48
 49 **(4.7)**. A 1N solution of NaOH (1.4 mL) and allyl bromide (0.13 mL, 1.1 mmol) was added to a
 50
 51 suspension of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3**
 52
 53 (0.38 g, 1.00 mmol) in H₂O (20 mL), and the mixture was stirred at room temperature for 6 h. The
 54
 55 product was filtered off and recrystallized from CHCl₃/Et₂O (0.28 g, 67% yield, m.p. 148–150 °C). MS
 56
 57 ESI + 417.2 (M+H)⁺, ESI – 415.3 (M-H)⁻. ¹H (500 MHz; DMSO-*d*₆): 1.38 (d, *J* = 6.72 Hz, 6H, -CH(CH₃)₂);
 58
 59
 60

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2
3 3.28 (sept., $J = 7.03$ Hz, 1H, $-\underline{CH}(\text{CH}_3)_2$); 3.74 (d, $J = 7.03$ Hz, 2H, $-\text{S}-\underline{CH}_2-\text{CH}$); 4.77 (bs, 2H, $\text{NH}-\underline{CH}_2-$);
4
5 5.02 (dd, $J = 9.78$ Hz, $J = 0.92$ Hz, 1H, $\underline{CH}_\alpha\text{H}_\beta=\text{CH}-$); 5.24 (dd, $J = 16.96$ Hz, $J = 1.53$ Hz, 1H, $\text{CH}_\alpha\text{H}_\beta=\text{CH}-$);
6
7 5.95 (ddt, $J = 17.1$ Hz, $J = 10.1$ Hz, $J = 7.03$ Hz, 1H, $\text{CH}_2=\underline{CH}-\text{CH}_2-$); 7.32 (ddd, $J = 7.39$ Hz, $J = 4.89$ Hz, $J =$
8
9 1.22 Hz, 1H, H_{Ar}); 7.49 (d, $J = 8.56$ Hz, 2H, H_{Ar}); 7.84 (td, $J = 7.64$ Hz, $J = 1.83$ Hz, 1H, H_{Ar}); 7.92 (d, $J =$
10
11 7.95 Hz, 1H, H_{Ar}); 8.06 (d, $J = 8.25$ Hz, 2H, H_{Ar}); 8.27 (bs, 1H, $-\underline{NH}-\text{CH}_2-$); 8.65 (ddd, $J = 4.86$ Hz, $J = 1.83$
12
13 Hz, $J = 0.92$ Hz, 1H, H_{Ar}); 12.58 (bs, 1H, $-\underline{NH}-$). ^{13}C (125 MHz; $\text{DMSO}-d_6$): 21.6; 25.8; 33.0; 42.9; 116.7;
14
15 120.0; 122.4; 126.5; 127.8; 134.9; 137.1; 137.5; 139.8; 149.4; 155.7; 160.1.
16
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21 **5[3-(Dimethylamino)-1-propyl]thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

22 **d]pyrimidine (4.8).** K_2CO_3 (0.6 g) and 3-chloro-N,N-dimethylpropylamine hydrochloride (182 mg, 1.15
23
24 mmol) were added to a solution of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-
25
26 d]pyrimidine-5-thiol **3** (0.38 g, 1 mmol) in DMSO (10 mL), and the mixture was stirred at 60 °C for 24
27
28 h. The reaction mixture was evaporated at a temperature below 50 °C, and the residue was
29
30 partitioned between CHCl_3 and H_2O . The product was purified stepwise (5%, 8% and 10%) with
31
32 MeOH in CHCl_3 by column chromatography. Chromatography provided (after evaporation under
33
34 vacuum) an amorphous colorless glass foam (0.33 g, 72% yield). MS ESI + 231.6 ($\text{M}+2\text{H}$) $^{2+}$ 50%, 462.1
35
36 ($\text{M}+\text{H}$) $^+$ 100%, ESI – 460.1 ($\text{M}-\text{H}$) $^-$. ^1H (500 MHz; CDCl_3): 1.34 (d, $J = 6.72$ Hz, 6H, $-\text{CH}(\underline{\text{CH}}_3)_2$); 1.92 (pent.,
37
38 $J = 7.03$ Hz, 2H, $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$); 2.22 (s, 6H, $-\text{N}(\text{CH}_3)_2$); 2.44 (t, $J = 7.34$ Hz, 2H, $-\text{CH}_2-$); 3.09 (t, $J = 7.03$
39
40 Hz, 2H, $-\text{CH}_2-$); 3.29 (sept., $J = 6.72$ Hz, 1H, $-\underline{CH}(\text{CH}_3)_2$); 4.68 (bs, 2H, $\text{NH}-\underline{CH}_2-$); 6.87 (bs, 1H, $-\text{NH}-$);
41
42 7.18–7.20 (m, 1H, H_{Ar}); 7.27 (d, $J = 7.64$ Hz, 2H, H_{Ar}); 7.60 (d, $J = 7.95$ Hz, 1H, H_{Ar}); 7.70 (t, $J = 7.93$ Hz,
43
44 1H, H_{Ar}); 7.76 (d, $J = 7.64$ Hz, 2H, H_{Ar}); 8.60 (d, $J = 3.97$ Hz, 1H, H_{Ar}). ^{13}C (125 MHz; CDCl_3): 21.7; 26.2;
45
46 27.4; 28.9; 29.6; 43.9; 45.1; 58.5; 120.8; 122.2; 127.0; 127.9; 137.0; 138.0; 139.0; 149.3; 150.6; 156.9;
47
48 162.0.
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52
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54

55 **5-[2-(Dimethylamino)-1-ethyl]thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

56
57 **d]pyrimidine (4.9).** K_2CO_3 (0.6 g) and 2-chloro-N,N-dimethylethylamine hydrochloride (166 mg, 1.15
58
59
60

mmol) were added to a solution of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidin-5-thiol **3** (0.38 g, 1 mmol) in DMSO (10 mL), and the mixture was stirred at 60 °C for 24 h. The reaction mixture was evaporated at a temperature below 50 °C, and the residue was partitioned between CHCl₃ and H₂O. The product was purified stepwise (5%, 8% and 10%) with MeOH in CHCl₃ by column chromatography. Chromatography provided (after evaporation under vacuum) an amorphous colorless glass foam (0.30 g, 67% yield). MS ESI + 224.5 (M+2H)²⁺ 70%, 448.1 (M+H)⁺ 100%, ESI – 446.1 (M-H). ¹H (500 MHz; CDCl₃): 1.31 (d, *J* = 7.03 Hz, 6H, -CH-(CH₃)₂); 2.24 (s, 6H, 2x -CH₃); 2.66 (t, *J* = 7.64 Hz, 2H, -CH₂-); 3.20 (t, *J* = 7.34, 2H, -CH₂-); 3.27 (sept.; *J* = 7.03 Hz, 1H, -CH-(CH₃)₂); 4.62 (bs, 2H, -NH-CH₂-); 6.97 (bs, 1H, -NH-CH-); 7.15–7.16 (m, 1H, ArH); 7.17 (d, *J* = 7.95 Hz, 2H, ArH); 7.55 (d, *J* = 8.25 Hz, 1H, ArH); 7.66 (dd, *J* = 7.64 Hz, *J* = 1.83 Hz, 1H, ArH); 7.69 (d, *J* = 7.95 Hz, 2H, ArH); 8.55 (d, *J* = 3.97 Hz, 1H, ArH). ¹³C (125 MHz; CDCl₃): 21.6; 26.2; 28.0; 43.9; 44.9; 59.0; 120.8; 122.2; 126.9; 127.9; 137.0; 138.0; 138.9; 149.3; 150.6; 156.9; 161.6.

5-(1-Hydroxy-3-methyl-2-butyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine (4.10). The precursor 2-bromo-3-methyl-1-butanol was prepared from 3-methylbutanal by bromination with a bromine-dioxane complex (1:1) according to the literature.⁴⁵ The obtained 2-bromo-3-methylbutanal was then reduced to the desired 2-bromo-3-methyl-1-butanol in the usual manner using NaBH₄ and was used for alkylation without purification.

K₂CO₃ (0.6 g) and 2-bromo-3-methyl-1-butanol (0.25 g, 1.5 mmol) were added to a solution of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (0.38 g, 1 mmol) in DMSO (10 mL), and the mixture was stirred at room temperature for 48 h. The reaction mixture was kept at a temperature below 50 °C. The residue was partitioned between CHCl₃ and H₂O, and the product was purified stepwise (2%, 3% and 4%) with MeOH in CHCl₃ by column chromatography. Chromatography provided (after evaporation under vacuum) an amorphous colorless glass foam (0.31 g, 67% yield). MS ESI + 463.1 (M+H)⁺, ESI – 461.1 (M-H). ¹H (500 MHz; CDCl₃): 0.92 (d, *J* = 6.72 Hz, 3H, -CH₃); 0.97 (d, *J* = 6.72 Hz, 3H, -CH₃); 1.23 (d, *J* = 6.72 Hz, 3H, -CH₃); 1.24 (d, *J* = 7.03 Hz, 3H, -

1
2
3 CH₃); 1.83 (sex., $J = 6.72$ Hz, 1H, -CH-CH-(CH₃)₂); 3.14–3.27 (m, 3H, -CH(CH₃)₂, -CH₂-); 3.68–3.71 (m, 1H,
4 -CH-); 4.66 (bs, 2H, NH-CH₂-); 7.04 (bs, 1H, -NH-CH₂-); 7.18–7.22 (m, 3H, H_{Ar}); 7.61 (d, $J = 7.95$ Hz, 1H,
5 H_{Ar}); 7.70 (dd, $J = 7.64$ Hz, $J = 1.83$ Hz, 1H, H_{Ar}); 7.73 (d, $J = 7.95$ Hz, 2H, H_{Ar}); 8.59 (d, $J = 4.89$ Hz, 1H,
6 H_{Ar}), 11.9 (bs, 1H, -NH-). ¹³C (125 MHz; CDCl₃): 18.0; 18.3; 21.4; 21.6; 26.1; 33.5; 35.7; 44.1; 78.4;
7
8
9
10
11
12 120.9; 122.2; 127.1; 128.0; 137.0; 138.3; 138.6; 149.3; 157.0; 163.7.
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14
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17 **5-(Methoxymethyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine**

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19 **(4.11)**. Chloromethyl methyl ether (31 μ L, 0.41 mmol) was added to a mixture of 3-isopropyl-7-[4-(2-
20 pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidin-5-thiol **3** (0.14 g, 0.37 mmol) and K₂CO₃ (0.10 g)
21 in DMF (3 mL), and then the mixture was stirred at room temperature for 4 h. The reaction mixture
22 was evaporated at a temperature below 50 °C, and the residue was partitioned between CHCl₃ and
23 H₂O. The product was purified stepwise (1%, 2% and 3% MeOH) in CHCl₃ by column chromatography.
24
25 The product was crystallized from DCM (0.11 g, 70% yield, m.p. 160–162 °C). MS ESI + 421.1 (M+H)⁺,
26 ESI – 419.1 (M-H)⁻. ¹H (500 MHz; DMSO-*d*₆): mixture of two tautomeric forms. 1.36–1.40 (m, 6H, -
27 CH(CH₃)₂); 3.22–3.29 (m, 4H, -CH(CH₃)₂, -CH₃); 4.74–4.81 (m, 2H, NH-CH₂-); 5.31–5.36 (m, 2H, -S-CH₂-);
28 7.32–7.35 (m, 1H, H_{Ar}); 7.47–7.53 (m, 2H, H_{Ar}); 7.85–7.88 (m, 1H, H_{Ar}); 7.90–7.95 (m, 1H, H_{Ar}); 8.02–
29 8.10 (m, 2H, H_{Ar}); 8.65–8.66 (m, 1H, H_{Ar}); 12.23 (bs, -NH-, form I); 13.81 (bs, -NH-, form II). ¹³C (125
30 MHz; DMSO-*d*₆): mixture of two tautomeric forms. 21.6; 21.7; 24.8; 26.2; 42.6; 43.2; 55.9; 73.4; 120.0
31
32 120.6; 122.3; 122.4; 126.3; 126.6; 127.6; 128.0; 137.1; 137.7; 139.4; 139.9; 148.7; 148.9; 149.4;
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34 153.9; 155.6; 155.8; 159.2.
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50 **5-(2-Hydroxycyclopentyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

51 **d]pyrimidine (4.12)**. A solution of 1,2-epoxycyclopentane (0.10 mL, 1.15 mmol) in MeOH (2 mL) was
52 added to a stirred mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-
53 d]pyrimidine-5-thiol **3** (0.38 g, 1 mmol) and tetramethylammonium hydroxide (catalytic amount, 80
54 mg) in MeOH (6 mL), and the mixture was stirred at 40 °C for 7 h. The reaction mixture was
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3 evaporated at a temperature below 50 °C, and the residue was partitioned between 2-
4 methyltetrahydrofuran and H₂O. The combined organic phase was dried with magnesium sulfate and
5
6 methyltetrahydrofuran and H₂O. The combined organic phase was dried with magnesium sulfate and
7 evaporated under vacuum. The product was purified stepwise (2%, 3% and 4%) with MeOH in CHCl₃
8
9 by column chromatography. Crystallization from CHCl₃ gave 0.25 g (54% yield, m.p. 148–150 °C). MS
10
11 ESI + 461.1 (M+H)⁺, ESI – 459.1 (M-H)⁻. ¹H (500 MHz; CDCl₃): 1.17 (d, *J* = 6.42 Hz, 3H, -CH₃); 1.20 (d, *J* =
12
13 6.72 Hz, 3H, -CH₃); 1.45–1.53 (m, 1H, -S-CH-CH_αH_β-); 1.64–1.78 (m, 3H, -CH₂-CH₂-CH₂- + HO-CH-CH_αH_β-
14
15); 2.02–2.12 (m, 2H, -S-CH-CH_αH_β- + HO-CH-CH_αH_β-); 3.17 (sept., *J* = 7.03 Hz, 1H, -CH(CH₃)₂); 3.60–3.65
16
17 (m, 1H, -S-CH-CH₂); 4.20–4.24 (m, 1H, HO-CH-CH₂-); 4.67 (bs, 2H, NH-CH₂-); 7.17–7.25 (m, 3H, H_{Ar});
18
19 7.60 (d, *J* = 7.64 Hz, 1H, H_{Ar}); 7.68–7.74 (m, 3H, H_{Ar}); 8.58–8.59 (m, 1H, H_{Ar}); 12.13 (bs, 1H, NH). ¹³C
20
21 (125 MHz; CDCl₃): 21.3; 21.8; 22.7; 25.8; 29.7; 34.4; 44.2; 50.6; 82.7; 120.8; 122.3; 127.1; 128.2;
22
23 137.0; 138.3; 138.4; 149.3; 156.8; 164.4.

30 **5-(2-Aminocyclohexyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

31 **d]pyrimidine (4.13).** 1,2-Cyclohexenimine (0.60 mL, 5 mmol; prepared according to the literature⁴⁶)
32
33 was added dropwise at room temperature to a stirred mixture of 3-isopropyl-7-[4-(2-
34
35 pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (0.38 g, 1.0 mmol) and 1 mL of aq.
36
37 48% HBr solution in DMF (10 mL). The mixture was stirred for 24 h. The reaction mixture was then
38
39 neutralized with an aq. solution of Na₂CO₃, and the crude product was isolated as a precipitate. The
40
41 product was purified, stepwise (5%, 8% and 11%) with MeOH in CHCl₃, with a trace amount of aq.
42
43 NH₄OH, by column chromatography. Chromatography provided a product crystallized from CHCl₃
44
45 (0.06 g, 13% yield, m.p. 165–185 °C). MS APCI + 474.4 (M+H)⁺, APCI – 442.2 (M-H)⁻. ¹H (500 MHz;
46
47 DMSO-*d*₆): 1.15–1.31 (m, 4H, H-cyclohexyl); 1.38 (d, *J* = 7.03 Hz, 6H, -CH(CH₃)₂); 1.44–1.49 (m, 1H, H-
48
49 cyclohexyl); 1.59–1.67 (m, 1H, H-cyclohexyl); 1.89–1.92 (m, 1H, H-cyclohexyl); 2.20–2.23 (m, 1H, H-
50
51 cyclohexyl); 2.64 (td, *J* = 10.09 Hz, *J* = 3.97 Hz, 1H, H-cyclohexyl); 3.23–3.28 (m, 3H, -CH(CH₃)₂, -CH₂-);
52
53 3.36–3.45 (m, 1H, H-cyclohexyl); 4.72–4.81 (m, 2H, NH-CH₂-); 7.33 (ddd, *J* = 7.34 Hz, *J* = 4.86 Hz, *J* =
54
55 1.22, 1H, H_{Ar}); 7.48 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 7.86 (td, *J* = 7.34 Hz, *J* = 1.83 Hz, 1H, H_{Ar}); 7.93 (d, *J* = 7.95
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3 Hz, 1H, H_{Ar}); 8.06 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 8.29 (bs, 1H, -NH-); 8.65 (ddd, *J* = 4.89 Hz, *J* = 1.53 Hz, *J* =
4
5 0.92 Hz, 1H, H_{Ar}). ¹³C (125 MHz; DMSO-*d*₆): 21.5; 21.6; 24.3; 25.7; 30.6; 32.8; 35.0; 42.9; 52.2; 52.6;
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7 79.1; 120.0; 122.4; 126.5; 127.7; 137.1; 137.4; 139.8; 149.4; 155.7; 160.8.
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12 **3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-5-methylsulfanyl-1(2)H-pyrazolo[4,3-d]pyrimidine (4.14).**

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14 This compound was prepared according to the literature.³⁰
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19 **5-(2-Acetylamino-1-ethyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

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21 **d]pyrimidine (4.15).** This compound was isolated by column chromatography as a byproduct of the
22
23 synthesis of 5-(2-ureido-1-ethyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-
24
25 d]pyrimidine (4.27). The isolation yielded an amorphous colorless glass foam (0.08 g, 20% yield). MS
26
27 ESI + 232.5 (M+2H)²⁺ 100%, 463.1 (M+H)⁺ 60% , ESI – 461.1 (M-H)⁻. NMR: mixture of tautomers: ¹H
28
29 (500 MHz, CDCl₃) δ 1.36 (d, *J* = 7.0 Hz, 6H, -CH(CH₃)₂); 1.77–1.78 (m, 3H, -CH₃); 3.18 (t, *J* = 5.8 Hz, 2H, -
30
31 CH₂-CH₂-); 3.31 (sept, *J* = 7.0 Hz, 1H, -CH(CH₃)₂); 3.47 (d, *J* = 5.5 Hz, 2H, -CH₂-CH₂-); 4.73 (d, *J* = 3.7 Hz,
32
33 2H, NH-CH₂-); 7.08 (bs, 1H, -NH); 7.18–7.21 (m, 1H, H_{Ar}); 7.36 (d, *J* = 8.3 Hz, 2H, H_{Ar}); 7.61 (d, *J* = 7.9
34
35 Hz, 1H, H_{Ar}); 7.69–7.73 (m, 1H, H_{Ar}); 7.82 (d, *J* = 8.3 Hz, 2H, H_{Ar}); 8.60 (d, *J* = 4.9 Hz, 1H, H_{Ar}). ¹³C (125
36
37 MHz, CDCl₃): 21.8; 23.1; 26.3; 30.3; 40.5; 44.2; 120.7; 122.3; 127.1; 128.1; 137.1; 138.2; 138.8; 149.3;
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39 150.1; 156.7; 161.8; 171.3.
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46 **5-(2-Hydroxy-1-butyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

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48 **d]pyrimidine (4.16).** A solution of 1,2-epoxybutane (0.1 mL, 1.15 mmol) in MeOH (2 mL) was added
49
50 to a stirred mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-
51
52 thiol **3** (0.38 g, 1 mmol) and tetramethylammonium hydroxide (catalytic amount, 60 mg) in MeOH (6
53
54 mL), and the mixture was stirred at room temperature for 10 h. The product was precipitated after
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56 adding water and then purified stepwise (1%, 2%, and 3%) with MeOH in CHCl₃ by column
57
58 chromatography. Chromatography yielded (after evaporation under vacuum) an amorphous colorless
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3 glass foam (0.28 g, 62% yield). MS ESI + 449.2 (M+H)⁺ 20%, 471.3 (M+Na)⁺ 100%, ESI – 447.2 (M-H)⁻.
4
5 ¹H (500 MHz; CDCl₃): 0.89 (t, *J* = 7.34 Hz, 3H, -CH₃); 1.18 (d, *J* = 5.83 Hz, 6H, -CH(CH₃)₂); 1.51–1.66 (m,
6
7 2H, -CH-CH₂-CH₃); 3.09 (dd, *J* = 14.98 Hz, *J* = 7.03, 1H, -S-CH_αH_βCH-); 3.18 (sept., *J* = 6.72 Hz, 1H, -
8
9 CH(CH₃)₂); 3.27 (bd, *J* = 14.67 Hz, 1H, -S-CH_αH_βCH-); 3.89–3.91 (m, 1H, -CH-OH); 4.64 (bs, 2H, -NH-
10
11 CH₂-); 7.16–7.19 (m, 3H, H_{Ar}); 7.57 (d, *J* = 7.95 Hz, 1H, H_{Ar}); 7.67–7.71 (m, 3H, H_{Ar}); 8.56 (d, *J* = 3.67 Hz,
12
13 1H, H_{Ar}). ¹³C (125 MHz; CDCl₃): 9.8; 21.50; 21.56; 26.1; 29.4; 37.6; 44.0; 74.3; 120.9; 122.2; 127.0;
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15 127.9; 137.0; 138.1; 138.5; 149.2; 156.9; 163.5.
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21 **5-[2-(1-Imidazolyl)-1-ethyl]thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**
22 **d]pyrimidine (4.17)**. The precursor for synthesis, N-(2-chloroethyl)imidazole, was prepared by
23
24 stirring 20 g sodium imidazolide in 1,2-dichloroethane (80 mL) at room temperature for 3 days. After
25
26 adding water, the product was extracted in 1,2-dichloroethane and was then obtained by the
27
28 evaporation of the organic phase under vacuum (15 torr). This crude product was used in subsequent
29
30 synthesis.
31
32
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34 K₂CO₃ (0.2 g) and N-(2-chlorethyl)imidazole (143 mg, 1.1 mmol) were added to a solution of
35
36 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (0.38 g, 1 mmol)
37
38 in DMF (8 mL), and the reaction mixture was stirred at room temperature for 24 h. The crude product
39
40 was precipitated by adding water and purified stepwise (3%, 4% and 5%) with MeOH in CHCl₃ by
41
42 column chromatography. The crystallization from CHCl₃ provided a colorless product (0.21 g, 45%
43
44 yield, m.p. 208–211 °C). MS ESI + 471.1 (M+H)⁺, ESI – 469.1 (M-H)⁻. NMR: mixture of tautomers, ¹H
45
46 (500 MHz, DMSO-*d*₆): δ 1.39 (d, *J* = 6.7 Hz, 6H, -CH(CH₃)₂); 3.29–3.41 (m, 3H, -CH(CH₃)₂ + -CH₂-CH₂-);
47
48 4.26 (bs, 2H, -CH₂-CH₂-); 4.75–4.79 (m, 2H, NH-CH₂-); 6.88 (s, 1H, H_{Ar}); 7.17 (s, 1H, H_{Ar}); 7.33 (s, 1H,
49
50 H_{Ar}); 7.44–7.59 (m, 3H, H_{Ar}); 7.85–8.13 (m, 5H, H_{Ar} + -NH-); 8.65 (s, 1H, H_{Ar}); 12.26 (s, 1H, -NH-). ¹³C
51
52 (125 MHz, DMSO-*d*₆): δ 21.7; 26.4; 31.1; 42.6; 43.2; 45.7; 119.2; 120.1; 122.5; 126.5; 126.7; 127.5;
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54 128.0; 128.4; 137.2; 137.7; 139.4; 148.8; 149.5; 155.7; 159.7.
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3 **5-(3-Amino-2-hydroxy-1-propyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-**
4 **pyrazolo[4,3-d]pyrimidine (4) (R/S) mixture(4.18) and (R) antipode (4.31).** K₂CO₃ (0.17 g) and 3-
5 benzylidenamino-1-chloropropan-2-ol (0.55 g, 3 mmol; R or R/S prepared from R or R/S –
6 epichlorohydrin according to the literature⁴⁷) were added to a solution of 3-isopropyl-7-[4-(2-
7 pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (0.38 g, 1 mmol) in MeOH (10 mL),
8 and the mixture was stirred at 40 °C for 24 h. The reaction mixture was then acidified by conc. aq.
9 HCl and stirred at 45 °C for 5 h. The crude product was concentrated under vacuum, then alkalized by
10 adding 1 g Na₂CO₃ in 10 mL water and crystallized from a water solution at 4-8 °C. The product was
11 then purified stepwise (5%, 8% and 10%) with MeOH in CHCl₃, with a trace amount of aq. NH₄OH, by
12 column chromatography. Crystallization from MeOH afforded the product (0.30 g; 67% yield; m.p.
13 124–128 °C, (R/S)-antipode; m.p. 120–125 °C, (R)-antipode). MS ESI + 450.1 (M+H)⁺ 100%, 472.1
14 (M+Na)⁺ 20%, ESI – 448.1 (M-H)⁻, 484.1 (M+Cl)⁻ 40%, α_D (R)-antipode = -4.8°, (c = 1.37 g/100mL,
15 DMSO, 22 °C). ¹H (500 MHz; CD₃OD): 1.40 (d, *J* = 7.03 Hz, 6H, -CH(CH₃)₂); 2.68 (dd, *J* = 13.14 Hz, *J* =
16 7.64 Hz, 1H, NH₂-CH_αH_β-CH); 2.82 (dd, *J* = 13.14 Hz, *J* = 3.67 Hz, 1H, NH₂-CH_αH_β-CH); 3.23 (dd, *J* = 14.37
17 Hz, *J* = 6.42 Hz, 1H, -S-CH_αH_β-CH); 3.30 (dd, *J* = 14.37 Hz, *J* = 5.50 Hz, 1H, -S-CH_αH_β-CH); 3.38 (sept., *J* =
18 7.03 Hz, 1H, -CH(CH₃)₂); 3.87–3.91 (m, 1H, -CH₂-CH-CH₂-); 4.84 (bs, 2H, NH-CH₂-); 7.31 (ddd, *J* = 7.34
19 Hz, *J* = 5.04 Hz, *J* = 0.92 Hz, 1H, H_{Ar}); 7.49 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 7.77 (d, *J* = 8.25 Hz, 1H, H_{Ar}); 7.84
20 (td, *J* = 7.49 Hz, *J* = 1.83 Hz, 1H, H_{Ar}); 7.88 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 8.56 (d, *J* = 4.58 Hz, 1H, H_{Ar}). ¹³C
21 (125 MHz; CD₃OD): 22.2; 27.0; 36.1; 44.8; 46.7; 72.7; 122.5; 123.7; 128.3; 128.4; 129.1; 138.9; 139.1;
22 139.4; 141.0; 150.2; 158.5; 163.9.

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25 **5-(2-Hydroxy-2-methyl-1-propyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-**
26 **pyrazolo[4,3-d]pyrimidine (4.19).** A solution of 1,2-epoxy-2-methylpropane (0.12 mL, 1.3 mmol) in
27 MeOH (2 mL) was added to a stirred mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-
28 pyrazolo[4,3-d]pyrimidine-5-thiol **3** (0.38 g, 1 mmol) and tetramethylammonium hydroxide (catalytic
29 amount, 40 mg) in MeOH (6 mL), and the mixture was stirred at room temperature for 7 h. The crude
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3 product was isolated as a precipitate after adding water. The product was purified stepwise (2%, 3%
4 and 4%) with MeOH in CHCl₃ by column chromatography. Chromatography yielded (after
5 evaporation under vacuum) an amorphous colorless glass foam (0.28 g, 62% yield). MS ESI + 449.1
6
7
8 (M+H)⁺, ESI – 447.1 (M-H)⁻. ¹H (500 MHz; CDCl₃ + drop of DMSO-*d*₆): 1.23 (d, *J* = 6.72 Hz, 6H, -
9 CH(CH₃)₂); 1.35 (s, 6H, -C(CH₃)₂); 3.18–3.22 (m, 3H, -CH(CH₃)₂, -CH₂-); 4.69 (bd, *J* = 4.89 Hz, 2H, NH-
10 CH₂-); 7.19 (ddd, *J* = 7.34 Hz, *J* = 4.89 Hz, *J* = 0.92, 1H, H_{Ar}); 7.24 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 7.27 (bs, 1H, -
11 NH-CH₂-); 7.61 (d, *J* = 7.95 Hz, 1H, H_{Ar}); 7.71 (td, *J* = 7.79 Hz, *J* = 1.83 Hz, 1H, H_{Ar}); 7.75 (d, *J* = 8.25 Hz,
12 2H, H_{Ar}); 8.60 (d, *J* = 4.89 Hz, 1H, H_{Ar}). ¹³C (125 MHz; CDCl₃ + drop of DMSO-*d*₆): 21.5; 26.2; 29.3; 44.0;
13 44.5; 71.6; 120.8; 122.2; 127.0; 128.0; 137.0; 137.7; 138.3; 138.6; 149.4; 150.2; 157.0; 163.7.
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25 **5-(2-Guanidino-1-ethyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**
26 **d]pyrimidine (4.20).** 1H-pyrazole-1-carboxamide hydrochloride (0.143 g, 0.95 mmol) was added to
27 a solution of 5-(2-amino-1-ethyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-
28 d]pyrimidine (**4.35**) (0.35 g, 0.84 mmol) and diethylamine (0.30 mL, 1.7 mmol) in DMF (2 mL), and the
29 reaction mixture was stirred at room temperature for 2 h. The reaction mixture was evaporated at a
30 temperature below 50 °C, and the residue was partitioned between CHCl₃ and H₂O. The combined
31 organic phase was dried with sodium sulfate and evaporated under vacuum. The product was
32 purified stepwise (10%, 12% and 14%) with MeOH in CHCl₃, with a trace amount of aq. NH₄OH, by
33 column chromatography. Chromatography yielded (after evaporation in vacuo) an amorphous
34 colorless glass foam (0.225 g, 58% yield). MS ESI + 231.7 (M+2H)²⁺ 100%, 462.1 (M+H)⁺ 30% , ESI –
35 460.1 (M-H)⁻. NMR: mixture of tautomers: ¹H (500 MHz, DMSO-*d*₆) δ 1.35 (d, *J* = 7.0 Hz, 6H, -
36 CH(CH₃)₂); 3.16 (d, *J* = 4.6 Hz, 1H); 3.20 (t, *J* = 6.3 Hz, 2H, -CH₂-CH₂-); 3.24 (sept., *J* = 6.9 Hz, 1H, -
37 CH(CH₃)₂); 3.37 (s, 2H); 3.43–3.47 (m, 2H, -CH₂-CH₂-); 4.76 (d, *J* = 5.2 Hz, 2H, NH-CH₂-); 7.33 (dd, *J* =
38 7.0, 5.2 Hz, 1H, H_{Ar}); 7.46 (s, 1H, -NH); 7.50 (d, *J* = 7.9 Hz, 2H, H_{Ar}); 7.84–7.88 (m, 2H, H_{Ar} + -NH); 7.94
39 (d, *J* = 7.9 Hz, 1H, H_{Ar}); 8.06 (d, *J* = 7.9 Hz, 2H, H_{Ar}); 8.64 (d, *J* = 4.3 Hz, 1H, H_{Ar}); 8.94 (s, 1H, -NH); 13.05
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(s, 1H, -NH). ^{13}C (125 MHz, DMSO- d_6) δ 21.7; 26.3; 29.4; 40.5; 42.9; 48.6; 120.1; 120.7; 122.5; 126.6; 127.6; 127.8; 137.2; 137.5; 139.7; 139.8; 148.4; 148.9; 149.5; 155.7; 156.9; 159.7.

5-(Carbamoylmethyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-

d]pyrimidine (4.21). K_2CO_3 (0.09 g) and 2-chloroacetamide (51 mg, 0.54 mmol) were added to a solution of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (200 mg, 0.54 mmol) in DMF (6 mL). The reaction mixture was stirred at room temperature for 2 h and evaporated at a temperature below 50 °C. The residue was triturated with water and subsequently crystallized (0.13 g, 56% yield, m.p. 220–235 °C). MS ESI + 434.2 (M+H) $^+$, ESI – 432.3 (M-H) $^-$. ^1H (500 MHz; DMSO- d_6): 1.36 (d, $J = 7.03$ Hz, 6H, $-\text{CH}(\text{CH}_3)_2$); 3.28 (sept., $J = 7.03$ Hz, 1H, $-\text{CH}(\text{CH}_3)_2$); 3.73 (s, 2H, $-\text{S}-\text{CH}_2-$); 4.77 (bs, 2H, $-\text{NH}-\text{CH}_2-$); 7.03 (bs, 1H, $-\text{C}(\text{O})-\text{NH}_\alpha\text{H}_\beta$); 7.33 (ddd, $J = 6.42$ Hz, $J = 4.89$ Hz, $J = 0.92$ Hz, 1H, H_{Ar}); 7.43 (bs, 1H, $-\text{C}(\text{O})-\text{NH}_\alpha\text{H}_\beta$); 7.51 (d, $J = 7.95$ Hz, 2H, H_{Ar}); 7.88 (td, $J = 7.64$ Hz, $J = 1.53$ Hz, 1H, H_{Ar}); 7.93 (d, $J = 7.95$ Hz, 1H, H_{Ar}); 8.06 (d, $J = 7.95$ Hz, 2H, H_{Ar}); 8.35 (bs, 1H, $-\text{NH}-$); 8.65 (bd, $J = 4.28$ Hz, 1H, H_{Ar}). ^{13}C (125 MHz; DMSO- d_6): 21.6; 25.8; 34.4; 42.9; 120.0; 122.4; 126.5; 128.0; 137.1; 137.5; 139.8; 149.4; 155.7; 160.2; 170.3.

5-(3-Amino-1-propyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-

d]pyrimidine (4.22). K_2CO_3 (0.35 g) and 3-bromopropylamine hydrochloride (0.40 g, 1.8 mmol) were added to a solution of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (0.38 mg, 1 mmol) in DMF (10 mL), and the reaction mixture was stirred at 45 °C for 24 h. DMF was evaporated under vacuum, and the product was purified stepwise (5% and 10%) with MeOH in CHCl_3 with a trace amount of aq. NH_4OH by column chromatography. Chromatography yielded (after evaporation under vacuum) an amorphous colorless glass foam (0.30 g, 69% yield). ESI + 434.1 (M+H) $^+$, ESI – 432.1 (M-H) $^-$. ^1H (500 MHz; DMSO- d_6): 1.37 (d, $J = 7.03$ Hz, 6H, $-\text{CH}(\text{CH}_3)_2$); 1.80 (pent., $J = 7.03$ Hz, 2H, $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$); 2.70 (t, $J = 7.03$ Hz, 2H, $-\text{CH}_2-$); 3.09 (t, $J = 7.03$ Hz, 2H, $-\text{CH}_2-$); 3.27 (sept., $J = 7.03$ Hz, 1H, $-\text{CH}(\text{CH}_3)_2$); 4.77 (bs, 2H, $-\text{NH}-\text{CH}_2-$); 7.22 (ddd, $J = 7.34$ Hz, $J = 4.89$ Hz, $J = 0.92$

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3 Hz, 1H, H_{Ar}); 7.48 (d, *J* = 8.56 Hz, 2H, H_{Ar}); 7.85 (dt, *J* = 7.64 Hz, *J* = 1.53 Hz, 1H, H_{Ar}); 7.92 (d, *J* = 7.95
4 Hz, 1H, H_{Ar}); 8.06 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 8.47 (appt. bt, 1H, -NH-); 8.64 (ddd, *J* = 4.58 Hz, *J* = 1.53 Hz,
5 *J* = 0.92 Hz, 1H, H_{Ar}). ¹³C (125 MHz; DMSO-*d*₆): 21.7; 25.9; 27.5; 31.3; 39.7; 42.9; 120.0; 122.4; 123.3;
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10 126.5; 127.8; 137.1; 137.4; 138.6; 139.9; 145.8; 149.4; 150.2; 155.7; 160.6.
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14 **5-(3-Hydroxy-1-propyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

15 **d]pyrimidine (4.23).** K₂CO₃ (0.1 g) and 3-bromopropanol (54 μL, 0.6 mmol) were added to a solution
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17 of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (200 mg, 0.54
18 mmol) in DMF (6 mL), and the mixture was stirred at room temperature for 3 h. The reaction mixture
19 was evaporated at a temperature below 50 °C, and the residue was partitioned between CHCl₃ and
20 H₂O. The combined organic phase was dried (Na₂SO₄) and evaporated under vacuum. The product
21 was purified stepwise (2%, 3% and 4%) with MeOH in CHCl₃ by column chromatography. The product
22 was crystallized from CHCl₃/Et₂O (0.15 g, 66% yield, m.p. 150–152 °C). MS ESI + 435.2 (M+H)⁺, ESI –
23 433.3 (M-H)⁻. ¹H (500 MHz; CDCl₃ + DMSO-*d*₆): 1.22 (d, *J* = 7.03 Hz, 6H, -CH(CH₃)₂); 1.72–1.77 (m, 2H, -
24 CH₂-CH₂-CH₂-); 3.14 (t, *J* = 5.50 Hz, 2H, -S-CH₂-); 3.19 (sept., *J* = 7.03 Hz, 1H, -CH(CH₃)₂); 3.50–3.52 (m,
25 2H, -CH₂-OH); 4.62 (bs, 2H, -NH-CH₂-); 4.77 (bs, 1H, -OH); 7.05–7.07 (m, 1H, H_{Ar}); 7.31 (d, *J* = 7.95 Hz,
26 2H, H_{Ar}); 7.53–7.59 (m, 3H, H_{Ar} + -NH-CH₂-); 7.81 (d, *J* = 7.03 Hz, 2H, H_{Ar}); 8.47 (d, *J* = 4.58 Hz, 1H, H_{Ar});
27 11.66 (bs, 1H, -NH-). ¹³C (125 MHz; CDCl₃ + DMSO-*d*₆): 21.3; 25.7; 26.4; 32.6; 43.7; 58.5; 119.9; 121.8;
28 126.6; 128.2; 136.4; 138.2; 138.6; 149.1; 156.1; 162.5.
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48 **5-(2-Amino-2-methyl-1-propyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

49 **d]pyrimidine (4.24).** 2,2-Dimethylaziridine (1.50 mL, 20 mmol; prepared according to the literature⁴⁸)
50 was added dropwise to a stirred mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-
51 pyrazolo[4,3-d]pyrimidine-5-thiol **3** (3.8 g, 10 mmol), DMF (3 mL), aq. 48% solution HBr (3 mL) and
52 H₂O (10 mL) at room temperature, and the mixture was stirred for 24 h. The reaction mixture was
53 then neutralized with an aq. solution of Na₂CO₃, and the crude product was isolated as a precipitate.
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3 The product was purified stepwise (3%, 5%, 7% and 10%) with MeOH in CHCl₃ with a trace amount of
4 aq. NH₄OH by column chromatography. Chromatography yielded (after evaporation under vacuum)
5 an amorphous colorless glass foam (1.6 g, 36% yield). MS ESI + 448,2 (M+H)⁺, ESI – 446,2 (M-H)⁻.
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7 NMR: ¹H (500 MHz; DMSO-*d*₆): 1.29(s, 6H, 2x –CH₃); 1.33 (d, *J* = 7.03 Hz, 6H, –CH–(CH₃)₂); 3.47 (bs, 1H,
8 –CH–(CH₃)₂); 3.65 (s, 2H, –CH₂–); 5.01 (d, *J* = 5.20 Hz, 2H, –NH–CH₂–); 7.66 (d, *J* = 8.56 Hz, 2H, H_{Ar}); 7.81
9 (t, *J* = 6,42 Hz, 1H, H_{Ar}); 8.10 (d, *J* = 8.56 Hz, 2H, H_{Ar}); 8.29 (d, *J* = 7.95 Hz, 1H, H_{Ar}); 8.41 (t, *J* = 7.49 Hz,
10 1H, H_{Ar}); 8.54 (bs, 2H, –NH₂); 8.80 (dd, *J* = 5,2 Hz, *J* = 0.92 Hz, 1H, H_{Ar}). ¹³C (125 MHz; DMSO-*d*₆): 21.8;
11 24.5; 25,1; 30.7; 34.0; 38.7; 43.5; 48.7; 53.9; 123.8; 124.6; 128.0; 128.2; 132.0; 141.3; 143.6; 144.4;
12 152.3; 159.7.
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25 **5-[(Oxazolidin-2-on-5-yl)methyl]thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**
26 **d]pyrimidine (4.25).** K₂CO₃ (0.15 g) and 5-chlormethyl-2-oxazolidinone (0.15 g, 1.1 mmol) were
27 added to a solution of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-
28 thiol **3** (0.38 mg, 1 mmol) in DMF (5 mL), and the reaction mixture was stirred at 45 °C for 24 h. The
29 crude product was precipitated by adding water and purified stepwise (4%, 5% and 6%) with MeOH
30 in CHCl₃ by column chromatography. Chromatography yielded (after evaporation under vacuum) an
31 amorphous colorless glass foam (0.35 g, 74% yield). ESI + 476.2 (M+H)⁺, ESI – 474.2 (M-H)⁻. ¹H (500
32 MHz; CD₃OD): 1.40 (d, *J* = 7.03 Hz, 6H, –CH(CH₃)₂); 3.28 (dd, *J* = 14.21 Hz, *J* = 7.34 Hz, 1H, –NH–CH_αH_β–
33 CH–); 3.35–3.39 (m, 2H, –CH(CH₃)₂ + –S–CH_αH_β–CH–); 3.54 (bt, *J* = 8.80 Hz, 1H, –S–CH_αH_β–CH–); 3.64 (dd, *J*
34 = 14.06 Hz, *J* = 4.89 Hz, 1H, –NH–CH_αH_β–CH–); 4.83 (bs, 2H, NH–CH₂–); 4.85–4.86 (m, 1H, –CH₂–CH–CH₂–);
35 7.32 (ddd, *J* = 7.47 Hz, *J* = 4.89 Hz, *J* = 1.22 Hz, 1H, H_{Ar}); 7.49 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 7.79 (d, *J* = 7.79
36 Hz, 1H, H_{Ar}); 7.84 (td, *J* = 7.34 Hz, *J* = 1.83 Hz, 1H, H_{Ar}); 7.89 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 8.56 (d, *J* = 4.89,
37 1H, H_{Ar}). ¹³C (125 MHz; CD₃OD): 22.2; 27.1; 35.1; 44.8; 45.7; 77.0; 122.5; 123.6; 128.3; 128.8; 138.8;
38 139.4; 141.1; 150.2; 158.5; 162.0; 162.4.
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3 **5-(2-Carbamoyl-1-ethyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**
4 **d]pyrimidine (4.26).** K₂CO₃ (0.2 g) and 3-chloropropionamide (65 mg, 0.6 mmol) were added to a
5 solution of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (200
6 mg, 0.54 mmol) in DMF (6 mL). The reaction mixture was then stirred at 50 °C for 8 h. The mixture
7 was evaporated at a temperature below 50 °C, and the residue was partitioned between EtOAc and
8 H₂O. The combined organic phase was dried (Na₂SO₄), and the product was crystallized from EtOAc
9 (0.11 g, 46% yield, m.p. 137–140 °C). MS ESI + 448.2 (M+H)⁺ 100%, 470.2 (M+Na)⁺ 30%, ESI – 446.3
10 (M-H)⁻ 100%, 893.3 (2M-H)⁻ 30%. ¹H (500 MHz; DMSO-*d*₆): 1.37 (d, *J* = 7.03 Hz, 6H, -CH(CH₃)₂); 2.51 (t,
11 *J* = 7.03 Hz, 2H, O=C-CH₂-); 3.22 (t, *J* = 7.03 Hz, 2H, -S-CH₂-); 3.27 (sept., *J* = 6.72 Hz, 1H, -CH(CH₃)₂);
12 4.77 (bs, 2H, -NH-CH₂-); 6.80 (bs, 1H, -C(O)-NH_αH_β); 7.28 (bs, 1H, -C(O)-NH_αH_β); 7.32 (ddd, *J* = 7.41 Hz,
13 *J* = 4.74 Hz, *J* = 1.22 Hz, 1H, H_{Ar}); 7.50 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 7.85 (td, *J* = 7.64 Hz, *J* = 1.83 Hz, 1H,
14 H_{Ar}); 7.92 (d, *J* = 7.95 Hz, 1H, H_{Ar}); 8.05 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 8.47 (bs, 1H, -NH-); 8.65 (ddd, *J* = 4.89
15 Hz, *J* = 1.53 Hz, *J* = 0.92 Hz, 1H, H_{Ar}). ¹³C (125 MHz; DMSO-*d*₆): 21.6; 26.0; 35.2; 42.9; 120.0; 122.4;
16 125.3; 126.5; 127.8; 132.0; 137.1; 137.4; 140.0; 149.4; 155.7; 160.4; 172.7.
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36 **5-(2-Ureido-1-ethyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine**
37 **(4.27).** A solution of potassium cyanate (0.13 g, 1.6 mmol) in 1 mL of water was added dropwise to a
38 solution of 5-(2-amino-1-ethyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-
39 d]pyrimidine **4.35** (0.35 g, 0.84 mmol) in AcOH (6 mL), and the reaction mixture was stirred at room
40 temperature for 4 h. The reaction mixture was evaporated at a temperature below 50 °C, and the
41 residue was partitioned between CHCl₃ and H₂O. The combined organic phase was dried (Na₂SO₄)
42 and evaporated under vacuum. The product was purified stepwise (3%, 5%, 7% and 10%) with MeOH
43 in CHCl₃ by column chromatography. Chromatography yielded (after evaporation under vacuum) an
44 amorphous colorless glass foam (0.12 g, 31% yield). MS ESI + 232.5 (M+2H)²⁺ 100%, 463.1 (M+H)⁺
45 60%, ESI – 461.1 (M-H)⁻. ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.36 (d, *J* = 6.7 Hz, 6H, -CH(CH₃)₂); 3.04–3.09
46 (m, 2H, -CH₂-CH₂-); 3.24–3.28 (m, 3H, -CH(CH₃)₂ + -CH₂-CH₂-); 4.78 (s, 2H, NH-CH₂-); 5.49 (s, 2H, -NH₂);
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3 6.16 (bs, 1H, -NH); 7.33–7.35 (m, 1H, H_{Ar}); 7.52 (d, *J* = 7.3 Hz, 2H, H_{Ar}); 7.87 (t, *J* = 7.0 Hz, 1H, H_{Ar});
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5 7.94–7.96 (m, 1H, H_{Ar}); 8.08 (d, *J* = 6.7 Hz, 2H, H_{Ar}); 8.65 (s, 1H, H_{Ar}); 12.21 (s, 1H, -NH). ¹³C NMR (125
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7 MHz, DMSO-*d*₆) 21.7; 26.3; 31.0; 35.8; 43.2; 62.8; 79.2; 120.1; 122.5; 126.6; 128.1; 137.2; 139.6;
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9 148.8; 149.5; 155.7; 158.5; 160.4.

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14 **5-(3-Methylamino-1-ethyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

15 **d]pyrimidine (4.28).** 2-Bromo-N-methylethylamine hydrobromide (0.66 g, 3.0 mmol; prepared
16 according to the literature⁴⁹) was added to a mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-
17 1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (0.38 g, 1.0 mmol) in DMF (8 mL) and NaHCO₃ (0.50 g), and
18 the reaction mixture was stirred at room temperature for 2 days. The reaction mixture was
19 evaporated at a temperature below 50 °C, and the residue was partitioned between CHCl₃ and H₂O.
20 The combined organic phase was dried (Na₂SO₄) and evaporated under vacuum. The product was
21 purified stepwise (6%, 8% and 10%) with MeOH in CHCl₃ with a trace amount of aq. NH₄OH by
22 column chromatography. Chromatography yielded (after evaporation under vacuum) an amorphous
23 colorless glass foam (0.07 g, 16% yield). MS ESI + 434.1 (M+H)⁺, ESI – 432.1 (M-H)⁻. ¹H (500 MHz,
24 CDCl₃): δ 1.30 (d, *J* = 7.0 Hz, 6H, -CH(CH₃)₂); 2.42 (s, 3H, -NH-CH₃); 3.00 (t, *J* = 6.0 Hz, 2H, -CH₂-CH₂-);
25 3.22 (m, 3H, -CH(CH₃)₂ + -CH₂-CH₂-); 4.65 (s, 2H, NH-CH₂-); 7.16 (ddd, *J* = 7.34 Hz, *J* = 4.89 Hz, *J* = 0.92
26 Hz, 1H, H_{Ar}); 7.29 (d, *J* = 8.3 Hz, 2H, H_{Ar}); 7.43 (bs, 1H, -NH); 7.56 (d, *J* = 7.9 Hz, 1H, H_{Ar}); 7.67 (td, *J* =
27 7.7 Hz, *J* = 1.6 Hz, 1H, H_{Ar}); 7.74 (d, *J* = 8.1 Hz, 2H, H_{Ar}); 8.59 (d, *J* = 4.6 Hz, 1H, H_{Ar}). ¹³C (125 MHz,
28 CDCl₃): δ 21.5; 21.7; 26.2; 29.4; 29.6; 34.7; 44.0; 50.7; 120.7; 122.2; 127.0; 127.8; 128.0; 137.0; 138.1;
29 138.6; 139.0; 149.3; 150.4; 156.9; 161.2.

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52 **5-(2-Hydroxy-1-propyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

53 **d]pyrimidine (4.29).** A methyloxirane solution (110 mg, 1.8 mmol) in MeOH (2 mL) was added to a
54 stirred mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3**
55 (0.38 g, 1 mmol) and tetramethylammonium hydroxide (catalytic amount, 40 mg) in MeOH (6 mL), and
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3 the mixture was stirred at room temperature for 6 h. The product was precipitated after adding
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5 water, and it was finally purified stepwise (1%, 2%, and 3%) with MeOH in CHCl₃ by column
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7 chromatography. The product was crystallized from DCM/Et₂O (0.30 g, 69% yield, m.p. 144–146 °C).
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9 MS ESI + 435.2 (M+H)⁺, ESI – 433.3 (M-H)⁻. ¹H (500 MHz; CDCl₃): 1.21 (d, *J* = 6.11 Hz, 6H, -CH(CH₃)₂);
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11 1.25 (d, *J* = 6.42 Hz, 3H, -CH₃); 3.06 (dd, *J* = 14.98 Hz, *J* = 6.72 Hz, -S-CH_αH_β); 3.18 (sept., *J* = 7.03 Hz,
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13 1H, -CH(CH₃)₂); 3.25 (d, *J* = 14.37 Hz, 1H, -S-CH_αH_β); 4.19–4.22 (m, 1H, CHOH); 4.67 (bs, 2H, -NH-CH₂-
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15); 7.18–7.23 (m, 3H, H_{Ar}); 7.58 (d, *J* = 7.95 Hz, 1H, H_{Ar}); 7.69–7.72 (m, 3H, H_{Ar}); 8.59 (d, *J* = 4.89 Hz, 1H,
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17 H_{Ar}). ¹³C (125 MHz; CDCl₃): 21.5; 21.6; 22.7; 26.1; 39.8; 44.0; 69.0; 120.9; 122.3; 127.0; 128.0; 137.1;
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19 138.1; 138.5; 149.2; 156.9; 163.4.
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26 **5-(2,3-Diamino-1-propyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-**

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28 **d]pyrimidine (4.30).** K₂CO₃ (0.15 g) and 2,3-di[(benzyloxycarbonyl)amino]-1-chloropropan (0.41 g, 1.1
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30 mmol) were added to a solution of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-
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32 d]pyrimidine-5-thiol **3** (0.38 g, 1 mmol) in DMF (5 mL). The crude di-Z-protected product was isolated
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34 as a precipitate after adding water and then was triturated with 33% HBr in AcOH (1 mL) at room
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36 temperature for 1 h. After evaporation under vacuum, the product was purified stepwise (10%, 12%
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38 and 15%) with MeOH in CHCl₃ with a trace amount of aq. NH₄OH by column chromatography.
39
40 Chromatography yielded (after evaporation under vacuum) an amorphous colorless glass foam (0.31
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42 g, 69% yield). MS ESI + 449.1 (M+H)⁺, ESI – 447.1 (M-H)⁻. ¹H (500 MHz; CDCl₃): 1.30 (d, *J* = 6.72 Hz, 6H,
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44 -CH(CH₃)₂); 2.51 (dd, *J* = 12.69 Hz, *J* = 7.03 Hz, 1H, NH₂-CH_αH_β-CH-); 2.75 (dd, *J* = 12.69 Hz, *J* = 3.67 Hz;
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46 1H, NH₂-CH_αH_β-CH-); 2.94–3.01 (m, 2H, -S-CH_αH_β-CH- + -S-CH₂-CH-CH₂-); 3.21–3.28 (m, 2H, -CH(CH₃)₂ +
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48 -S-CH_αH_β-CH-); 3.80 (bs, 4H, 2 x -NH₂); 4.62 (bs, 2H, -NH-CH₂-); 7.16 (ddd, *J* = 7.34 Hz, *J* = 4.89 Hz, *J* =
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50 0.92 Hz, 1H, H_{Ar}); 7.22 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 7.27 (bs, 1H, -NH-CH₂-); 7.58 (d, *J* = 7.95 Hz, 1H, H_{Ar});
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52 7.67 (td, *J* = 7.95 Hz, *J* = 1.83 Hz, 1H, H_{Ar}); 7.75 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 8.58 (d, *J* = 4.89 Hz, 1H, H_{Ar}). ¹³C
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54 (125 MHz; CDCl₃): 21.7; 21.8; 26.2; 36.6; 43.9; 46.6; 53.2; 120.7; 122.2; 124.8, 127.1; 127.8; 137.0;
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56 138.2; 138.9; 139.0; 146.6; 149.4; 150.8; 156.8; 161.4.
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5-(2,3-Dihydroxy-1-propyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-

d]pyrimidine (4.32). 3-Chloro-1,2-propanediol (0.13 mL, 1.5 mmol) was added to a solution of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (0.38 g, 1 mmol) in DMF (3 mL) and tetramethylammonium hydroxide (0.11 g), and the mixture was stirred at room temperature for 24 h. The crude product was precipitated after adding water. Then, the mixture was purified stepwise (2%, 3%, 4% and 5%) with MeOH in CHCl₃ by column chromatography. Chromatography yielded (after evaporation under vacuum) an amorphous colorless glass foam (0.24 g, 54% yield). MS ESI + 451.1 (M+H)⁺ 100%, 473.2 (M+Na)⁺ 30% , ESI – 449.2 (M-H)⁻. ¹H (500 MHz; CDCl₃ + drop of CD₃OD): 1.32 (d, *J* = 7.03 Hz, 6H, -CH(CH₃)₂); 3.24–3.31 (m, 3H, -CH(CH₃)₂, -CH₂-); 3.59 (dd, 1H, *J* = 11.62 Hz, *J* = 4.89 Hz, -CH_αH_β-); 3.65 (dd, 1H, *J* = 11.62 Hz, *J* = 4.89 Hz, -CH_αH_β-); 3.96 (pent., *J* = 5.20 Hz, 1H, -CH-); 4.71 (bs, 2H, NH-CH₂-); 7.22 (dd, *J* = 7.03 Hz, *J* = 5.20 Hz, 1H, H_{Ar}); 7.35 (d, *J* = 7.95 Hz, 2H, H_{Ar}); 7.63 (d, *J* = 7.03 Hz, 1H, H_{Ar}); 7.73 (dd, *J* = 7.34 Hz, *J* = 1.54 Hz, 1H, H_{Ar}); 7.77 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 8.58 (d, *J* = 4.58 Hz, 1H, H_{Ar}). ¹³C (125 MHz; CDCl₃ + drop of CD₃OD): 21.4; 21.5; 25.9; 34.1; 44.1; 64.4; 72.5; 121.1; 122.3; 127.2; 128.3; 137.3; 138.3; 138.6; 149.1; 157.0; 163.4.

5-(2-Hydroxy-1-ethyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine (4.33).

- a) K₂CO₃ (0.2 g) and 2-bromoethanol (45 μL, 0.6 mmol) were added to a solution of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (200 mg, 0.54 mmol) in DMF (6 mL), and the mixture was stirred at room temperature for 1 h. The reaction mixture was evaporated at a temperature below 50 °C, and the residue was partitioned between CHCl₃ and H₂O. The combined organic phase was dried (Na₂SO₄) and evaporated in vacuo. The product was crystallized from CHCl₃/Et₂O (0.18 g, 80% yield).
- b) A solution of oxirane (80 mg, 1.8 mmol) in 2 mL MeOH was added to a stirred mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (0.38 g, 1

mmol) and tetramethylammonium hydroxide (catalytic amount, 40 mg) in MeOH (6 mL), and the mixture was stirred for 16 h at room temperature. The product was isolated as a precipitate after adding water. The product was crystallized from CHCl₃/Et₂O (0.36 g, 86% yield).

The analytical sample was recrystallized with the same procedure (m.p. 145–150 °C; UV (MeOH, nm): 246 λ_{max}, 275 sh 316 sh). MS ESI + 443.2 (M+Na)⁺, ESI – 419.3 (M-H)⁻. ¹H (500 MHz; CDCl₃): 1.23 (d, *J* = 7.03 Hz, 6H, -CH(CH₃)₂); 3.12–3.16 (m, 2H, -CH₂-); 3.19 (sept., *J* = 7.03 Hz, 1H, -CH(CH₃)₂); 3.80–3.81 (m, 2H, -CH₂-); 4.63 (bs, 2H, NH-CH₂-); 7.06–7.08 (m, 1H, H_{Ar}); 7.31 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 7.47 (bs, 1H, -NH-); 7.54–7.61 (m, 2H, H_{Ar}); 7.81 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 8.48–8.49 (m, 1H, H_{Ar}); 11.71 (bs, 1H, -NH-). ¹³C (125 MHz; CDCl₃): 21.3; 26.0; 33.9; 43.8; 63.5; 119.9; 121.9; 126.6; 128.2; 136.5; 138.3; 138.6; 149.2; 156.1; 162.0.

5-(3-Hydroxy-2-butyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-

d]pyrimidine (4.34). An E/Z-2,3-dimethyloxirane solution (130 mg, 1.8 mmol, in MeOH, 2 mL) was added to a stirred mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (0.38 g, 1 mmol) and tetramethylammonium hydroxide (catalytic amount, 40 mg) in MeOH (6 mL), and the mixture was stirred at room temperature for 16 h. The product was precipitated after adding water, and it was finally purified stepwise (1%, 2%, and 3%) with MeOH in CHCl₃ by column chromatography. The product was crystallized from DCM/Et₂O (0.25 g, 54% yield, m.p. 168–172 °C). MS ESI + 449.2 (M+H)⁺, ESI – 447.3 (M-H)⁻. ¹H (500 MHz; CDCl₃): ¹H (500 MHz, CDCl₃) δ 1.26–1.29 (m, 6H, -CH-(CH₃)₂); 1.33 (d, *J* = 6.4 Hz, 3H, -CH-CH₃); 1.43 (d, *J* = 7.0 Hz, 3H, -CH-CH₃); 3.23 (sept., 1H, -CH-(CH₃)₂); 3.53 (dq, *J* = 7.03 Hz, *J* = 7.03 Hz, 1H, -CH-CH₃); 3.90 (dq, *J* = 6.11 Hz, *J* = 6.11 Hz, 1H, -CH-CH₃); 4.72 (bs, 2H, NH-CH₂-); 7.20–7.23 (m, 1H, H_{Ar}); 7.25–7.29 (m, 2H, H_{Ar}); 7.64 (d, *J* = 7.9 Hz, 1H, H_{Ar}); 7.73 (td, *J* = 7.7, 1.7 Hz, 1H, H_{Ar}); 7.76–7.78 (m, 3H, H_{Ar} + NH-CH₂-); 8.64 (d, *J* =

4.9 Hz, 1H, H_{Ar}); 11.75 (bs, 1H, -NH). ¹³C (125 MHz, CDCl₃ + DMSO-*d*₆) δ 17.5; 21.4; 21.5; 21.7; 26.1; 44.0; 48.3; 71.9; 120.1; 122.0; 126.8; 128.3; 136.6; 138.3; 138.6; 149.3; 156.3; 161.8.

5-(2-Amino-1-ethyl)thio-3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine (4.35).

- a) Aziridine (0.42 mL, 8 mmol) was added dropwise to a stirred mixture of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (1.0 g, 2.66 mmol) and aq. 48% solution HBr (3.1 mL) in DMF (20 mL), and the mixture was stirred at room temperature for 24 h. The reaction mixture was neutralized with an aq. solution of Na₂CO₃, and the crude product was isolated as a precipitate.
- b) 2-(Boc-amino)ethyl bromide (0.61 g, 2.71 mmol) was added to a solution of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)H-pyrazolo[4,3-d]pyrimidine-5-thiol **3** (1 g, 2.66 mmol) in DMF (30 mL) with K₂CO₃ (0.5 g). The reaction mixture was stirred at room temperature for 16 h. The product was isolated as a precipitate after adding water. The precipitate was dissolved in 25 mL of 2N aq. HCl, and the de-Bocylation reaction was conducted overnight at room temperature. The reaction mixture was neutralized with an aq. solution of Na₂CO₃, and the crude product was isolated as a precipitate.

The product was purified stepwise (3%, 5%, 7% and 10%) with MeOH in CHCl₃ with a trace amount of aq. NH₄OH by column chromatography. Chromatography yielded (after evaporation under vacuum) an amorphous colorless glass foam (process a: 0.72 g, 65% yield; process b: 0.75 g; 67% yield). MS ESI + 420.2 (M+H)⁺ 100%, 839.3 (2M+H)⁺ 15% , ESI – 418.2 (M-H)⁻. ¹H (500 MHz; DMSO-*d*₆): 1.37 (d, *J* = 7.03 Hz, 6H, -CH(CH₃)₂); 2.90 (t, *J* = 6.72 Hz, 2H, NH₂-CH₂-); 3.13 (t, *J* = 6.72 Hz, 2H, -CH₂-S-); 3.28 (sept., *J* = 7.03 Hz, 1H, -CH(CH₃)₂); 4.77 (bs, 2H, NH-CH₂-); 7.32 (ddd, *J* = 7.34 Hz, *J* = 4.89 Hz, *J* = 0.92 Hz, 1H, H_{Ar}); 7.49 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 7.85 (td, *J* = 7.95 Hz, *J* = 1.83 Hz, 1H, H_{Ar}); 7.92 (d, *J* = 8.25 Hz, 1H, H_{Ar}); 8.06 (d, *J* = 8.25 Hz, 2H, H_{Ar}); 8.47 (bt, *J* = 5.81 Hz, 1H, -NH-CH₂-); 8.65 (ddd, *J* = 4.74 Hz, *J*

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3 = 1.53 Hz, $J = 0.92$ Hz, 1H, H_{Ar}). ^{13}C (125 MHz; DMSO- d_6): 21.6; 25.8; 32.7; 40.9; 42.9; 120.0; 122.4;
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5 126.5; 127.8; 137.1; 137.4; 138.5; 139.9; 149.4; 155.7; 160.3.
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10 **Kinase Inhibition Assay**

11 CDK/cyclin complexes were assayed as previously described.⁵⁰
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17 **Protein Crystallization, Diffraction Data Collection, and Structure Determination**

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19 The protein sample for crystallization was prepared by mixing 12 mg/ml CDK2/cycA2
20 complex in 40 mM HEPES at pH 7.5, 200 mM NaCl, and 0.02% monothioglycerol with 100
21 mM **4.35** in DMSO to the final inhibitor concentration of 2 mM, followed by 30 min of
22 incubation on ice and purification by centrifugation ($16000 \times g$, 10 min, 4 °C). The
23 CDK2/cycA2 crystal in complex with the inhibitor **4.35** was obtained at 18 °C using the
24 sitting-drop vapor diffusion technique and condition No. 21 from the Morpheus kit
25 (Molecular Dimensions, USA), containing 10% w/v PEG 20000, 20% v/v PEG MME 550,
26 0.03 M sodium fluoride, 0.03 M sodium bromide, 0.03 M sodium iodide, and 0.1 M
27 bicine/Trizma base at pH 8.5. The droplet contained 100 nl of the protein sample, 170 nl of
28 the reservoir solution, and 30 nl of a seed stock prepared by crushing crystals prepared in
29 condition No. 93 from the Morpheus kit (Molecular Dimensions, USA) in 30 μ l of the
30 precipitant solution, which contained 10% w/v PEG 20000, 20% v/v PEG MME 550, 0.02 M
31 1,6-hexanediol, 0.02 M 1-butanol, 0.02 M (*RS*)-1,2-propanediol, 0.02 M 2-propanol, 0.02 M
32 1,4-butanediol, 0.02 M 1,3-propanediol, and 0.1 M bicine/Trizma base at pH 8.5. The crystal
33 was harvested after 3 weeks and flash-cooled in liquid nitrogen without additional
34 cryoprotection. A complete dataset at 2.15 Å resolution was collected at 100 K at the
35 beamline MX14.1 of BESSY, Berlin, Germany.⁵¹ The dataset was processed using the
36 program XDS⁵² and its graphical interface XDSGUI.⁵³ The structure was determined by
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3 molecular replacement with the program Molrep⁵⁴ using the structure of CDK2/cycA2
4 available in the PDB under the code 5LMK⁵⁵ as a search model. Model refinement was
5 performed using the program REFMAC 5.8.0158⁵⁶ from the CCP4 package⁵⁷ in combination
6 with manual adjustments with Coot software.⁵⁸ MolProbity server⁵⁹ was used to evaluate of
7 the final model quality. The data collection and refinement statistics are listed in
8 **Supplementary Table 1**. All the figures representing structures were created using PyMOL.⁶⁰
9 Atomic coordinates and structural factors were deposited in the PDB under the accession code
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24 **Cell Culture**

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26 Lymphoma cell lines were obtained from the German Collection of Microorganisms and Cell
27 Cultures (DSMZ), American Tissue Culture Collection (ATCC) or European Collection of
28 Authenticated Cell Cultures and cultured in media according to providers' instructions. HBL2
29 cells were kindly provided by Prof. Martin Dreyling from the University of Munich,
30 Germany. UPF1H and UPF7U are a mantle cell lymphoma cell lines derived by Dr. Pavel
31 Klener (Institute of Pathological Physiology, First Faculty of Medicine, Charles University,
32 Czech Republic). UPF1H was established from the peripheral blood of a patient with
33 treatment-refractory mantle cell lymphoma. Whole exome sequencing confirmed the clonal
34 identity of UPF1H cells to patient lymphoma cells. UPF7U was derived from leukemized
35 blood of a patient with 2nd relapse of mantle cell lymphoma after failure of ibrutinib. Exome
36 sequencing confirmed that UPF7U kept majority of somatic mutations with the primary
37 lymphoma cells, from which it was derived.
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Cell Viability Assays

For the viability assays, cells were treated in triplicate with six different doses of each compound for 72 h. After treatments, Calcein AM solution was added for 1 h, and the fluorescence of live cells was measured at 485 nm/538 nm (excitation/emission) using a Fluoroskan Ascent microplate reader (Labsystems). The GI₅₀ value, the drug concentration lethal to 50% of the cells, was calculated from the dose response curves that resulted from the assays.

Chemicals

Dinaciclib, venetoclax and ibrutinib were purchased from MedChemExpress, and CR8 was purchased from Merck.

Immunoblotting

Cell lysates were prepared, and then proteins were separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. After blocking, overnight incubation with specific primary antibodies, and incubation with peroxidase-conjugated secondary antibodies, the peroxidase activity was detected with SuperSignal West Pico reagents (Thermo Scientific) using a CCD camera LAS-4000 (Fujifilm). The specific antibodies were purchased from Cell Signaling (anti-PARP, clone 46D11; anti-caspase-7; anti-caspase-3, clone 3G2; anti-Mcl-1, clone D35A5; anti-XIAP; anti-Bcl-xl, clone 54H6; anti-Rb, clone 4H1; anti-pRb S780, clone D59B7; anti-Rb S807/811, clone D20B12; anti-cleaved caspase-9 Asp330; anti-Bax, clone D2E11), Merck (anti-Bcl-2; anti- α -tubulin, clone DM1A), Santa Cruz Biotechnology (anti- β -actin, clone C4; anti-c-myc, clone 9E10), Bethyl Laboratories, USA (anti-phospho-RNA polymerase II S2 and S5), and Millipore (anti-RNA polymerase II, clone ARNA-3). The anti-PCNA (clone PC-10) antibody was generously gifted by Dr. B. Vojtěšek (Masaryk Memorial

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3 Cancer Institute, Brno, Czech Republic). All primary antibodies were diluted in PBS
4 containing 5% powdered milk and 0.1% Tween 20. Peroxidase-conjugated rabbit anti-mouse
5 immunoglobulin or porcine anti-rabbit immunoglobulin antisera (Cell Signaling) were used as
6 the secondary antibodies and visualized with ECL reagents (GE-Healthcare Life Sciences).
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14 **In Vivo Efficacy**

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16 All aspects of the animal study met the accepted criteria for the care and experimental use of
17 laboratory animals. The protocols were reviewed by the Institutional Animal Care and Use
18 Committee and approved by the Ministry of Education, Youth and Sports of the Czech
19 Republic under number 2844/2017-2. Immunodeficient NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ
20 mice (Jackson Laboratory, referred to as “NOD-SCID-gamma” or “NSG” mice) were
21 maintained in individually ventilated cages. The cells were injected subcutaneously (s.c.) (5-
22 10 mil. per mouse) into 8-12 week old female NSG mice; numbers of experimental animals
23 are indicated in the legends to Figures 4 and 6. Compound **4.35** dissolved in PBS was
24 administered intravenously (i.v.) via the tail vein. Therapy was initiated when all animals
25 developed palpable tumors. Tumors were measured every day in two perpendicular
26 dimensions with a digital caliper. In vivo experiments were terminated when at least one
27 mouse in any group developed a s.c. tumor ≥ 2 cm in any diameter. For the analysis of
28 pharmacodynamic markers, palpable tumor-bearing mice were injected with different doses of
29 **4.35** (5 and 10 mg/kg) and euthanized after 24 h. Subsequently, tumors were excised, filtered
30 through 45 μm nylon mesh, and evaluated for protein expression by immunoblotting analysis.
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54 **ASSOCIATED CONTENT**

55 **Supporting Information**

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3 The Supporting Information is available free of charge on the ACS Publications website at
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5 DOI: 10.1021/acs.jmedchem ...
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8 Computational analysis of compound binding to CDK2, crystal parameters and
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10 refinement statistics, kinase selectivity profile of **4.35**, NCI60 screening results,
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12 additional in vitro data, physicochemical and pharmacological properties of **4.35**,
13
14 additional in vivo data, additional methods, NMR spectra of prepared compounds,
15
16 supplementary references.
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19 Molecular formula strings (CSV)
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21 **Accession codes**

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24 PDB ID Code: 6GVA (complex of **4.35** with CDK2/cyclin A2). Authors will release the
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26 atomic coordinates and experimental data upon article publication.
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30 **AUTHOR INFORMATION**

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40 **Author Contributions**

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42 # these authors contributed equally; L.H., T.P. and A.Š. prepared and characterized
43
44 compounds, R.J., D.T., M.A. and M.P. performed biochemical and cellular experiments, P.K.,
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46 D.T. and L.D. performed animal experiments, J.Ši. and L.U. performed PK analyses, J.Šk.,
47
48 P.P. and P.Ř. performed crystallographic experiments, M.N. performed computational study,
49
50 R.J., P.K., M.S. and V.K. designed the study, analyzed data and drafted the manuscript.
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13
14 Germany) to collect diffraction data is gratefully acknowledged.
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19 **ABBREVIATIONS**

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21 DLBCL–ABC, Diffuse large B–cell lymphoma, subtype – activated B–cell; DLBCL–GCB,
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23 Diffuse large B–cell lymphoma, subtype – germinal center B–cell; MCL – mantle cell
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25 lymphoma; BL – Burkitt lymphoma; CML, chronic myelogenous leukemia; ALL, acute
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27 lymphoblastic leukemia; AML, acute monocytic leukemia
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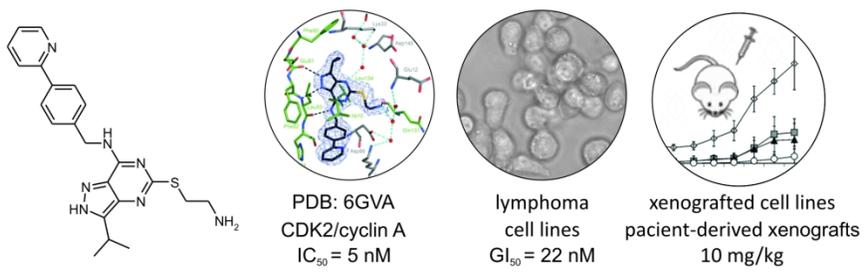


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