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Inhibition of the cellular function of perforin by 1-amino-2, 4-dicyanopyrido[1,2-*a*]benzimidazoles

Dani M. Lyons [a], Kristiina M. Huttunen [a,b], Kylie A. Browne [c], Annette Ciccone [c], Joseph A. Trapani [c], William A. Denny [a,*], Julie A. Spicer [a]

[a] Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand
[b] School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland
[c] Cancer Immunology Program, Peter MacCallum Cancer Centre, St. Andrew's Place, East Melbourne, Victoria 3002, Australia

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ABSTRACT

A high throughput screen showed the ability of a 1-amino-2,4-dicyanopyrido[1,2-*a*]benzimidazole analogue to directly inhibit the lytic activity of the pore-forming protein perforin. A series of analogues were prepared to study structure–activity relationships (SAR) for the this activity, either directly added to cells or released in situ by KHYG-1 NK cells, at non-toxic concentrations. These studies showed that the pyridobenzimidazole moiety was required for effective activity, with strongly basic centres disfavoured. This class of compounds was relatively unaffected by the addition of serum, which was not the case for a previous class of direct inhibitors.

1. Introduction

Perforin (PRF) is a pore-forming member of the membrane-attack-complex/PRF (MACPF) protein family[1], and is secreted, along with a range of serine proteases (granzymes), by both cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells.[2] Together, perforin and granzymes comprise a principal mechanism of CTL/NK cytotoxicity by which the immune systems of higher organisms protect themselves against pathogens and transformed cells. The critical role of perforin in this process is its ability, upon binding calcium, to assemble into aggregates of 12–18 molecules that form trans-membrane pores of 10–20 nm in diameter in the plasma membrane of the target cell, facilitating the entry of granzymes, which can trigger apoptosis.[3,4] Initially, the calcium-dependent membrane binding of perforin monomers is mediated by their C terminal C2 domains.[5] Successive perforin monomers then assemble into a cylindrical pore via a series of salt bridges linking Arg213 on the 'front' of one monomer with Glu343 on the 'back' of the next.[6] The structure of mouse perforin monomer at a resolution of 2.75 Å has recently been reported as a bent and twisted four-stranded β-sheet MACPF domain flanked by two clusters of α-helices that insert into membranes as β-strands upon pore formation.[7] In the same report, cryo-electron microscopy also confirmed the distribution of individual perforin sub-domains within an assembled pore structure.

Apart from well-recognised disease syndromes due to mutation and/or down-regulation of perforin expression, there is also much evidence linking inappropriate perforin activity to several human pathologies, including cerebral malaria, type 1 diabetes, juvenile idiopathic arthritis and postviral myocarditis,[7] as well as therapy-induced conditions such as allograft rejection and graft-versus-host disease.[8,9] Perforin is therefore a potentially druggable target, especially as it is encoded by a single-copy gene in both mice and humans.

However, there have been few reports of small-molecule inhibitors of perforin function, as distinguished from compounds that affect it indirectly by inhibiting cell respiration or perforin processing.[10] Enzastaurin (**1**) (Fig. 1), a bisindolylmaleimide structurally related to staurosporine and currently in Phase II trial for cancer, has recently been reported[11] to inhibit perforin release from NK cells, but is thought to act via the PKC/PI3K/AKT pathway. We recently reported[10] a series of dihydrofuro[3,4-*c*]pyridinones (e.g., **2**) (Fig. 1) as direct inhibitors of the cytolytic effects of perforin in cells, and demonstrated limited structure–activity effects across the series. The most active compound in the series (**2**) had a IC_{50} of 1 μM for inhibition of the lysis of Jurkat cells by added perforin, and at a concentration of 20 μM showed a 60% inhibition in the killing of K562 cells by perforin released from KHYG-1 NK cells, with little toxicity to the latter. However, the cellular activity of

Abbreviations: CDI, carbonyldiimidazole; CTL, cytotoxic T lymphocytes; DMF, dimethylformamide; LRMS, low-resolution mass spectrometry; NK, natural killer cells; MACPF, membrane-attack-complex/PRF; PRF, perforin; SEM, standard error of the mean; THF, tetrahydrofuran.

* Corresponding author. Tel.: +64 9 923 6144; fax: +64 9 923 7502.
E-mail address: b.denny@auckland.ac.nz (W.A. Denny).

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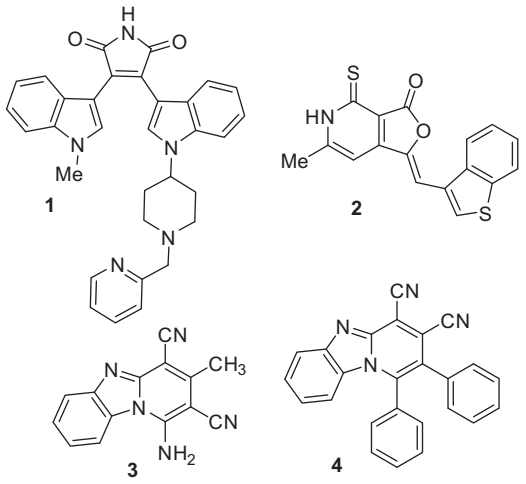


Figure 1. Inhibitors of perforin.

these compounds was later shown to be adversely affected by increasing concentrations of serum, limiting their further development. In the present paper we discuss the discovery of this new class of direct perforin inhibitors, the 1-amino-2,4-dicyanopyrido[1,2-*a*]benzimidazoles, and initial structure–activity relationship (SAR) studies on this new class.

2. Results and discussion

2.1. Chemistry

The lead compound (**5**) was selected from a high throughput screen[12] of 100,000 compounds sourced from commercial libraries, using a 384-well plate format. Compounds were screened (at 20 μM) by incubation with sheep red blood cells, which in the absence of an inhibitor are lysed by perforin, generating turbidity that can be measured by the change in absorbance at 650 nm. Compound **5** was one of a few hits that were confirmed in an assay measuring its potency for inhibiting the lysis of Jurkat human leukaemia cells by added mouse perforin, where it showed an IC_{50} of about 5 μM. This activity was reproduced by a re-synthesized sample prepared by the method given in Scheme 1. An advantage of this lead compared with **2** was a relative lack of potentially reactive substituents, including sulfur, but a potential disadvantage was low aqueous solubility.

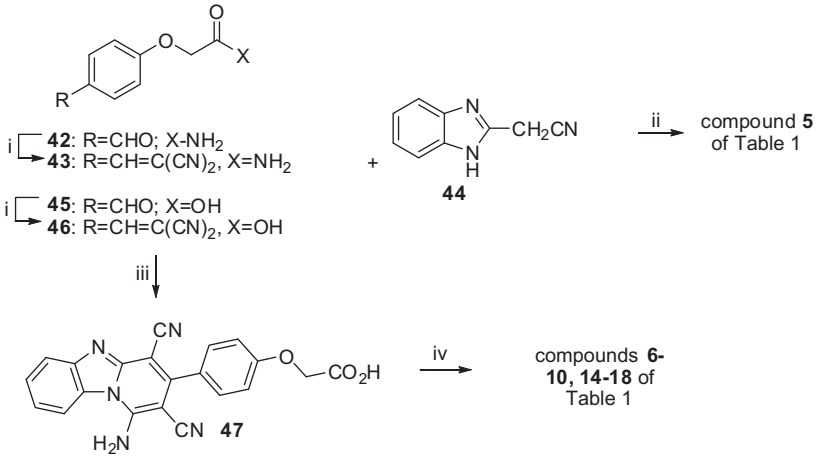
The synthesis of this general class of compounds has been reported by Bogdanowicz-Szwed and Czarny[13] from the reaction of 2-(1*H*-benzo[*d*]imidazol-2-yl)acetonitrile (**44**) with arylmalononitriles, and[14] by Elnagdi et al., by reaction of **44** with a mixture of formaldehyde and cyanothioacetamide to yield (e.g.) **3** (Fig. 1). Chao et al.[15] reported the synthesis of related 7-cyanopyrido[1,2-*a*]benzimidazoles (e.g., **4**) (Fig. 1) in moderate yield by the multi-component reaction of pyridine, chloroacetonitrile, malononitrile, and aromatic aldehyde in refluxing acetonitrile. Related compounds have been reported as potential inhibitors of transcription.[16]

An authentic sample of lead compound **5** was synthesized in order to confirm the original perforin inhibitory data. Reaction of aldehyde **42**, malononitrile and 3-aminopropanoic acid in EtOH provided compound **43**. (Note: we surveyed a variety of catalytic bases for use in this key Knoevenagel condensation and found that 3-aminopropanoic acid was the best). The dicyanovinyl intermediate **43** was then heated under reflux with 2-(1*H*-benzo[*d*]imidazol-2-yl)acetonitrile (**44**) and piperidine in EtOH to give compound **5** in good overall yield. To explore the SAR of the oxyacetamide side chain we prepared both N-alkylated (**6**–**10**) and hydroxyalkyl (**14**–**18**) compounds. The key intermediate **47** for these was synthesized in a similar manner, from 2-(4-formylphenoxy)acetic acid (**45**) (Scheme 1). Compound **47** was then treated with 1,1′-carbonyldiimidazole (CDI) in anhydrous DMF and subsequently reacted with various amines to provide compounds **6**–**10** and **14**–**18** in variable yields.

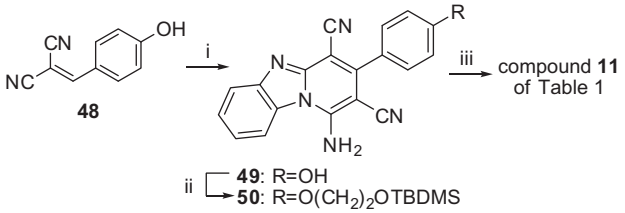
To replace the acetamide moiety, we prepared compound **11**, which carries an oxyethanol side chain (Scheme 2). Reaction of 2-(4-hydroxybenzylidene)malononitrile (**48**), **44** and piperidine in EtOH provided **49**, which was then reacted with *tert*-butyldimethylsilyl (TBDMS)-protected bromoethanol in the presence of Cs_2CO_3 in anhydrous DMF. Removal of the protecting group by tetrabutylammonium fluoride (TBAF) in THF then gave **11**.

Compounds **12** and **13**, containing carbon-linked side chains, were obtained from ethyl 3-(4-formylphenyl)propanoate (**51**) in a four-step sequence in moderate to good yields (Scheme 3). The first two steps were carried out as for **5**, affording ester **53**, which was then converted to the corresponding carboxylic acid **54** by hydrolysis with NaOH in EtOH, followed by CDI-mediated amide formation to give **12** and **13**.

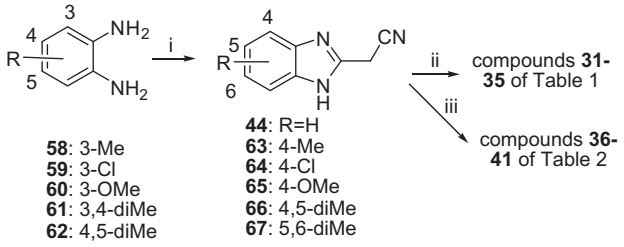
For amides directly attached to the phenyl ring (compounds **19**–**30**, Scheme 4), the key intermediate **57** was prepared from methyl 4-(2,2-dicyanovinyl)benzoate (**55**) by reaction with **44** and piperidine in EtOH and then hydrolysing the resulting ester **56** to give **57**. This was then reacted with CDI followed by amines in anhydrous DMF to give the required compounds.



Scheme 1. Reagents and conditions: (i) malononitrile, 3-aminopropanoic acid, EtOH, 20 °C, 24 h; (ii) piperidine, EtOH, reflux, 3 h; (iii) compound **44**, piperidine, EtOH, reflux, 3 h; (iv) CDI/DMF, 1 h, 20 °C, then various amines, 1 h, 20 °C.



Scheme 2. Reagents and conditions: (i) compound **44**, EtOH, piperidine, reflux, 2 h; (ii) $Br(CH_2)_2OTBDMS$, Cs_2CO_3, dry DMF, 90 °C (N_2), 16 h. (iii) TBAF, THF, 20 °C, 30 min.



Scheme 5. Reagents and conditions: (i) cyanoacetamide, 4 Å MS, xylene, reflux, 24 h; (ii) compound **43**, piperidine (catalytic), EtOH, reflux, 24 h; (iii) compound **42**, piperidine (catalytic), EtOH, reflux, 24 h.

Compounds **31**–**35**, with the same oxyacetamide side chain as the lead compound **5** but bearing various substituents on the benzoimidazole ring (Scheme 5), were prepared from substituted benzenediamines **58**–**62**, which were reacted with cyanoacetamide in xylene to form the benzoimidazolylacetonitriles **63**–**67**. Treatment of these with the dicyanovinyl intermediate **43** and piperidine in EtOH as above gave the desired compounds. Finally, similar treatment of the benzoimidazolylacetonitriles **44**, **63–67** with aldehyde **42** in the same way gave the analogous bicyclic analogues **36**–**41** (Table 2).

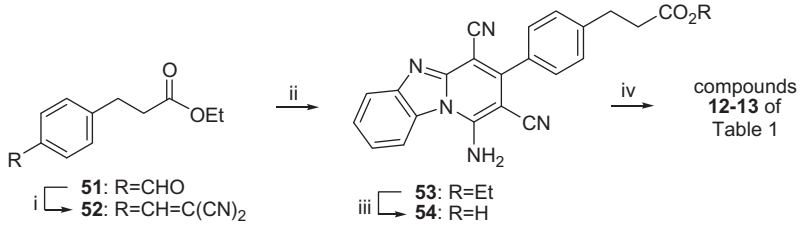
2.2. Structure–activity relationships

Table 1 provides structural data for a series of analogues of **5**, and their potencies for inhibiting the lysis of labelled Jurkat human leukaemia cells by added perforin. Compounds **6**–**10** use variants of the O-linked side chain of **5** to explore bulk tolerance with alkyl chains. While the NHMe compound **6** retains activity, there appears to be some activity loss with the longer members (**7**–**10**). Compound **11**, with a small $O(CH_2)_2OH$ side chain, also showed good potency. Compounds **12** and **13** used side chains similar to those in **6** and **7**, respectively, but using a CH_2 linker, and were both completely inactive. This suggests the oxygen linkage may be important for activity. Compounds **14–18** look at potentially solubilising chains (diol **17** has an aqueous solubility of 180 μM, compared to 16 μM for **5**). The 2-hydroxyethylacetamide **14** retains activity, but a longer derivative (**16**) or those with branched chains (**15**,**17**) do not. However, the (larger) pyridyl (**18**) did retain weak activity.

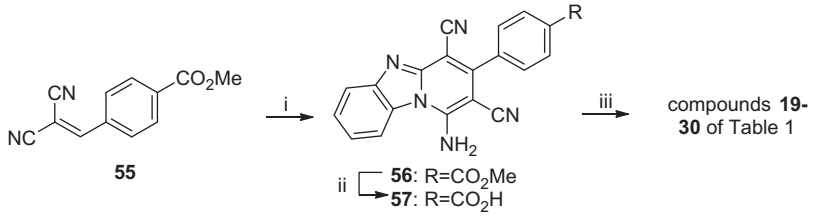
Given the above results with more bulky substituents, the sidechain was therefore pared right back to a primary amide (**19**). This compound, and its NMe analogue (**20**) showed excellent activity (IC_{50}s = 3.00 and 1.04 μM, respectively), a significant improvement on the lead compound **5** (5.19 μM). With improved solubility still a priority, a series of potentially solubilising sidechains were then introduced, linked directly to the amide (diol **26** and dimethylamine **27** have aqueous solubilities of 51 μM and 68 μM, respectively, compared with 25 μM for **20**). With the exception of the more bulky $CONMe_2$ analogue **21** and the two strongly basic analogues **27** (calcd pK_a 8.69) and **28** (calcd pK_a 8.85) all of the CONH-linked neutral compounds were at least as potent as the lead **5** in the Jurkat assay (IC_{50}s from 1.04 to 5.5 μM). The two weak bases **29** (calcd pK_a 6.84) and **30** (calcd pK_a 5.74) were less active (IC_{50}s 10 μM; comparable with **18**).

Compounds **31–35** explore additional substitution in the pyrido ring. The compounds showed activity, but overall this was little different to that of **5**. The substituent changes were not extensive, but were sufficiently-varied electronically to suggest no dramatic electronic influence on biological activity.

To further evaluate the importance of elements of the pyridobenzimidazole moiety, the bicyclic compounds **36**–**41**, both the

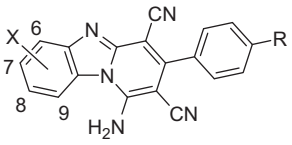


Scheme 3. Reagents and conditions: (i) malononitrile, 3-aminopropanoic acid, EtOH, 20 °C, 16 h; (ii) compound **44**, piperidine, EtOH, reflux, 3 h; (iii) aqueous NaOH, EtOH, reflux, 1 h; (iv) CDI, dry DMF, 1 h, RNH_2, 20 °C, 16 h.



Scheme 4. Reagents and conditions: (i) compound **44**, piperidine, EtOH, reflux, 3 h; (ii) NaOH, EtOH, reflux, 1 h; (iii) CDI, dry DMF, RNH_2, 20 °C, 16 h.

Table 1
Structures and perforin inhibitory activities of substituted 9-amino-6,8-dicyano-7-phenylpyrido[1,2-a]benzimidazole analogues



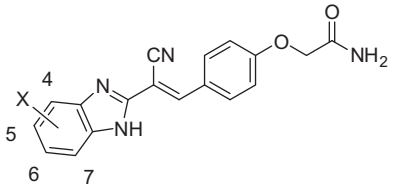
No.	R	X	IC_{50}[a] (μM)
5	OCH_2CONH_2	H	5.19 ± 0.31
6	$OCH_2CONHMe$	H	4.56 ± 0.88
7	OCH_2CONMe_2	H	6.89 ± 0.51
8	$OCH_2CONHEt$	H	7.44 ± 0.72
9	OCH_2CONH^nPr	H	13.9 ± 1.0
10	OCH_2CONH^iPr	H	13.2 ± 0.8
11	OCH_2CH_2OH	H	2.87 ± 0.37
12	$CH_2CH_2CONHMe$	H	>20
13	$CH_2CH_2CONMe_2$	H	>20
14	$OCH_2CONHCH_2CH_2OH$	H	8.25 ± 1.50
15	$OCH_2CONHCH_2CH(Me)OH$	H	>20
16	$OCH_2CONHCH_2CH_2CH_2OH$	H	>20
17	$OCH_2CONHCH_2CH(OH)CH_2OH$	H	>20
18	$OCH_2CONHCH_2CH_2$(4-pyridyl)	H	~10
19	$CONH_2$	H	3.00 ± 0.60
20	CONHMe	H	1.04 ± 0.14
21	$CONMe_2$	H	>20
22	CONHEt	H	3.16 ± 0.10
23	$CONHCH_2CH_2OH$	H	3.08 ± 0.65
24	$CONHCH_2CH_2CH_2OH$	H	4.18 ± 0.39
25	$CONHCH_2CH(CH_3)OH$	H	5.55 ± 1.08
26	$CONHCH_2CH(OH)CH_2OH$	H	3.24 ± 0.77
27	$CONHCH_2CH_2NMe_2$	H	>20
28	$CONHCH_2CH_2$Npiperidyl	H	>20
29	$CONHCH_2CH_2$Nmorpholyl	H	10.0 ± 1.6
30	$CONHCH_2CH_2$(4-pyridyl)	H	10.5
31	OCH_2CONH_2	6-Me	6.87 ± 1.79
32	OCH_2CONH_2	6-Cl	5.67 ± 0.60
33	OCH_2CONH_2	6-OMe	7.49 ± 1.71
34	OCH_2CONH_2	6,7-DiMe	5.21 ± 0.60
35	OCH_2CONH_2	7,8-DiMe	3.53 ± 0.29
2			0.97

[a] Testing was carried out over a range of doses, with the lysis of Jurkat cells by perforin measured by ^{51}Cr release. Values are the average of at least three independent IC_{50} determinations plus SEM. For some compounds great inter-experiment variability was observed, possibly related to their relative insolubility.

parent **36** and analogues **37**–**41** were prepared (Table 2). These targets explored the same limited set of substitution patterns on the left-hand ring as did compounds **31–35**. They effectively represent loss of the benzo ring of the pyrido[1,2-*a*]benzimidazole, together with its 1-amino and 8-cyano substituents. Table 2 shows that the majority of these pyridobenzimidazole compounds lost activity in the Jurkat assay.

Selected examples from Table 1 were also evaluated for their ability to inhibit the effect of perforin released by KHYG-1 NK cells, as shown in Table 3. Some of the compounds (e.g., **9**,**10**,**13**) were almost as effective as the dihydrofuro[3,4-*c*]pyridinone **2** (although not as effective as some of the analogues of that series[10]) in the absence of added mouse serum, and without significant toxicity to the KHYG-1 cells. However, there was little correlation between the potencies of the compounds in the Jurkat lysis assay and their ability to inhibit the effect of perforin released by KHYG-1 NK cells (in the absence of serum), as shown by the equation relating Jurkat IC_{50} and the percentage of KHYG-1 killing: $\log [IC_{50}\text{Jurkat}] = -1.2 \times \log[100 - (\%\text{KHYG-1 inhib})] + 2.9$ ($n = 21$, $R = 0.43$, $F = 4.6$, $p = 0.05$). This overall view is reinforced by specific examples (e.g., **24**: Jurkat IC_{50} 4.18 μM but no KHYG-1 inhibition; **13**: Jurkat IC_{50} >20 μM) but 49% KHYG-1 inhibition. This may well be due to the additional barriers to compound distribution in the latter

Table 2
Structures and perforin inhibitory activities of bicyclic analogues



No.	X	IC_{50}[a] (μM)
36	H	>20
37	4-Me	>20
38	4-Cl	6.72 ± 1.84
39	4-OMe	>20
40	4,5-DiMe	>20
41	5,6-DiMe	5.14 ± 1.21

[a] As for Table 1.

Table 3
Capacity of selected compounds to inhibit perforin delivered by KHYG-1 NK cells

No.	Jurkat IC_{50} (μM)	KHYG-1 inhibition (% at 20 μM)		KHYG-1 viability[c] (%)
		No serum[a]	10% serum[b]	
5	5.19	18	2	86
2	0.97	60	6	88
6	4.56	41	42	84
7	6.9	34	25	78
8	7.44	0	11	77
9	13.9	41	22	90
10	13.2	52	22	70
11	3.37	15	16	85
13	>20	49	64	96
14	8.25	0	6	92
19	3.00	7	6	79
20	1.04	9	12	84
22	3.17	19	12	53
23	3.70	4	12	89
24	4.18	0	7	86
25	5.55	0	0	68
26	3.94	0	0	84
29	10	1	5	85
30	10.5	15	20	62
31	6.88	10	8	88
34	5.21	36	7	60
35	3.53	38	5	62

[a] Inhibition by compound (20 μM) of the perforin-induced lysis of K562 target cells when co-incubated with KHYG-1 human NK cells (see Section 4). Percent inhibition calculated compared to untreated control.
[b] As for a, but in the presence of 10% mouse serum.
[c] Viability of KHYG-1 NK cells after 24 h by Trypan blue exclusion assay (see Section 4). All results are the average of at least three separate determinations.

cell–cell assay. One encouraging aspect of the study was that the activities of the bulk (9 of 12) of the compounds in Table 3 showing significant (15% or greater) inhibition were relatively unaffected by the addition of serum, which was not the case for the dihydrofuro[3,4-*c*]pyridinone series (e.g., **2**).

3. Conclusions

Molecules with the ability to directly inhibit the function of perforin are of potential use in the treatment of some autoimmune diseases and therapy-induced conditions characterised by undesired perforin secretion, but to date only one class, the dihydrofuro[3,4-*c*]pyridinones have been reported.[10] Compound **5** in the present paper was one of a small number of compounds from a high throughput screen to show confirmatory inhibition of the lytic activity of

perforin in a cell-based assay, and as a novel and accessible structure with no obvious structural alerts, warranted further evaluation.

Analogues of **5** with both O-linked and CONH-linked side chains showed moderate inhibition of the lysis of Jurkat human leukaemia cells by added perforin, with the latter side chains appearing slightly more effective. Compounds with strongly basic centres (**27,28**), prepared in order to improve solubility were poorly tolerated. Additional substitution in the pyrido ring of the pyridobenzimidazole appeared to have little effect on activity, but the complete pyrido[1,2-*a*]benzimidazole moiety appeared important, with four out of six analogues lacking the benzo ring being inactive.

Selected compounds also showed good inhibition of the lytic activity of perforin released by KHYG-1 NK cells at non-toxic concentrations, although there was limited correlation between these effects and the potencies of the compounds in the Jurkat lysis assay. Encouragingly the compounds were relatively unaffected by the addition of serum, which was not the case for the dihydrofuro[3,4-*c*]pyridinone series.

4. Experimental section

4.1. Chemistry

Analyses were performed by the Microchemical Laboratory, University of Otago, Dunedin, NZ. Many samples tenaciously held water or DMF (verified by NMR). Melting points were determined using an Electrothermal Model 9200 and are as read. NMR spectra were measured in CD_3SOCD_3 (unless otherwise specified) on a Bruker Advance 400 spectrometer and referenced to Me_4Si. Mass spectra were recorded either on a Varian VG 7070 spectrometer at nominal 5000 resolution or a Finnigan MAT 900Q spectrometer. All final compound purities were determined to be >95% by HPLC on an Alltech Alltima C18 column (3.2 × 150 mm, 5 μm) eluting with 5–80% MeCN/45 mM NH_4HCO_2. pK_a values were calculated using ACD pK_a software (version 8.0; Advanced Chemistry Development Inc., Toronto, Canada),

4.1.1. 2-[4-(1-Amino-2,4-dicyano-6-methylpyrido[1,2-*a*]benzimidazol-3-yl)phenoxy]acetamide (5) (Scheme 1)

A solution of 2-(4-formylphenoxy)acetamide (**42**) (1.0 g, 5.59 mmol) and malononitrile (1.11 g, 17 mmol) in ethanol (170 mL) was stirred at 20 °C. 3-Aminopropanoic acid (0.20 g, 2.23 mmol) was added at the resulting solution was stirred at 20 °C overnight. The solution was concentrated under reduced pressure and the remaining solution was diluted with water and cooled to 0 °C. The resulting precipitate was washed with water and hexane and was dried to give 2-(4-(2,2-dicyanovinyl)phenoxy)acetamide[17] (**43**) as a pale yellow powder (0.89 g, 70%). ^{1}H NMR δ ppm 8.41 (s, 1H), 7.97 (d, J = 8.9 Hz, 2H), 7.64 (br s, 1H), 7.46 (br s, 1H), 7.18 (d, J = 9.0 Hz, 2H), 4.60 (s, 2H). LRMS (APCI$^+$) for $C_{12}H_{10}N_3O_2$ (M+H)$^+$: Calcd 228, Found 228.

A mixture of **43** (0.50 g, 2.20 mmol) and 2-(1*H*-benzo[*d*]imidazol-2-yl)acetonitrile (**44**) (0.17 g, 1.10 mmol) in ethanol (7 mL) was stirred at 20 °C. Piperidine (160 μL, 1.65 mmol) was added and the solution was heated to reflux for 3 h. The solution was cooled and the precipitate formed was collected, washed with EtOH and hexanes and dried to give 2-[4-(1-amino-2,4-dicyano-6-methylpyrido[1,2-*a*]benzimidazol-3-yl)phenoxy]acetamide (**5**) as a lime green powder (0.21 g, 50%), mp 290–293 °C. ^{1}H NMR δ ppm 8.68 (br s, 2H), 8.61 (d, J = 8.4 Hz, 1H), 7.87 (d, J = 8.0 Hz, 1H), 7.66 (br s, 1H), 7.63–7.54 (m, 3H), 7.47 (br s, 1H) 7.49–7.40 (m, 1H), 7.15 (d, J = 8.7 Hz, 2H), 4.55 (s, 2H). ^{13}C NMR δ ppm 66.54, 77.01, 85.62, 114.51 (2C), 114.86, 115.74, 115.99, 118.73, 121.75, 126.34, 127.12, 128.21, 130.25 (2C), 144.54, 147.39, 152.15, 152.56, 158.95, 169.45. Anal. Calcd for ($C_{21}H_{14}N_6O_2$): C, 65.96; H, 3.69; N, 21.98. Found: C, 65.51; H, 3.85; N, 21.80.

4.1.2. 2-[4-(1-Amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenoxy]-*N*-methylacetamide (6)

Reaction of 2-(4-formylphenoxy)acetic acid (**45**) (5.0 g, 27.75 mmol), malononitrile (5.51 g, 83.41 mmol) and 3-aminopropanoic acid (1.0 g, 11.22 mmol) in EtOH (875 ml) as for the preparation of **43** gave 2-(4-(2,2-dicyanovinyl)phenoxy)acetic acid (**46**) as light brown solid (2.65 g, 42%) which was used directly. ^{1}H NMR δ ppm 13.14 (br s, 1H), 8.40 (s, 1H), 7.96 (d, J = 8.9 Hz, 2H), 7.17 (d, J = 9.0 Hz, 2H), 4.85 (s, 2H).

A solution of **46** (0.50 g, 2.19 mmol), **44** (0.17 g, 1.09 mmol) in EtOH (7 mL) and piperidine (163 μL, 1.65 mmol) was heated to reflux for 3 h. The solution was cooled and the precipitate formed was collected and washed with EtOH and Et_2O. The crude material was stirred with 10% aqueous AcOH, filtered, and the precipitate was washed with water, EtOH and Et_2O and dried to give 2-(4-(1-amino-2,4-dicyanobenzo[4,5]imidazo[1,2-a]pyridin-3-yl)phenoxy) acetic acid (**47**) as a yellow powder (0.18 g, 43%), mp 296–299 °C. ^{1}H NMR δ ppm 13.07 (br s, 1H), 8.64 (br s, 2H), 8.60 (d, J = 8.4 Hz, 1H), 7.88 (d, J = 7.8 Hz, 1H), 7.65–7.58(m, 1H), 7.57 (d, J = 8.8, 2H), 7.46–7.42 (m, 1H), 7.13 (d, J = 8.8 Hz, 2H), 4.80 (s, 2H). Anal. Calcd for ($C_{21}H_{13}N_5O_3{\cdot}0.75H_2O$): C, 65.79; H, 3.42; N, 18.27. Found: C, 63.76; H, 3.81; N, 17.38.

A solution of acid **47** (0.07 g, 0.18 mmol) in dry DMF (5 mL) was warmed gently and then stirred at 20 °C under N_2. CDI (0.04 g, 0.27 mmol) was added and the solution was stirred at 20 °C for 1 h. A solution of methylamine in MeOH (140 μL, 2.0 M) was added and the solution was stirred for another 1 h. The DMF was removed in vacuo and the residue was recrystallized from DMF/Et_2O to give **6** as a yellow powder (0.04 g, 50%), mp 310–314 °C. ^{1}H NMR δ ppm 8.30 (br s, 2H), 8.61 (d, J = 8.4 Hz, 1H), 8.14–8.07 (m, 1H), 7.87 (d, J = 7.8 Hz, 1H), 7.62–7.56 (m, 3H), 7.46–7.41 (m, 1H), 7.17 (d, J = 8.8 Hz, 2H), 4.59 (s, 2H), 2.73 (d, J = 0.4 Hz, 3H). Anal. Calcd for ($C_{22}H_{16}N_6O_2{\cdot}1.25H_2O$): C, 63.08; H, 4.05; N, 20.06. Found: C, 63.04; H, 4.38; N, 20.41.

4.1.3. 2-[4-(1-Amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenoxy]-*N*,*N*-dimethylacetamide (7)

Similar reaction of acid **47** (0.10 g, 0.26 mmol) in dry DMF (5 mL) with CDI, followed by treatment with dimethylamine in MeOH, and purification of the crude product by column chromatography (silica, 20% EtOH/EtOAc) gave **7** as a yellow powder (0.03 g, 30%), mp 298–302 °C. ^{1}H NMR δ ppm 8.70 (br s, 2H), 8.60 (d, J = 8.4 Hz, 1H), 7.86 (d, J = 7.8 Hz, 1H), 7.64–7.57 (m, 1H), 7.56 (d, J = 8.8 Hz, 2H), 7.44–7.37 (m, 1H), 7.11 (d, J = 8.9 Hz, 2H), 4.93 (s, 2H), 3.04 (s, 3H), 2.88 (s, 3H). HRMS (ESI$^+$) for $C_{23}H_{19}N_6O_2$ (M+H)$^+$: Calcd 411.1564. Found: 411.1564. HPLC purity, 99.1%.

4.1.4. 2-[4-(1-Amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenoxy]-*N*-ethylacetamide (8)

Similar reaction of acid **47** (0.20 g, 0.52 mmol) in dry DMF (10 mL) with CDI, followed by treatment with ethylamine in water gave **8** as a yellow powder (0.11 g, 49%), mp (EtOH/Et_2O) 297–301 °C. ^{1}H NMR δ ppm 8.66 (br s, 2H), 8.61 (d, J = 8.4 Hz, 1H), 8.16 (t, J = 5.6 Hz, 1H), 7.88 (d, J = 7.6 Hz, 1H), 7.64–7.56 (m, 3H), 7.41–7.48 (m, 1H), 7.17 (d, J = 8.8 Hz, 2H), 4.58 (s, 2H), 3.25–3.15 (m, 2H), 1.07 (t, J = 7.2 Hz, 3H). Anal. Calcd for ($C_{23}H_{18}N_6O_2{\cdot}1.75H_2O$): C, 62.51; H, 4.90; N, 19.02. Found: C, 62.47; H, 4.51; N, 19.04.

4.1.5. 2-[4-(1-Amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenoxy]-*N*-propylacetamide (9)

Similar reaction of acid **47** (0.20 g, 0.52 mmol) in dry DMF (10 mL) with CDI, followed by treatment with propylamine gave

9 as a yellow powder (0.14 g, 63%), mp (EtOH/Et_2O) 302–306 °C. ^1H NMR δ ppm 8.66 (br s, 2H), 8.61 (d, J = 8.4 Hz, 1H), 8.14 (t, J = 5.6 Hz, 1H), 7.88 (d, J = 7.7 Hz, 1H), 7.66–7.54 (m, 3H), 7.42–7.48 (m, 1H), 7.17 (d, J = 8.8 Hz, 2H), 4.59 (s, 2H), 3.12 (q, J = 6.7 Hz, 2H), 1.47 (sextet, J = 7.3 Hz, 2H), 0.85 (t, J = 7.4 Hz, 3H). Anal. Calcd for ($C_{24}H_{20}N_6O_2 \cdot 2H_2O$): C, 62.60; H, 5.25; N, 18.25. Found: C, 62.94; H, 4.80; N, 18.42.

4.1.6. 2-[4-(1-Amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenoxy]-*N*-*iso*propylacetamide (10)

Similar reaction of acid **47** (0.20 g, 0.52 mmol) in dry DMF (10 mL) with CDI, followed by treatment with *iso*propylamine gave **10** as a yellow powder (0.14 g, 63%), mp (EtOH/Et_2O) 321–325 °C. ^1H NMR δ ppm 8.65 (br s, 2H), 8.60 (d, J = 8.4 Hz, 1H), 7.93 (d, J = 7.8 Hz, 1H), 7.88 (d, J = 7.7 Hz, 1H), 7.64–7.54 (m, 3H), 7.41–7.48 (m, 1H), 7.17 (d, J = 8.8 Hz, 2H), 4.56 (s, 2H), 4.04–3.91 (m, 1H), 1.13 (s, 3H), 1.11 (s, 3H). Anal. Calcd for ($C_{24}H_{20}N_6O_2 \cdot 1.5H_2O$): C, 63.85; H, 5.13; N, 18.61. Found: C, 63.88; H, 4.99; N, 18.78.

4.1.7. 2-[4-(1-Amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenoxy]-*N*-(2-hydroxyethyl)acetamide (14)

Similar reaction of acid **47** (0.20 g, 0.52 mmol) in dry DMF (10 mL) with CDI, followed by treatment with ethanolamine gave **14** as a yellow powder (0.12 g, 53%), mp (Et_2O/EtOH) 270–272 °C. ^1H NMR δ ppm 9.02 (br s, 2H), 8.62 (d, J = 8.3 Hz, 1H), 8.10 (t, J = 5.6 Hz, 1H), 7.84 (d, J = 7.7 Hz, 1H), 7.61–7.53 (m, 3H), 7.45–7.40 (m, 1H), 7.18 (d, J = 8.8 Hz, 2H), 4.71 (t, J = 5.5 Hz, 1H), 4.60 (s, 2H), 3.47 (q, J = 5.8 Hz, 2H), 3.21–3.31 (m, 2H). Anal. Calcd for ($C_{23}H_{18}N_6O_3 \cdot 1.25H_2O$): C, 61.53; H, 4.60; N, 18.72. Found: C, 61.45; H, 4.46: N, 18.63.

4.1.8. 2-[4-(1-Amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenoxy]-*N*-(2-hydroxypropyl)acetamide (15)

Similar reaction of acid **47** (0.20 g, 0.52 mmol) in dry DMF (10 mL) with CDI, followed by treatment with 1-aminopropan-2-ol, gave **15** as a yellow powder (0.07 g, 29%), mp (DMF/Et_2O) 294–297 °C. ^1H NMR δ ppm 8.68 (br s, 2H), 8.60 (d, J = 8.4 Hz, 1H), 8.03 (t, J = 5.8 Hz, 1H), 7.88 (d, J = 7.8 Hz, 1H), 7.67–7.54 (m, 3H), 7.48–7.41 (m, 1H), 7.17 (d, J = 8.8 Hz, 2H), 4.72 (d, J = 2.6 Hz, 1H), 4.62 (s, 2H), 3.79–3.65 (m, 1H), 3.20–3.04 (m, 2H), 1.04 (d, J = 6.2 Hz, 3H). Anal. Calcd for ($C_{24}H_{20}N_6O_3 \cdot 0.75H_2O$): C, 63.50; H, 4.77; N, 18.51. Found: C, 63.51; H, 4.72; N, 18.55.

4.1.9. 2-[4-(1-Amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenoxy]-*N*-(2-hydroxypropyl)acetamide (16)

Similar reaction of acid **47** (0.20 g, 0.52 mmol) in dry DMF (10 mL) with CDI, followed by treatment with 3-aminopropanol gave **16** as a yellow powder (0.03 g, 12%), mp 245–248 °C. ^1H NMR δ ppm 8.66 (br s, 2H), 8.61 (d, J = 8.4 Hz, 1H), 8.15 (t, J = 5.6 Hz, 1H), 7.88 (d, J = 7.6 Hz, 1H), 7.64–7.56 (m, 3H), 7.48–7.42 (m, 1H), 7.17 (d, J = 8.8 Hz, 2H), 4.59 (s, 2H), 3.42 (t, J = 6.3 Hz, 2H, interchangeable with D_2O), 3.23 (q, J = 6.6 Hz, 2H), 1.62 (quint, J = 6.6 Hz, 2H). Hydroxyl proton is not observed. Anal. Calcd for $C_{24}H_{20}N_6O_3 \cdot 2.25H_2O$: C, 59.93; H, 5.13; N, 17.47. Found: C, 59.84; H, 4.78; N, 17.46.

4.1.10. 2-[4-(1-Amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenoxy]-*N*-(2,3-dihydroxypropyl)acetamide (17)

Similar reaction of acid **47** (0.20 g, 0.52 mmol) in dry DMF (10 mL) with CDI, followed by treatment with 3-aminopropan-1,2-diol gave **17** as a yellow powder (0.013 g, 5%), mp (Me_2CO/Et_2O) 194–197 °C. ^1H NMR δ ppm 8.66 (br s, 2H), 8.61 (d, J = 8.4 Hz, 1H), 8.03 (t, J = 5.7 Hz, 1H), 7.88 (d, J = 7.6 Hz, 1H), 7.64–7.56 (m, 3H), 7.48–7.41 (m, 1H), 7.18 (d, J = 8.8 Hz, 2H), 4.63 (s, 2H), 3.58 (quint, J = 5.7 Hz, 1H, interchangeable with D_2O), 3.36–3.28 (m, 3H, interchangeable with D_2O), 3.12 (dd, J = 13.5, 6.9 Hz, 1H, interchangeable with D_2O). Hydroxyl protons are not observed. HRMS (ESI^+) for $C_{24}H_{21}N_6O_4$ $(M+H)^+$: Calcd 457.1619, Found 457.1627. HPLC purity, 94.5%.

4.1.11. 2-[4-(1-Amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenoxy]-*N*-(2-pyridin-4-ylethyl)acetamide (18)

Similar reaction of acid **47** (0.20 g, 0.52 mmol) in dry DMF (10 mL) with CDI, followed by treatment with 3-aminopropan-1,2-diol gave **18** as a yellow powder (0.07 g, 26%, mp (DMF/MeOH/Et_2O) 299–302 °C. ^1H NMR δ ppm 8.67 (br s, 2H), 8.61 (d, J = 8.4 Hz, 1H), 8.47 (d, J = 6.0 Hz, 2H), 8.26 (t, J = 5.7 Hz, 1H), 7.88 (d, J = 7.6 Hz, 1H), 7.64–7.55 (m, 3H), 7.48–7.41 (m, 1H), 7.25 (d, J = 6.0 Hz, 2H), 7.13 (d, J = 8.8 Hz, 2H), 4.58 (s, 2H), 3.45 (q, J = 6.7 Hz, 2H), 2.81 (t, J = 7.1 Hz, 2H). Anal. Calcd for ($C_{28}H_{21}N_7O_2$. 0.5DMF): C, 67.61; H, 4.71; N, 20.05. Found: C, 67.57; H, 4.53; N, 20.30.

4.1.12. 1-Amino-3-[4-(2-hydroxyethoxy)phenyl]pyrido[1,2-*a*]benzimidazole-2,4-dicarbonitrile (11) (Scheme 2)

A mixture of 2-(4-hydroxybenzylidene)malononitrile (**48**) (0.50 g, 2.94 mmol) and **44** (0.23 g, 1.47 mmol) in ethanol (9 mL) was stirred at 20 °C. Piperidine (220 μL, 2.21 mmol) was added and the solution was heated to reflux for 2 h. The solution was cooled and the precipitate formed was collected, washed with EtOH and hexanes and recrystallized from Me_2CO/Et_2O to give 1-amino-3-(4-hydroxyphenyl)pyrido[1,2-*a*]benzimidazole-2,4-dicarbonitrile (**49**) as a yellow powder (0.03 g, 7%), mp >300 °C. ^1H NMR δ ppm 10.00 (s, 1H), 8.53–8.65 (m, 3H), 7.86 (d, J = 7.7 Hz, 1H), 7.60 (t, J = 7.5 Hz, 1H), 7.48–7.40 (m, 3H), 6.96 (d, J = 8.6 Hz, 2H). Anal. Calcd for ($C_{19}H_{11}N_5O$): C, 70.15; H, 3.41; N, 21.53. Found: C, 69.96; H, 3.37; N, 21.51.

A mixture of **49** (0.20 g, 0.62 mmol), (2-bromoethoxy)(*tert*-butyl)dimethylsilane (0.22 g, 0.92 mmol) and Cs_2CO_3 (0.15 g, 0.46 mmol) in dry DMF (6 mL) were heated at 90 °C overnight under N_2. The solution was cooled, diluted with water, and extracted with EtOAc. The organic extracts were washed, dried and evaporated. The crude product (**50**) was dissolved in THF (56 mL) and treated at 20 °C with a solution of tetrabutylammonium fluoride in THF (1.48 mL, 1.0 M) for 30 min. The reaction was quenched by the addition of water and the THF was removed under reduced pressure. The aqueous solution was extracted with EtOAc and the combined extracts were washed with water and brine, dried (Na_2SO_4) and evaporated to give **11** as a yellow powder (0.03 g, 18%), mp (EtOAc) 302–305 °C. ^1H NMR δ ppm 8.63 (br s, 2H), 8.60 (d, J = 8.4 Hz, 1H), 7.87 (d, J = 7.6 Hz, 1H), 7.64–7.58 (m, 1H), 7.56 (d, J = 8.8 Hz, 2H), 7.47–7.41 (m, 1H), 7.15 (d, J = 8.8 Hz, 2H), 4.90 (t, J = 5.4 Hz, 1H), 4.11 (t, J = 4.9 Hz, 2H), 3.77 (q, J = 7.5 Hz, 2H). HRMS (ESI^+) for $C_{21}H_{16}N_5O_2$ $(M+H)^+$: Calcd 370.1299, Found 370.1292. HPLC purity, 94.8%.

4.1.13. 3-[4-(1-Amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenyl]-*N*-methylpropanamide (12) (Scheme 3)

A solution of ethyl 3-(4-formylphenyl)propanoate (**51**) (5.56 g, 27.0 mmol) and malononitrile (5.35 g, 81.0 mmol) in EtOH (850 mL) was stirred at 20 °C. 3-Aminopropanoic acid (0.96 g, 10.80 mmol) was added and the resulting solution was stirred at 20 °C overnight, then the solvent volume was reduced and the remaining solution was diluted with water and cooled to 4 °C. The precipitate collected, washed with water and hexanes, and was dried to give ethyl 3-(4-(2,2-dicyanovinyl)phenyl)propanoate (**52**) as a pale yellow solid (5.11 g, 74%) which was used directly. ^1H NMR δ ppm 8.48 (s, 1H), 7.88 (d, J = 8.3 Hz, 2H), 7.49 (d, J = 8.3 Hz, 2H), 4.04 (q, J = 7.1 Hz, 2H), 2.95 (t, J = 7.5 Hz, 2H), 2.67 (t, J = 7.5 Hz, 2H), 1.15 (t, J = 7.1 Hz, 3H). LRMS ($APCI^-$) for $C_{15}H_{13}N_2O_2$ $(M-H)^-$: Calcd 254, Found 254.

A mixture of **52** (5.11 g, 20.10 mmol) and **44** (1.58 g, 10.10 mmol) in ethanol (59 mL) was stirred at 20 °C. Piperidine (1.50 mL, 15.0 mmol) was added and the solution was heated to reflux for 3 h. The solution was cooled and diluted with Et_2O to precipitate a product that was collected, washed with Et_2O and was dried to give ethyl 3-[4-(1-amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenyl]propanoate (**53**) as a yellow powder (2.99 g, 72%), mp 258–260 °C. ^{1}H NMR δ ppm 8.64 (br s, 2H), 8.61 (d, *J* = 8.4 Hz, 1H), 7.87 (d, *J* = 7.7 Hz, 1H), 7.64–7.57 (m, 1H), 7.53 (d, *J* = 8.2 Hz, 2H), 7.49–7.41 (m, 3H), 4.07 (q, *J* = 7.1 Hz, 2H), 2.98 (t, *J* = 7.5 Hz, 2H), 2.72 (t, *J* = 7.6 Hz, 2H), 1.17 (t, *J* = 7.1 Hz, 3H). Anal. Calcd for ($C_{24}H_{19}N_5O_2{\cdot}0.25H_2O$): C, 70.40; H, 4.68; N, 17.10. Found: C, 69.64; H, 4.79; N, 17.23.

A solution of ester **53** (0.20 g, 0.49 mmol) in a mixture of EtOH (50 mL) and aqueous NaOH (1.2 mL, 2.0 M) was stirred under reflux for 1 h, then cooled, diluted with water and the EtOH removed under reduced pressure. The resulting solution was acidified to pH 1 (concd HCl) and the precipitate formed was collected, washed with water, EtOH, Et_2O, then dried to give 3-[4-(1-amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenyl]propanoic acid (**54**) as a yellow powder (0.18 g, 97%) mp 314–317 °C. ^{1}H NMR δ ppm 8.69 (br s, 2H), 8.62 (d, *J* = 8.4 Hz, 1H), 7.88 (d, *J* = 7.7 Hz, 1H), 7.65–7.58 (m, 1H), 7.54 (d, *J* = 8.2 Hz, 2H), 7.50–7.41 (m, 3H), 2.96 (t, *J* = 7.6 Hz, 2H), 2.65 (t, *J* = 7.7 Hz, 2H). Acid proton not observed. HRMS (ESI$^+$) for $C_{22}H_{16}N_5O_2$ (M+H)$^+$: Calcd 382.1299, Found 382.1308.

A solution of acid **54** (0.10 g, 0.26 mmol) in dry DMF (5 mL) was stirred at 20 °C under N_2. CDI (0.06 g, 0.39 mmol) was added and the solution was stirred for 1 h. A solution of methylamine in MeOH (180 μL, 2.0 M) was added and the solution was stirred at 20 °C overnight. The DMF was removed under reduced pressure and the crude material was purified by column chromatography on silica gel, eluting with 20% EtOH/EtOAc, then recrystallized from Me_2CO/Et_2O to give **12** as yellow needles (0.02 g, 23%) mp 290–293 °C. ^{1}H NMR δ ppm 8.67 (br s, 2H), 8.61 (d, *J* = 8.4 Hz, 1H), 7.88 (d, *J* = 7.8 Hz, 1H), 7.81–7.75 (m, 1H), 7.64–7.58 (m, 1H), 7.53 (d, *J* = 8.2 Hz, 2H), 7.48–7.41 (m, 3H), 2.97–2.91 (m, 2H), 2.59 (d, *J* = 4.6 Hz, 3H), 2.50–2.43 (m, 2H). HRMS (ESI$^+$) for $C_{23}H_{19}N_6O$ (M+H)$^+$: Calcd 395.1615, Found 395.1617. HPLC purity, 97.6%.

4.1.14. 3-[4-(1-Amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)phenyl]-*N*-methylpropanamide (13)

Similar reaction of acid **54** (0.10 g, 0.26 mmol) in dry DMF (10 mL) with CDI, followed by treatment with of dimethylamine in MeOH (180 μL, 2.0 M), and purification of the product by column chromatography on silica gel eluting with 20% EtOH/EtOAc gave **13** as yellow needles (0.03 g, 23%), mp (Me_2CO/Et_2O) 280–282 °C. ^{1}H NMR δ ppm 8.75 (br s, 2H), 8.61 (d, *J* = 8.4 Hz, 1H), 7.88 (d, *J* = 7.7 Hz, 1H), 7.64–7.58 (m, 1H), 7.53 (d, *J* = 8.2 Hz, 2H), 7.50–7.41 (m, 3H), 2.96 (s, 3H), 2.96–2.91 (m, 2H), 2.84 (s, 3H), 2.74–7.68 (m, 2H). HRMS (ESI$^+$) for $C_{24}H_{21}N_6O$ (M+H)$^+$: Calcd 409.1771, Found 409.1780. HPLC purity, 95.6%.

4.1.15. 4-(9-Amino-6,8-dicyanopyrido[1,2-*a*]benzimidazol-7-yl)benzamide (19) (Scheme 4)

A mixture of methyl 4-(2,2-dicyanovinyl)benzoate (**55**) (0.50 g, 2.36 mmol) and **44** (0.19 g, 1.18 mmol) in EtOH (7 mL) was stirred at 20 °C. Piperidine (175 μL, 1.77 mmol) was added and the solution was heated to reflux for 3 h. The solution was cooled and the precipitate formed was collected, washed with Et_2O and dried to give methyl 4-(9-amino-6,8-dicyanopyrido[1,2-*a*]benzimidazol-7-yl)benzoate (**56**) as a yellow powder (0.11 g, 24%), mp >300 °C. ^{1}H NMR δ ppm 8.77 (br s, 2H), 8.63 (d, *J* = 8.4 Hz, 1H), 8.17 (d, *J* = 8.5 Hz, 2H), 7.90 (d, *J* = 7.7 Hz, 1H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.66–7.60 (m, 1H), 7.50–7.44 (m, 1H), 3.92 (d, *J* = 8.3 Hz, 3H). Anal. Calcd for ($C_{21}H_{13}N_5O_2$): C, 68.66; H, 3.57; N, 19.06. Found: C, 68.45; H, 3.63; N, 18.91.

A solution of **56** (1.0 g, 2.72 mmol) and NaOH (0.54 g, 14.0 mmol) in EtOH (75 mL) was heated to reflux for 1 h, then cooled and the EtOH removed under reduced pressure. The residue was diluted with water, acidified to pH 1 (concd HCl) and the precipitate collected, washed with water, EtOH and Et_2O and dried to give 4-(9-amino-6,8-dicyanopyrido[1,2-*a*]benzimidazol-7-yl)benzoic acid (**57**) as a yellow powder (0.96 g, 100%), mp >345 °C. ^{1}H NMR δ ppm 13.24 (br s, 1H), 8.77 (br s, 2H), 8.63 (d, *J* = 8.4 Hz, 1H), 8.15 (d, *J* = 8.4 Hz, 2H), 7.90 (d, *J* = 7.6 Hz, 1H), 7.74 (d, *J* = 8.5 Hz, 2H), 7.66–7.60 (m, 1H), 7.51–7.44 (m, 1H). Anal. Calcd for ($C_{20}H_{11}N_5O_2{\cdot}1.25H_2O$): C, 67.13; H, 3.24; N, 19.57. Found: C, 63.94; H, 3.52; N, 18.35.

A solution of **57** (0.20 g, 0.57 mmol) in dry DMF (10 mL) was stirred at 20 °C under N_2. CDI (0.14 g, 0.85 mmol) was added and the solution was stirred for 1 h, then a solution of NH_3 in MeOH (400 μL, 7.0 M) was added and the solution was stirred at 20 °C for 1 h. The DMF was removed under reduced pressure and the residue was recrystallized from DMF/Et_2O to give **19** as a yellow powder (0.10 g, 48%), mp >340 °C. ^{1}H NMR δ ppm 8.74 (br s, 2H), 8.63 (d, *J* = 8.4 Hz, 1H), 8.14 (br s, 1H), 8.06 (d, *J* = 8.4 Hz, 2H), 7.90 (d, *J* = 7.7 Hz, 1H), 7.69 (d, *J* = 8.4 Hz, 2H), 7.66–7.60 (m, 1H), 7.52 (br s, 1H), 7.50–7.43 (m, Hz, 1H). Anal. Calcd for ($C_{20}H_{12}N_6O$. 1.5DMF·0.5H_2O): C, 63.08; H, 4.97; N, 22.52. Found: C, 62.71; H, 5.26; N, 22.53.

4.1.16. 4-(9-Amino-6,8-dicyanopyrido[1,2-*a*]benzimidazol-7-yl)-*N*-methylbenzamide (20)

Similar reaction of acid **57** (0.20 g, 0.57 mmol) and CDI (0.14 g, 0.85 mmol) in dry DMF (10 mL) at 20 °C under N_2 for 1 h, followed by treatment with methylamine in MeOH (1.42 mL, 2.0 M) at 20 °C for 1 h, gave **20** as a yellow powder (0.14 g, 67%), mp (DMF/Et_2O) >320 °C. ^{1}H NMR δ ppm 8.78 (br s, 2H), 8.63 (d, *J* = 8.4 Hz, 1H), 8.64–8.57 (m, 1H), 8.01 (d, *J* = 8.4 Hz, 2H), 7.88 (d, *J* = 8.1 Hz, 1H), 7.70 (d, *J* = 8.3 Hz, 2H), 7.61 (t, *J* = 7.7 Hz, 1H), 7.49–7.42 (m, 1H), 2.84 (d, *J* = 4.5 Hz, 3H). Anal. Calcd for ($C_{21}H_{14}N_6O$.0.75DMF): C, 66.30; H, 4.61; N, 22.45. Found: C, 66.21; H, 4.40; N, 22.52.

4.1.17. 4-(9-Amino-6,8-dicyanopyrido[1,2-*a*]benzimidazol-7-yl)-*N*,*N*-dimethylbenzamide (21)

Similar reaction of acid **57** (0.20 g, 0.57 mmol) and CDI (0.14 g, 0.85 mmol) in dry DMF (10 mL) at 20 °C under N_2 for 1 h, followed by treatment with dimethylamine in MeOH (1.42 mL, 2.0 M) at 20 °C for 1 h. The crude material was purified by preparative HPLC on a Synergi Max-RP C12 column eluting with 30–98% MeCN/45 mM NH_4HCO_2 to give **21** as a yellow powder (0.03 g, 14%), mp >315 °C. ^{1}H NMR δ ppm 8.73 (br s, 2H), 8.63 (d, *J* = 8.4 Hz, 1H), 7.89 (d, *J* = 7.6 Hz, 1H), 7.68 (d, *J* = 8.4 Hz, 2H), 7.65–7.59 (m, 3H), 7.50–7.43 (m, 1H), 3.04 (s, 3H), 2.97 (s, 3H). HRMS (ESI$^+$) for $C_{22}H_{16}N_6NaO$ (M+H)$^+$: Calcd 403.1278, Found 403.1283.

4.1.18. 4-(9-Amino-6,8-dicyanopyrido[1,2-*a*]benzimidazol-7-yl)-*N*-ethylbenzamide (22)

Similar reaction of acid **57** (0.20 g, 0.57 mmol) and CDI (0.14 g, 0.85 mmol) in dry DMF (10 mL) at 20 °C under N_2 for 1 h, followed by treatment with ethylamine in water (225 μL, 70%) at 20 °C for 1 h, gave **22** as a yellow powder (0.15 g, 68%), mp >325 °C. ^{1}H NMR δ ppm 8.75 (br s, 2H), 8.67–8.59 (m, 2H), 8.03 (d, *J* = 8.3 Hz, 2H), 7.90 (d, *J* = 8.0 Hz, 1H), 7.70 (d, *J* = 8.3 Hz, 2H), 7.66–7.59 (m, 1H), 7.50–7.43 (m, 1H), 3.39–3.26 (m, 2H), 1.17 (t, *J* = 7.2 Hz, 3H). Anal. Calcd for ($C_{22}H_{16}N_6O{\cdot}1.25H_2O$): C, 65.58; H, 4.63; N, 20.86. Found: C, 65.69; H, 4.42; N, 21.00.

4.1.19. 4-(9-Amino-6,8-dicyanopyrido[1,2-*a*]benzimidazol-7-yl)-*N*-(2-hydroxyethyl)benzamide (23)

Similar reaction of acid **57** (0.20 g, 0.57 mmol) and CDI (0.14 g, 0.85 mmol) in dry DMF (10 mL) at 20 °C under N_2 for 1 h, followed

by treatment with ethanolamine (215 μL, 2.84 mmol) at 20 °C for 1 h, gave **23** as a yellow powder (0.20 g, 87%), mp (Et_2O) 285–290 °C. ^{1}H NMR δ ppm 8.80 (br s, 2H), 8.65 (d, J = 8.3 Hz, 1H), 8.60 (t, J = 5.6 Hz, 1H), 8.04 (d, J = 8.5 Hz, 2H), 7.87 (d, J = 7.7 Hz, 1H), 7.69 (d, J = 8.4 Hz, 2H), 7.63–7.56 (m, 1H), 7.48–7.40 (m, 1H), 4.73 (t, J = 5.6 Hz, 1H), 3.56 (q, J = 6.0 Hz, 2H), 3.38 (q, J = 6.0 Hz, 2H). Anal. Calcd for ($C_{22}H_{16}N_6O_2 \cdot 75H_2O$): C, 64.46; H, 4.30; N, 20.50. Found: C, 64.15; H, 4.40; N, 20.57.

4.1.20. 4-(9-Amino-6,8-dicyanopyrido[1,2-*a*]benzimidazol-7-yl)-*N*-(3-hydroxypropyl)benzamide (24)

Similar reaction of acid **57** (0.20 g, 0.57 mmol) and CDI (0.14 g, 0.85 mmol) in dry DMF (10 mL) at 20 °C under N_2 for 1 h, followed by treatment with 3-aminopropanol (215 μL, 2.83 mmol) at 20 °C for 1 h, gave **24** as a yellow powder (0.15 g, 63%), mp >300–304 °C. ^{1}H NMR δ ppm 8.75 (br s, 2H), 8.65–8.59 (m, 2H), 8.02 (d, J = 8.4 Hz, 2H), 7.90 (d, J = 7.7 Hz, 1H), 7.70 (d, J = 8.4 Hz, 2H), 7.66–7.60 (m, 1H), 7.50–7.43 (m, 1H), 4.40 (br s, 1H), 3.50 (t, J = 6.3 Hz, 2H), 3.37 (q, J = 6.6 Hz, 2H), 1.73 (quint, J = 6.7 Hz, 2H). Anal. Calcd for ($C_{23}H_{18}N_6O_2 \cdot 0.5H_2O$): C, 65.86; H, 4.57; N, 20.04. Found: C, 65.85; H, 4.54; N, 20.06.

4.1.21. 4-(9-Amino-6,8-dicyanopyrido[1,2-*a*]benzimidazol-7-yl)-*N*-(2-hydroxypropyl)benzamide (25)

Similar reaction of acid **57** (0.20 g, 0.57 mmol) and CDI (0.14 g, 0.85 mmol) in dry DMF (10 mL) at 20 °C under N_2 for 1 h, followed by treatment with 1-aminopropan-2-ol (220 μL, 2.84 mmol) at 20 °C for 1 h, gave **25** as a yellow powder (0.14 g, 59%), mp (DMF/Et_2O) >300 °C. ^{1}H NMR δ ppm 8.75 (br s, 2H), 8.63 (d, J = 8.4 Hz, 1H), 8.58 (t, J = 5.7 Hz, 1H), 8.05 (d, J = 8.4 Hz, 2H), 7.90 (d, J = 7.6 Hz, 1H), 7.70 (d, J = 8.4 Hz, 2H), 7.66–7.59 (m, 1H), 7.50–7.43 (m, 1H), 4.76 (d, J = 3.6 Hz, 1H), 3.89–3.78 (m, 1H), 3.28–3.30 (m, 2H), 1.10 (d, J = 6.2 Hz, 3H). Anal. Calcd for ($C_{23}H_{18}N_6O_2$. 1.25DMF): C, 64.03; H, 5.37; N, 20.24. Found: C, 64.10; H, 5.34; N, 20.27.

4.1.22. 4-(1-Amino-2,4-dicyanopyrido[1,2-*a*]benzimidazol-3-yl)-*N*-(2,3-dihydroxypropyl)benzamide (26)

Similar reaction of acid **57** (0.20 g, 0.57 mmol) and CDI (0.14 g, 0.85 mmol) in dry DMF (10 mL) at 20 °C under N_2 for 1 h, followed by treatment with 3-aminopropan-1,2-diol (0.26 g, 2.84 mmol) at 20 °C for 1 h, gave **26** as a yellow powder (0.05 g, 19%), mp (Et_2O) >300 °C. ^{1}H NMR δ ppm 8.70 (br s, 2H), 8.64 (d, J = 8.4 Hz, 1H), 8.56 (t, J = 5.7 Hz, 1H), 8.04 (d, J = 8.4 Hz, 2H), 7.87 (d, J = 7.7 Hz, 1H), 7.70 (d, J = 8.5 Hz, 2H), 7.63–7.57 (m, 1H), 7.52–7.50 (m, 1H), 4.81 (d, J = 5.0 Hz, 1H), 4.55 (t, J = 5.8 Hz, 1H), 3.69 (sextet, J = 5.5 Hz, 1H), 3.49–3.42 (m, 1H), 3.39 (d, J = 5.5 Hz, 2H), 3.32–3.21 (m, 1H). Anal. Calcd for ($C_{23}H_{18}N_6O_3 \cdot 2H_2O$): C, 59.73; H, 4.79; N, 18,17. Found: C, 60.06; H, 4.44; N, 18.18.

4.1.23. 4-(9-Amino-6,8-dicyanopyrido[1,2-*a*]benzimidazol-7-yl)-*N*-[2-(dimethylamino)ethyl]benzamide (27)

Similar reaction of acid **57** (0.20 g, 0.57 mmol) and CDI (0.14 g, 0.85 mmol) in dry DMF (10 mL) at 20 °C under N_2 for 1 h, followed by treatment with *N*,*N*-dimethylethylenediamine (90 μL, 0.85 mmol) at 20 °C for 1 h, gave **27** as a yellow powder (0.22 g, 93%), mp (Et_2O) >177–180 °C. ^{1}H NMR δ ppm 9.21 (br s, 2H), 8.76–8.67 (m, 2H), 8.00 (d, J = 8.4 Hz, 2H), 7.72–7.64 (m, 3H), 7.47–7.40 (m, 1H), 7.32–7.25 (m, 1H), 3.57 (q, J = 6.0 Hz, 2H), 3.00 (t, J = 6.1 Hz, 2H), 2.64 (s, 6H). Anal. Calcd for ($C_{24}H_{21}N_7O \cdot 1.5H_2O$): C, 63.99; H, 5.37; N, 21.76. Found: C, 64.10; H, 5.70; N, 21.69.

4.1.24. 4-(9-Amino-6,8-dicyanopyrido[1,2-*a*]benzimidazol-7-yl)-*N*-(2-piperidin-1-ylethyl)benzamide (28)

Similar reaction of acid **57** (0.20 g, 0.57 mmol) and CDI (0.14 g, 0.85 mmol) in dry DMF (10 mL) at 20 °C under N_2 for 1 h, followed by treatment with 4-(2-aminoethyl)piperidine (405 μL, 2.84 mmol) at 20 °C for 1, gave **28** as yellow needles (0.03 g, 11%), mp (Me_2CO/Et_2O) 201–204 °C. ^{1}H NMR δ ppm 8.76–8.68 (m, 2H), 8.29 (br s, 2H), 8.00 (d, J = 8.4 Hz, 2H), 7.73–7.64 (m, 3H), 7.47–7.40 (m, 1H), 7.32–7.25 (m, 1H), 3.58 (q, J = 6.1 Hz, 2H), 3.08–2.79 (m, 6H), 1.73–1.66 (quint, J = 5.3 Hz, 4H), 1.54–1.45 (m, 2H). Anal. Calcd for ($C_{27}H_{25}N_7O \cdot H_2O$): C, 67.34; H, 5.65; N, 20.36. Found: C, 66.90; H, 5.50; N, 20.42.

4.1.25. 4-(9-Amino-6,8-dicyanopyrido[1,2-*a*]benzimidazol-7-yl)-*N*-(2-morpholin-4-ylethyl)benzamide (29)

Similar reaction of acid **57** (0.20 g, 0.57 mmol) and CDI (0.14 g, 0.85 mmol) in dry DMF (10 mL) at 20 °C under N_2 for 1 h, followed by treatment with 4-(2-aminoethyl)morpholine (375 μL, 2.84 mmol) at 20 °C for 1 h, gave **29** as a yellow powder (0.14 g, 52%), mp (Me_2CO/Et_2O), 288–291 °C. ^{1}H NMR δ ppm 8.75–8.54 (m, 4H), 8.01 (d, J = 8.4 Hz, 2H), 7.86 (d, J = 7.8 Hz, 1H), 7.70 (d, J = 8.4 Hz, 2H), 7.62–7.55 (m, 1H), 7.46–7.39 (m, 1H), 3.62 (t, J = 4.3 Hz, 4H), 3.47 (q, J = 6.3 Hz, 2H), 2.68–2.48 (m, 6H). Anal. Calcd for ($C_{26}H_{23}N_7O_2$): C, 67.08; H, 4.98; N, 21.06. Found: C, 66.80; H, 5.00; N, 20.79.

4.1.26. 4-(9-Amino-6,8-dicyanopyrido[1,2-*a*]benzimidazol-7-yl)-*N*-(2-pyridin-4-ylethyl)benzamide (30)

Similar reaction of acid **57** (0.20 g, 0.57 mmol) and CDI (0.14 g, 0.85 mmol) in dry DMF (10 mL) at 20 °C under N_2 for 1 h, followed by treatment with 4-(2-aminoethyl)pyridine (236 μL, 1.98 mmol) at 20 °C for 1 h, gave **30** as yellow needles (0.02 g, 11%), mp (DMF/MeOH) >315 °C. ^{1}H NMR δ ppm 8.79 (br s, 2H), 8.79 (t, J = 5.5 Hz, 1H), 8.63 (d, J = 8.4 Hz, 1H), 8.49 (d, J = 6.0 Hz, 2H), 7.98 (d, J = 8.4 Hz, 2H), 7.89 (d, J = 7.7 Hz, 1H), 7.70 (d, J = 8.4 Hz, 2H), 7.66–7.59 (m, 1H), 7.50–7.43 (m, 1H), 7.31 (d, J = 5.6 Hz, 2H), 3.59 (q, J = 6.6 Hz, 2H), 2.93 (t, J = 7.1 Hz, 2H). HRMS (ESI$^+$) for $C_{27}H_{19}N_7NaO$ $(M+H)^+$: Calcd 480.1543, Found 480.1550. HPLC purity, 98.2%.

4.1.27. (*E*,*Z*)-2-(4-(2-(1*H*-Benzo[*d*]imidazol-2-yl)-2-cyanovinyl) phenoxy)acetamide (36) (Scheme 5)

A solution of **44** (0.32 g, 2.01 mmol) and **43** (0.30 g, 1.67 mmol) were heated under reflux for 15 h in EtOH (20 ml) with a catalytic amount of piperidine to give **36** as a yellow solid (75%), mp (EtOH/Et_2O) 252–254 °C, without further purification. ^{1}H NMR δ ppm 12.96 (br s, 1H), 8.27 (s, 1H), 8.00 (d, J = 8.9 Hz, 2H), 8.65–8.56 (m, 2H), 7.59 (br s, 1H), 7.41 (br s, 1H), 7.27–7.21 (m, 2H), 7.17 (d, J = 9.0 Hz, 2H), 4.57 (s, 2H). HRMS (ESI$^+$) for $C_{18}H_{15}N_4O_2$ $(MH)^+$: Calcd 319.1190, Found 319.1185. Anal. Calcd for ($C_{18}H_{14}N_4O_2$): C, 67.91; H, 4.43; N, 17.60. Found: C, 68.13; H, 4.49; N, 17.77. HPLC purity, 98.7% (*E*/*Z* ratio 35:64).

4.1.28. 2-(4-(1-Amino-2,4-dicyano-6-methylbenzo[4,5]imidazo[1,2-*a*]pyridin-3-yl)phenoxy)acetamide (31)

A solution of 3-methylbenzene-1,2-diamine (**58**) (1.50 g, 12.28 mmol) and cyanoacetamide (2.06 g, 24.56 mmol) in xylene (25 ml) was heated under reflux for 15 h in the presence of 4 Å molecular sieves. The hot solvent was decanted and the residue was extracted with Et_2O/MeOH and filtrated. The filtrate was evaporated to dryness to give 2-(4-methyl-1*H*-benzo[*d*]imidazol-2-yl)acetonitrile (**63**) as a brown solid (1.62 g, 77%) which was used directly. ^{1}H NMR δ ppm 9.80 (br s, 1H), 7.46–7.35 (m, 1H), 7.20 (t, J = 7.7 Hz, 1H), 7.09 (d, J = 7.4 Hz, 1H), 4.15 (s, 2H), 2.59 (s, 3H). LRMS (APCI$^+$) for $C_{10}H_{10}N_3$ (MH^+): Calcd 172, Found 172.

A solution of **63** (0.30 g, 1.75 mmol), **43** (0.48 g, 2.10 mmol) and a catalytic amount of piperidine in EtOH (20 ml) was heated under reflux for 15 h. The cooled solution was filtered and the precipitate was washed with EtOH and purified by flash chromatography on silica gel, eluting with 5–30% MeOH/CH_2Cl_2 to give **31** as a green

solid (0.14 g, 20%), mp 289–293 °C. ^{1}H NMR δ ppm 8.59 (br s, 2H), 8.41 (d, J = 8.4 Hz, 1H), 7.61 (br s, 1H), 7.58 (d, J = 8.8 Hz, 2H), 7.43 (d, J = 7.4, 1H), 7.42 (br s, 1H), 7.37–7.31 (m, 1H), 7.16 (d, J = 8.8 Hz, 2H), 4.55 (s, 2H), 2.65 (s, 3H). HRMS (ESI$^+$) for $C_{22}H_{17}N_6O_2$ (MH^+): Calcd 397.1408, Found 397.1406. Anal. Calcd for ($C_{22}H_{16}N_6O_2 \cdot 0.9H_2O$): C, 64.04; H, 4.35; N, 20.37. Found: C, 63.96; H, 4.17; N, 20.02.

4.1.29. (*E,Z*)-2-(4-(2-Cyano-2-(4-methyl-1*H*-benzo[*d*]imidazol-2-yl)vinyl)phenoxy)acetamide (37)

Similar reaction of **63** (0.30 g, 1.75 mmol) and **42** (0.35 g, 1.93 mmol) under reflux for 15 h in EtOH (20 ml) with a catalytic amount of piperidine gave **37** as a light brown solid (0.10 g, 16%), mp (EtOH) 264–268 °C, without further purification. ^{1}H NMR δ ppm 12.16–13.10 (m, 1H), 8.36–8.21 (m, 1H), 8.00 (d, J = 8.4 Hz, 2H), 7.59 (br s, 1H), 7.41 (br s, 1H), 7.30–7.54 (m, 1H), 7.17 (d, J = 8.6 Hz, 2H), 7.22–7.10 (m, 1H), 7.09–7.01 (m, 1H), 4.57 (s, 2H), 2.56 (s, 3H). HRMS (ESI$^-$) for $C_{19}H_{15}N_4O_2$ $(M-H)^-$: Calcd 331.1200, Found 331.1191. HPLC purity, 97.2% (*E*/*Z* ratio 51:46).

4.1.30. 2-(4-(1-Amino-6-chloro-2,4-dicyanobenzo[4,5]imidazo[1,2-*a*]pyridin-3-yl)phenoxy)acetamide (32)

Similar reaction of 3-chlorobenzene-1,2-diamine (**59**) (1.50 g, 10.52 mmol) with cyanoacetamide (1.77 g, 21.04 mmol) in xylene (25 ml), and purification of the crude product by flash chromatography on silica gel, eluting with 15–50% EtOAc/hexanes, provided 2-(4-chloro-1*H*-benzo[*d*]imidazol-2-yl)acetonitrile (**64**) as a yellow-orange solid (0.82 g, 41%), which was used directly. ^{1}H NMR ($CDCl_3$) δ ppm 9.87 (br s, 1H), 7.39 (d, J = 7.9 Hz, 1H), 7.32 (t, J = 7.8 Hz, 1H), 7.23 (d, J = 7.9 Hz, 1H), 4.21 (s, 2H). LRMS (APCI$^+$) for $C_9H_7ClN_3$ (MH^+): Calcd 192, Found 192.

A solution of **64** (0.40 g, 2.11 mmol), **43** (0.40 g, 1.76 mmol) and a catalytic amount of piperidine in EtOH (20 ml) was heated under reflux for 15 h, and the product was purified by preparative HPLC on an Agilent Zorbax SB-C18 column, eluting with 10–95% MeCN/45 mM NH_4HCO_2, to give **32** as a yellowish solid (65 mg, 9%), mp 328–332 °C. ^{1}H NMR δ ppm 8.73 (br s, 2H), 8.58 (d, J = 8.4 Hz, 1H), 7.70 (d, J = 7.8 Hz, 1H), 7.61 (br s, 1H), 7.58 (d, J = 8.8 Hz, 2H), 7.42 (t, J = 8.1 Hz, 1H), 7.41 (br s, 1H), 7.17 (d, J = 8.8 Hz, 2H), 4.56 (s, 2H). HRMS (ESI$^-$) for $C_{21}H_{12}ClN_6O_2$ $(M-H)^-$: Calcd 415.0716, Found 415.0714. Anal. Calcd for ($C_{21}H_{13}ClN_6O_2 \cdot 1.0H_2O$): C, 58.00; H, 3.48; N, 19.33. Found: C, 57.71; H, 3.78; N, 19.74.

4.1.31. (*E,Z*)-2-(4-(2-(4-Chloro-1*H*-benzo[*d*]imidazol-2-yl)-2-cyanovinyl)phenoxy)acetamide (38)

Similar reaction of **64** (0.35 g, 1.83 mmol) and **42** (0.30 g, 1.66 mmol) in EtOH (20 ml) with a catalytic amount of piperidine gave **38** as a yellow solid (0.46 g, 79%), mp (EtOH) 268–271 °C, without further purification. ^{1}H NMR δ ppm 13.27 (br s, 1H), 8.41–8.27 (m, 1H), 8.02 (d, J = 8.9 Hz, 2H), 7.59 (br s, 1H), 7.41 (br s, 1H), 7.63–7.49 (m, 1H), 7.32 (d, J = 7.6 Hz, 1H), 7.25 (t, J = 7.8 Hz, 1H), 7.17 (d, J = 8.9 Hz, 2H), 4.58 (s, 2H). HRMS (ESI$^+$) for $C_{18}H_{14}ClN_4O_2$ $(MH)^+$: Calcd 353.0800, Found 353.0799. Anal. Calcd for ($C_{18}H_{13}ClN_4O_2$): C, 61.28; H, 3.71; N, 15.88. Found: C, 61.66; H, 3.73; N, 16.21. HPLC purity, 97.8% (*E*/*Z* ratio 42:56).

4.1.32. 2-(4-(1-Amino-2,4-dicyano-6-methoxybenzo[4,5]imidazo[1,2-*a*]pyridin-3-yl)phenoxy)acetamide (33)

Similar reaction of 3-methoxybenzene-1,2-diamine (**60**) (1.50 g, 10.86 mmol) with cyanoacetamide (1.83 g, 21.71 mmol) in xylene (25 ml) and purification of the product by flash chromatography on silica gel, eluting with 50–100% EtOAc/hexanes, gave 2-(4-methoxy-1*H*-benzo[*d*]imidazol-2-yl)acetonitrile (**65**) as a pale yellow solid (1.53 g, 75%), which was used directly. ^{1}H NMR ($CDCl_3$) δ ppm 9.69 (br s, 1H), 7.35–7.06 (m, 2H), 6.74 (t, J = 7.9 Hz, 1H), 4.14 (s, 2H), 4.01 (s, 3H). LRMS (APCI$^+$) for $C_{10}H_{10}N_3O$ (MH^+): Calcd 188, Found 188.

A solution of **65** (0.40 g, 2.11 mmol), **43** (0.40 g, 1.76 mmol) and a catalytic amount of piperidine in EtOH (20 ml) was heated under reflux for 15 h, and the product was purified by flash chromatography on silica gel, eluting with 3% MeOH/CH_2Cl_2, to give **33** as a brown solid (0.29 g, 40%), mp 284–286 °C. ^{1}H NMR δ ppm 8.57 (br s, 2H), 8.17 (d, J = 8.4 Hz, 1H), 7.61 (br s, 1H), 7.58 (d, J = 8.8 Hz, 2H), 7.47 (br s, 1H), 7.45–7.39 (m, 1H), 7.37 (t, J = 8.3 Hz, 1H), 7.16 (d, J = 8.8 Hz, 2H), 4.55 (s, 2H), 4.01 (s, 3H). HRMS (ESI$^-$) for $C_{22}H_{15}N_6O_3$ $(M-H)^-$: Calcd 411.1211, Found 411.1216. Anal. Calcd for ($C_{22}H_{16}N_6O_3 \cdot 0.9MeOH \cdot 0.5H_2O$): C, 60.75; H, 4.46; N, 18.89. Found: C, 61.09; H, 4.61; N, 18.66.

4.1.33. (*E,Z*)-2-(4-(2-Cyano-2-(4-methoxy-1*H*-benzo[*d*]imidazol-2-yl)vinyl)phenoxy)acetamide (39)

Similar reaction of **65** (0.35 g, 1.84 mmol) and **42** (0.30 g, 1.66 mmol) in EtOH (20 ml) with a catalytic amount of piperidine gave **39** as a yellow solid (0.32 g, 41%), mp (EtOH) 241–244 °C, without further purification. ^{1}H NMR δ ppm 12.94 (br s, 1H), 8.38–8.22 (m, 1H), 7.97 (d, J = 8.9 Hz, 2H), 7.58 (br s, 1H), 7.41 (br s, 1H), 7.24–7.13 (m, 2H), 7.16 (d, J = 8.9 Hz, 2H), 6.84–6.72 (m, 1H), 4.57 (s, 2H), 3.96 (s, 3H). HRMS (ESI$^+$) for $C_{19}H_{17}N_4O_3$ $(MH)^+$: Calcd 349.1295, Found 349.1298. Anal. Calcd for ($C_{19}H_{16}N_4O_3 \cdot 0.2H_2O$): C, 64.84; H, 4.70; N, 15.92. Found: C, 64.65; H, 4.60; N, 16.12. HPLC purity, 99.2% (*E*/*Z* ratio 32:67).

4.1.34. 2-(4-(1-Amino-2,4-dicyano-6,7-dimethyl benzo[4,5]imidazo[1,2-*a*]pyridin-3-yl)phenoxy)acetamide (34)

Similar reaction of 3,4-dimethylbenzene-1,2-diamine (**61**) (1.50 g, 11.01 mmol) with cyanoacetamide (1.85 g, 22.03 mmol) in xylene (25 ml) gave 2-(4,5-dimethyl-1*H*-benzo[*d*]imidazol-2-yl)acetonitrile (**66**) as a brown solid (2.03 g, 99%) which was used directly ^{1}H NMR ($CDCl_3$) δ ppm 9.98 (br s, 1H), 7.35–7.26 (m, 1H), 7.12 (d, J = 8.4 Hz, 1H), 4.11 (s, 2H), 2.47 (s, 3H), 2.39 (s, 3H). LRMS (APCI$^+$) for $C_{11}H_{12}N_3$ (MH^+): Calcd 186, Found 186.

A solution of **66** (0.30 g, 1.62 mmol), **43** (0.44 g, 1.94 mmol) and a catalytic amount of piperidine in EtOH (20 ml) was heated under reflux for 15 h, to give an intermediate (0.35 g, 1.01 mmol) that was treated further with malononitrile (0.13 g, 2.02 mmol) and catalytic piperidine in EtOH (10 mL) under reflux overnight to close the middle ring. The cooled reaction mixture was filtered and washed with EtOH to give **34** as a yellow-green solid (0.15 g, 22%), mp (EtOH) 283–286 °C. ^{1}H NMR δ ppm 8.51 (br s, 2H), 8.30 (d, J = 8.8 Hz, 1H), 7.60 (br s, 1H), 7.57 (d, J = 8.8 Hz, 2H), 7.41 (br s, 1H), 7.24 (d, J = 8.6 Hz, 1H), 7.15 (d, J = 8.8 Hz, 2H), 4.55 (s, 2H), 2.57 (s, 3H), 2.44 (s, 3H). HRMS (ESI$^+$) for $C_{23}H_{19}N_6O_2$ $(MH)^+$: Calcd 411.1564, Found 411.1558. HPLC purity, 97.1%.

4.1.35. (*E,Z*)-2-(4-(2-Cyano-2-(4,5-dimethyl-1*H*-benzo[*d*]imidazol-2-yl)vinyl)phenoxy)acetamide (40)

Similar reaction of **66** (0.34 g, 1.84 mmol) and **42** (0.30 g, 1.66 mmol) in EtOH (20 ml) with a catalytic amount of piperidine gave **40** as a yellow solid (0.11 g, 19%), mp (EtOH) 275–278 °C, without further purification. ^{1}H NMR δ ppm 12.74 (br s, 0.5H, observe *E*- and *Z*- isomers separately), 12.45 (br s, 0.5H, observe *E*- and *Z*- isomers separately), 8.33–8.18 (m, 1H), 7.98 (d, J = 8.9 Hz, 2H), 7.59 (br s, 1H), 7.41 (br s, 1H), 7.41–7.20 (m, 1H), 7.16 (d, J = 8.9 Hz, 2H), 7.09–7.02 (m, 1H), 4.57 (s, 2H), 2.48 (s, 3H), 2.34 (s, 3H). HRMS (ESI$^+$) for $C_{20}H_{19}N_4O_2$ $(MH)^+$: Calcd 347.1503, Found 347.1497. Anal. Calcd for ($C_{20}H_{18}N_4O_2 \cdot 0.2H_2O$): C, 68.64; H, 5.30; N, 16.01. Found: C, 68.25; H, 5.04; N, 15.92. HPLC purity 96.3% (*E*/*Z* ratio 57:39).

4.1.36. 2-(4-(1-Amino-2,4-dicyano-7,8-dimethylbenzo[4,5] imidazo[1,2-*a*]pyridin-3-yl)phenoxy)acetamide (35)

Similar reaction of 4,5-dimethylbenzene-1,2-diamine (**62**) (1.50 g, 11.01 mmol) with cyanoacetamide (1.85 g, 22.03 mmol) in xylene (25 ml) gave 2-(5,6-dimethyl-1*H*-benzo[*d*]imidazol-2-yl)acetonitrile (**67**) as a brown solid (0.86 g, 42%), which was used directly ^{1}H NMR ($CDCl_3$) δ ppm 9.48 (br s, 1H), 7.43–7.29 (m, 2H), 4.10 (s, 2H), 2.37 (s, 6H). LRMS ($APCI^+$) for $C_{11}H_{12}N_3$ (MH^+) Calcd 186, Found 186.

A solution of **67** (0.61 g, 3.30 mmol), **43** (0.50 g, 2.20 mmol) and a catalytic amount of piperidine in EtOH (20 ml) was heated under reflux for 15 h to give an intermediate (0.62 g, 1.79 mmol) that was treated further with malononitrile (0.24 g, 3.58 mmol) and catalytic piperidine in EtOH (20 ml) overnight to close the middle ring. The cooled reaction mixture was filtered and washed with EtOH and the product was purified by flash chromatography, eluting with 3–20% MeOH/CH_2Cl_2, to give **35** as a brown solid (0.04 g, 4%), mp >305 °C. ^{1}H NMR δ ppm 8.55 (br s, 2H), 8.43 (s, 1H), 7.64 (s, 1H), 7.60 (br s, 1H), 7.57 (d, J = 8.9 Hz, 2H), 7.41 (br s, 1H), 7.15 (d, J = 8.8 Hz, 2H), 4.55 (s, 2H), 2.45 (s, 3H), 2.42 (s, 3H). HRMS (ESI^+) for $C_{23}H_{19}N_6O_2$ $(MH)^+$: Calcd 411.1564, Found 411.1570. HPLC purity, 94.3%.

4.1.37. (*E,Z*)-2-(4-(2-Cyano-2-(5,6-dimethyl-1*H*-benzo[*d*] imidazol-2-yl)vinyl)phenoxy)acetamide (41)

Similar reaction of **67** (0.34 g, 1.84 mmol) and **42** (0.30 g, 1.66 mmol) in EtOH (20 ml) with a catalytic amount of piperidine gave **41** as a yellow solid (0.37 g, 64%), mp (EtOH) >320 °C, without further purification. ^{1}H NMR δ ppm 12.68 (br s, 1H), 8.20 (s, 1H), 7.97 (d, J = 8.9 Hz, 2H), 7.58 (br s, 1H), 7.41 (br s, 1H), 7.47–7.26 (m, 2H), 7.15 (d, J = 7.0 Hz, 2H), 4.57 (s, 2H), 2.33 (s, 6H). HRMS (ESI^+) for $C_{20}H_{19}N_4O_2$ $(MH)^+$: Calcd 347.1503, Found 347.1502. Anal. Calcd for ($C_{20}H_{18}N_4O_2 \cdot 0.1H_2O$): C, 68.99; H, 5.27; N, 16.09. Found: C, 68.76; H, 5.19; N, 16.16. HPLC purity, 95.7% (*E*/*Z* ratio 45:51).

4.1.38. Aqueous solubility

Suspensions of compounds in water (1 mL) were sonicated for 15 min, then centrifuged at 13,000 rpm for 6 min. Supernatants were re-centrifuged, then diluted two-fold with water to avoid precipitation during HPLC analysis. This was carried out using an Altima C18, 5u, 3.2 × 150 mm column, with a flow rate of 0.5 mL/min, and gradient elution from organic eluent (80% v/v MeCN/water) to aqueous eluent (45 nM ammonium formate, pH 3.5).

4.2. Biology

4.2.1. Inhibition of perforin-mediated lysis of Jurkat cells

The ability of the compounds to inhibit the lysis of nucleated (Jurkat T lymphoma) cells in the presence of 0.1% BSA, as measured by release of ^{51}Cr was measured. Jurkat target cells were labelled by incubation in medium with 100 μCi ^{51}Cr for 1 h. The cells were then washed three times to remove unincorporated isotope and resuspended at 1×10^5 cells per mL in RPMI buffer supplemented with 0.1% BSA. Each test compound was pre-incubated to concentrations of 20 μM, 10 μM, 5 μM, 2.5 μM and 1.25 μM with recombinant perforin for 30 min with DMSO as a negative control (final DMSO concentration ⩽ 1%). ^{51}Cr labelled Jurkat cells were then added and cells were incubated at 37 °C for 4 h. The supernatant was collected and assessed for its radioactive content on a gamma counter (Wallac Wizard 1470 automatic gamma counter). Each data point was performed in triplicate and an IC_{50} was calculated from the range of concentrations described to above. Compounds with an IC_{50} <1 μM were titrated down to lower concentrations in the same manner as above, to determine an accurate IC_{50}.

4.2.2. KHYG-1 mediated cytotoxicity assay

KHYG-1 cells were washed and resuspended in RPMI + 0.1% BSA at 4×10^5 cells/ml and 50 μL of KHYG-1 cells were dispensed to each well of a 96-well V-bottom plate. Test compounds were added to KHYG-1 cells at various concentrations up to 20 μM and incubated at RT for 20 min. 1×10^6 K562 target cells were labelled with 75 μCi ^{51}Cr in 200 μL RPMI for 90 min at 37 °C, cells were washed as described above and resuspended in 5 ml RPMI + 0.1% BSA. 50 μL of ^{51}Cr labelled K562 leukaemia target cells were added to each well of the KHYG-1 plate (effector–target 2:1) and incubated at 37 °C for 4 h. ^{51}Cr release was assayed using a Skatron Harvesting Press and radioactivity estimated on a Wallac Wizard 1470 Automatic Gamma counter (Turku, Finland). The percentage of specific cytotoxicity was calculated by the formula:

$$\%\text{specific lysis} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

and expressed as the mean of triplicate assays.

4.2.3. Toxicity to KHYG-1 NK cells

The toxicity assay was carried out in exactly the same manner as the killing assay above, but instead of adding the labelled K562 target cells, 100 μL of RPMI 0.1% BSA was added. Cells were incubated for 4 h at 37 °C then washed ×3 in RPMI + 0.1% BSA. Cells were then resuspended in 200 μL of complete medium and incubated for 18–24 h at 37 °C. Trypan blue was added to each well and viable (clear) cells counted as a percentage of total (clear + blue) cell number (% viability).

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