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Small molecule inhibitors of Nicotinamide N-Methyltransferase (NNMT)

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Chapter 4

Esterase-sensitive prodrug forms of a potent
NNMT inhibitor translate its biochemical potency
into cellular activity

Manuscript under review

Abstract

A recently discovered NNMT inhibitor with single digit nanomolar IC₅₀ values (compound **17u** from **Chapter 3**) was found to be highly potent in biochemical assays but lacking in cellular activity. In order to translate the observed potent inhibitory activity into strong cellular activity, a prodrug strategy was investigated. This prodrug strategy focused on the temporary protection of the amine and carboxylic acid moieties of the highly polar amino acid side chain present in bisubstrate inhibitor **17u**. The modification of the carboxylic acid into a range of esters in the absence or presence of a trimethyllock (TML) protecting group at the amine group yielded a range of candidate prodrugs. Based on stability in buffers combined with conformed esterase-dependent conversion to the parent compound, the isopropyl ester was selected as the preferred acid prodrug. The isopropyl ester and isopropyl ester-TML prodrugs demonstrated improved cell permeability, which importantly also translated into significantly enhanced cellular activity in assays designed to measure the enzymatic activity of NNMT in live cells.

1. Introduction

Nicotinamide *N*-methyltransferase (NNMT) is a small molecule methyltransferase enzyme responsible for the conversion of nicotinamide (NA, vitamin b3) to 1-methylnicotinamide (MNA). NNMT utilizes the cofactor *S*-adenosyl-L-methionine (SAM) as a methyl donor, which is converted to *S*-adenosyl-L-homocysteine (SAH) upon methylation of nicotinamide.¹ Under normal physiological conditions, NNMT is mainly expressed in the liver and in adipose tissue.² One of the primary roles of NNMT is the detoxification of xenobiotics. This function is achieved through NNMT's broad substrate recognition that allows for the methylation of different metabolites, including pyridines, quinolines, and other related heterocyclic aromatics.³

The overexpression of NNMT has been described in a wide variety of tissues and diseases, generally with detrimental effects. Elevated NNMT activity in cancer has been correlated with tumour aggressiveness and is proposed to promote migration, invasion, proliferation, leading to its potential a biomarker predictive of worsened clinical outcomes.^{4–11} The overexpression of NNMT has been shown to cause depletion of the cellular pool of SAM, distorting the SAM/SAH balance, subsequently leading to a hypomethylated state with downstream effects on gene expression beneficial for tumour growth and metastasis.¹² This process is supported by recent proteomics-based research revealing NNMT to be the master regulator of the differentiation of cancer-associated fibroblasts (CAFs).¹³ In another recent investigation upregulated MNA levels in the tumour microenvironment were found to lead to the inhibition of T-cell functions resulting in their deregulated killing capacity and increased tumour growth.¹⁴

Potent, selective, and cell-active NNMT inhibitors are valuable tools to probe the complex regulatory functions mediated by NNMT and to also investigate a number of different pharmacological hypotheses that suggest NNMT as a therapeutic target. Although the increase in reports describing the roles of NNMT in disease have led to an increase in the development of inhibitors of NNMT, to date very few cell-active inhibitors have been described. In this regard, the bisubstrate inhibitors of NNMT pioneered by our group and others exhibit very potent enzyme inhibition in biochemical assays but due to the polar nature of their structures, show only limited cellular activity.^{15–19} Recent work in our group yielded compound **17u** (see **Chapter 3**), which showed an IC₅₀ value of 3.7 nM and was found to be more potent than similar bisubstrate inhibitors reported by other groups. However, when tested against a range of human cancer cell lines, compound **17u** only showed significant antiproliferative effects at the high concentration tested of 100 μM, more than four orders of magnitude higher than the concentration needed for enzyme inhibition in the biochemical assay. The absence of cellular activity of compound **17u** is presumably due to the compound's poor cell permeability which is most likely caused by the presence of two

highly polar functional groups that are present in all of the potent bisubstrate inhibitors of NNMT; the carboxylic acid and amine moieties of the amino acid sidechain. Notably, previous structure activity relationship studies of the bisubstrate inhibitors revealed that both the carboxylic acid and amine moieties of the amino acid sidechain are required for potent inhibitory activity and attempts at replacing them with less polar bio-isosteres in all cases resulted in a significant loss of potency.

Therefore, a prodrug strategy was applied to the structure of compound **17u** with the aim of improving its cellular activity (Figure 1A). The carboxylic acid moiety was converted into a variety of esters, which can be cleaved by cellular esterases. In the case of the amino group a different prodrug strategy was applied. The derivatization of amines to give amides has not been widely used as a prodrug strategy due to the high chemical stability of amide linkages^{20,21} and the lack of amidase enzymes necessary for hydrolysis²². To circumvent these problems, the trimethyl-lock (TML) moiety²³ was selected for the prodrug form of the amine (Figure 1B). After esterase-mediated hydrolysis of the acetyl group, the liberated hydroxyl group of the TML moiety spontaneously cyclizes to form the corresponding lactone ring, with concomitant release of the free amine. Using these strategies, a series of prodrugs were prepared in which either one, or both, of the ester and TML-groups were incorporated.

To investigate the most suitable prodrug form of compound **17u**, the different ester and TML modified analogues were evaluated for their hydrolytic stability in buffer after which the most stable prodrugs were evaluated for the esterase-mediated release of the parent compound. After confirming the esterase mediated conversion to the active compound, the prodrugs were evaluated for cellular activity in a range of cellular assays and compared to the activity of the parent compound.

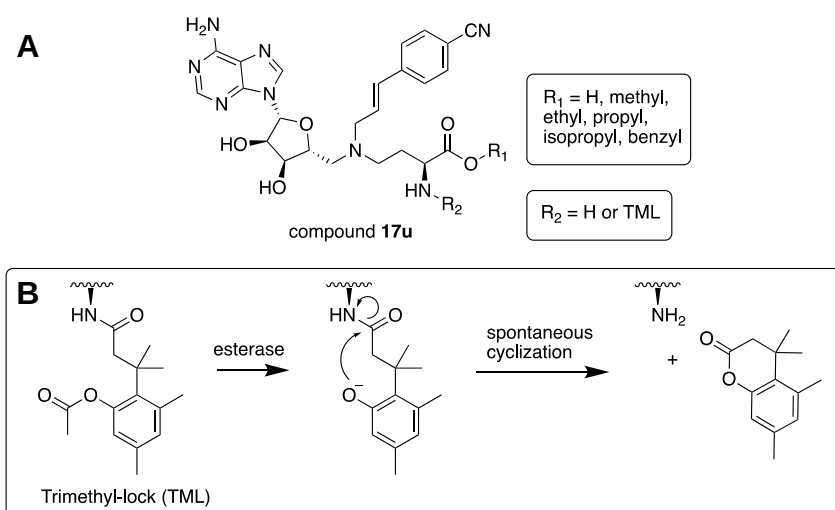
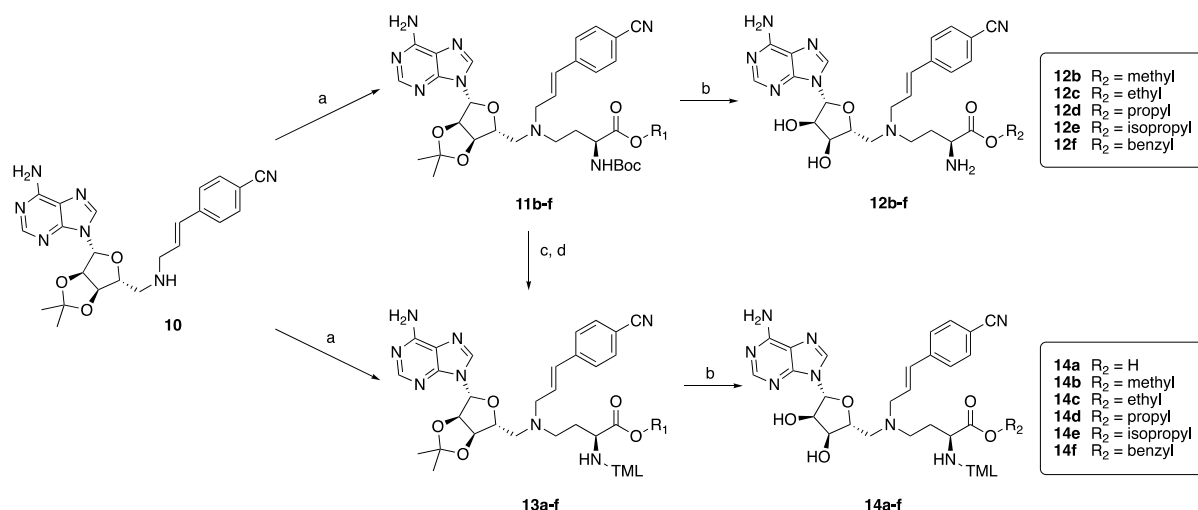


Figure 1. A) Prodrug strategy of compound **17u**. The carboxylic acid can be masked as an ester and the amine can be masked as an amide using the esterase-sensitive trimethyl-lock (TML). **B)** The mechanism of the trimethyl-lock cleavage. Deacetylation by esterases results in subsequent spontaneous lactonization releasing the free amine.

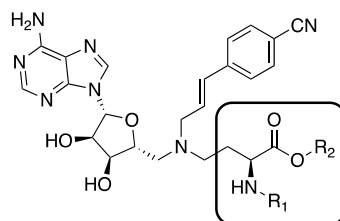


Scheme 2. Synthesis of prodrugs **12b-f** and **14a-f**. Reagents and conditions: (a) aldehydes **5b-f** or **9a**, $\text{NaBH}(\text{OAc})_3$, AcOH , DCE , rt, overnight (34–73%); (b) TFA , CH_2Cl_2 , H_2O , rt, 2h (70–93%); (c) TFA , CH_2Cl_2 , rt, 2h; (d) TML acid **7**, BOP, Et_3N , CH_2Cl_2 , rt, 2h (77–83%);

The aldehydes were subsequently coupled to intermediate **10** using reductive amination conditions forming intermediates **11b-f**, which can be deprotected to form ester prodrugs **12b-f** (**Scheme 2**). Compounds **11b-f** can alternatively be selectively Boc-deprotected using TFA/DCM to form intermediates **13b-f** and subsequently coupled to trimethyllock acid **7** with BOP and triethylamine. Compound **13a** was synthesized through coupling of aldehyde **9a** with compound **10**. The intermediates were then deprotected under acidic conditions to yield ester-TML dual prodrugs **14a-f**. Initially, the prodrugs were tested for their residual activity in the enzymatic activity assay. However, several prodrugs unexpectedly showed significant inhibition at fixed concentrations of 5 and 25 μM . In order to evaluate the validity of these results, the prodrugs were subsequently evaluated for their hydrolytic stability in both PBS buffer at pH 7.4 and Tris buffer at pH 8.4. Both these buffers and pHs have been used in the different biochemical and cellular assays described in this report. Compounds were dissolved in DMSO at a concentration of 40 mM and diluted with the respective buffer to a final concentration of 1 mM. Compounds were tested directly (t_0) and subsequently every 2 hours over a time period of 16 hours by HPLC. The formation of the parent compound was evaluated and normalized by measuring the peak area at 214 nm and comparing it to the initial timepoint. The results presented in Table 1 below show significant hydrolysis over time for most of the ester prodrugs. Only the isopropyl ester seems to be stable under these conditions. Interestingly, the stability of the prodrugs increased significantly in the presence of the trimethyllock (TML) moiety at the amine position. Even for the rather labile methyl ester **12b**, the TML group in **14b** results in a decrease in hydrolysis of the methyl ester. Benzyl ester

8f was found to be the least stable and due to its poor aqueous solubility, the benzyl ester-TML dual prodrug **14f** was not evaluated further.

Table 1. Stability data of the prodrugs in Tris buffer at pH 8.4 and in PBS buffer at pH 7.4. The values indicate the percentage of compound peak area present after 16 hours of incubation at room temperature.



compound	Ester R ₂ (R ₁ =H)	Tris	PBS	compound	Ester R ₂ (R ₁ =TML)	Tris	PBS
17u	H	n.a.	n.a.	14a	H	93.97	99.10
12b	Methyl	22.59	36.97	14b	Methyl	73.88	88.08
12c	Ethyl	54.84	68.36	14c	Ethyl	87.00	90.44
12d	Propyl	61.32	71.96	14d	Propyl	87.52	90.30
12e	Isopropyl	93.70	98.59	14e	Isopropyl	87.69	91.13
12f	Benzyl	21.60	33.43	14f	Benzyl	n.d.	n.d.

n.a. not applicable; n.d. not determined

The most stable esters were found to be the isopropyl ester (compound **12e**), the trimethyllock (compound **14a**) and the isopropyl-trimethyllock dual prodrug (compound **14e**). However, as compound **14a** was not found to improve the cellular activity of the parent compound (discussed below), this compound was not evaluated further. The next step was to establish whether compounds **12e** and **14e** can be converted to the parent compound in the presence of an esterase. Using commercially available pig liver esterase (PLE), both compounds were shown to be readily converted to the parent compound as measured by HPLC (Figure 2). Within 4 hours the prodrugs were fully converted to the parent compound, while no hydrolysis was observed in the absence of PLE. Of note is the sequential conversion of dual prodrug **14e** in which the TML is hydrolysed first followed by the isopropyl ester. No trace of compound **14a** could be observed in which the ester is cleaved and the TML is still in place. This finding suggests that the TML group hinders the esterase-mediated hydrolysis of the isopropyl ester moiety and only after deacetylation of the TML moiety followed by its spontaneous loss, can the ester moiety be cleaved by the esterase.

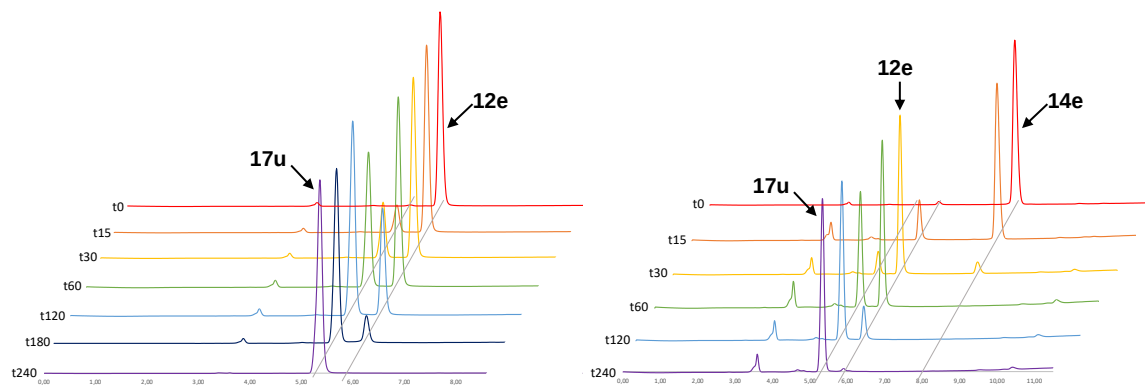


Figure 2. Esterase-mediated hydrolysis of isopropyl ester prodrug **12e** (left) and isopropyl-TML dual prodrug **14e** (right). The data shows clean conversion of the prodrugs to the parent compound **17u**. For the dual prodrug **14e**, conversion of the TML happens first followed by the hydrolysis of the isopropyl ester moiety.

The prodrugs shown in Table 1 were also subjected to a variety of cellular assays to screen for the most suitable prodrug form of compound **17u**. Initially, the prodrugs were tested in an MTT assay to evaluate their effect on cell viability against three different cancer cell lines: HSC-2 (oral cancer), T24 (bladder cancer), and A549 (lung cancer) (Figure 3). The results of these assays did not show an appreciable effect for ester prodrugs **12b-f** or TML prodrug **14a** compared to parent compound **17u**. Selected dual prodrugs **14b** and **14c** did show some activity, but only against HSC-2 cells, which overall seemed to be the more sensitive cell-line towards NNMT inhibition compared to the T24 and A549 cell lines. By comparison, when tested at the highest concentration evaluated (100 μ M) compound **14e** did cause reduction of cell viability in a time dependent manner.

Compound **14e** was then tested in a neon electroporation assay in which the cell membrane is made highly permeable by energy pulses without killing the cells. With this technique, the effect of poorly cell permeable compounds can be evaluated. As control measurements, cells were either treated with DMSO alone, were not electroporated, or were treated with the parent compound **17u**. The results depicted in the bar graph in Figure 4 indicate an effect of the treatment of compounds **17u** and **14e** on the cell viability of the electroporated A549 cells. Again, however, the effects on cell viability were only found at a concentration of 100 μ M, more than 10,000 times higher than the activity of the parent compound in biochemical assays (IC_{50} = 3.7 nM).

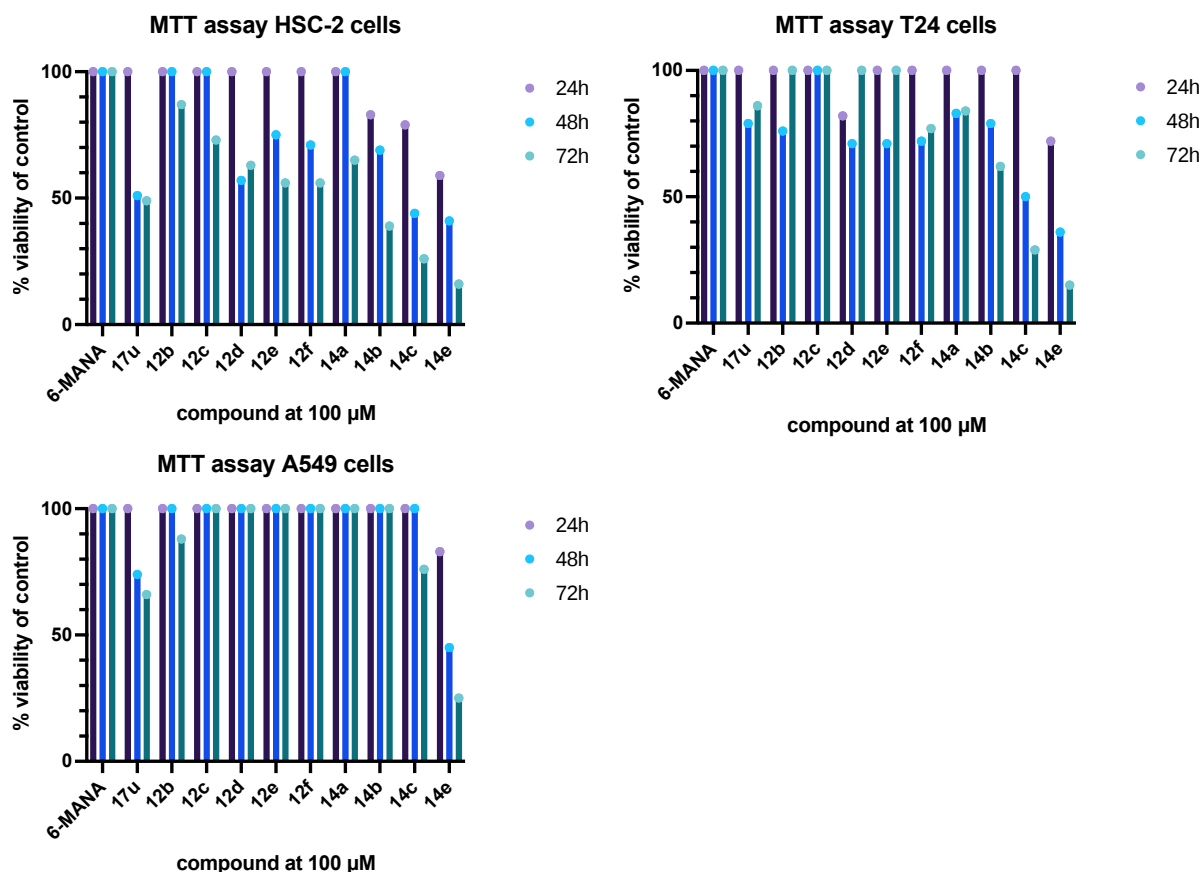


Figure 3. MTT cell viability data presented as percentage of DMSO control in three different cancer cell lines tested with 100 μ M compound after 24, 48 and 72 hours of incubation. Experiments were performed in triplicate.

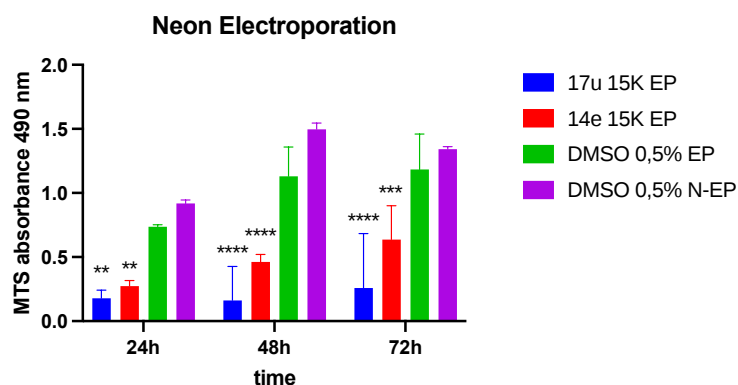


Figure 4. Effect of compounds **17u** and **14e** on the cell viability of A549 cells after neon electroporation. Absorbance of the water-soluble formazan product was measured at 490 nm using the MTS cell viability assay. The cell viability after treatment with compounds **17u** (blue) and **14e** (red) are significantly lower than the DMSO control (green) and non-electroporated DMSO control (purple).

The findings from both the conventional MTT and neon electroporation assays reveal that the prodrug inhibitors have little effect on cell viability unless tested at very high concentrations. One explanation for these finding may be that prodrugs are not effectively entering the cells. An alternative explanation could be that the compounds do enter the cells but that NNMT inhibition is simply not inherently toxic to these cells. If this is the case, the impact on cell viability observed when applying the compounds at the highest concentration tested (100 μ M) could instead be ascribed to a non-specific toxic effect.

To investigate the cellular activity of the prodrugs in a more direct manner, compounds **12b-f** and **14a-e** were screened in a cellular MNA assay and compared to parent compound **17u** at a fixed concentration of 10 μ M (Figure 5). In this assay the levels of MNA produced by an immortalized human microvascular endothelial cell line (HMEC-1) are quantified using a sensitive LC-MS assay. The results of the treatment of HMEC-1 cells with the different prodrugs show significant improvement of cellular activity over the parent compound for all prodrugs tested except for TML prodrug **14a**. These results indicate that masking the carboxylic acid is more important to promote cell permeability than masking the amine functionality. Of note is the comparable efficacy compared to reference compound 6-methylamino-nicotinamide (6-MANA), for which *in vivo* effects have been previously demonstrated²⁵, and the absence of any effect for 5-amino-1-methylquinolium (5-MQ), another reference compound used in cellular assays²⁶.

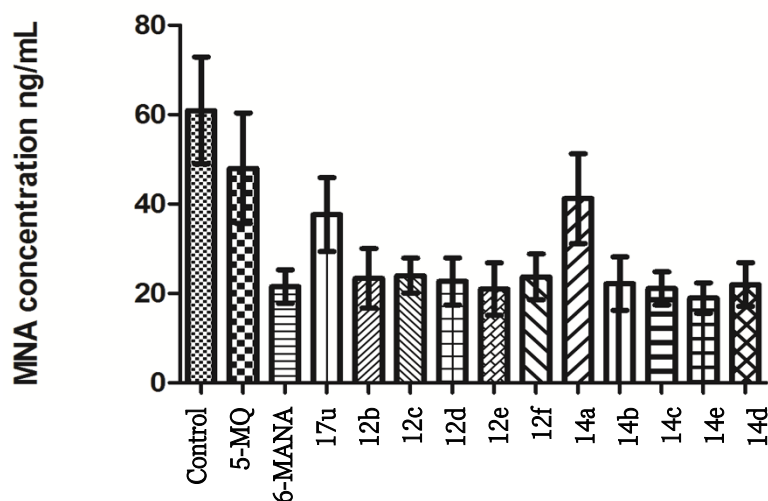


Figure 5. MNA concentrations in endothelial HMEC-1 cells after 24-hour incubation with 10 μ M of compounds 5-amino-methylquinolinium (5-MQ), 6-methylamino-nicotinamide (6-MANA), compound **17u** or prodrug compounds **12b-f** and **14a-e**.

From this data, the best prodrugs in terms of stability and potency are compounds **12e** and **14e**, corresponding to the isopropyl ester prodrugs with and without the TML group on the amine. The study was therefore continued focusing on compounds **12e** and **14e**, which were further evaluated for their effect on cellular levels of MNA in a dose-dependent manner. The results presented in Figure 6 demonstrate that parent compound **17u** and reference compound 6-MANA require higher concentrations to substantially decrease the levels of MNA in A549 lung cancer cells. In contrast, when the isopropyl ester **12e** was tested, a clear and significant decrease in MNA levels were observed. This effect is even further enhanced by the introduction of the TML moiety as present in compound **14e**. These findings indicate that the prodrug strategy here applied was able to convert a potent, but non-permeable inhibitor, into a compound with cellular activity. Notably, our findings also seem to suggest that the capacity for a small molecule to inhibit NNMT in cells does not *per se* lead to an impact on cell viability. This is in keeping with recent reports showing that the addition of NNMT inhibitors to cancer associated fibroblasts do not kill the cells but rather cause a reversion of cell morphology to one that more-closely resembles normal fibroblasts.¹³

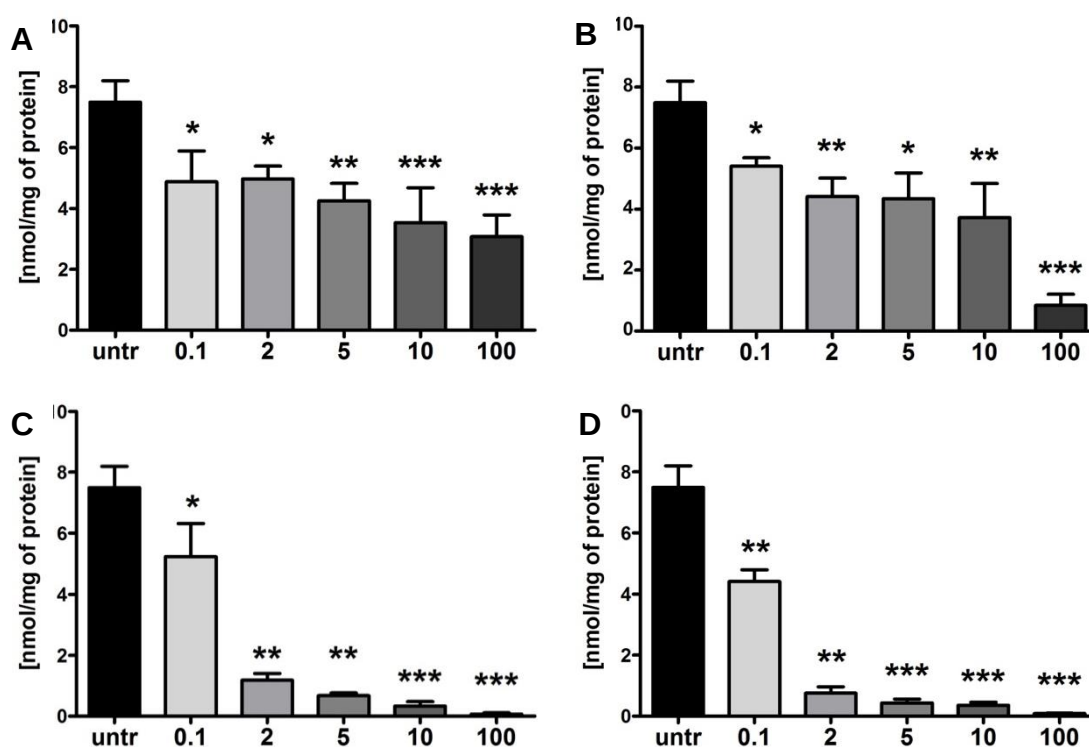


Figure 6. Concentration of MNA in A549 cells after 24-hour treatment with compounds 6-MANA (A), **17u** (B), **12e** (C) and **14e** (D).

3. Conclusion

In this report we describe a prodrug strategy to translate the potent activity of lead compound **17u** found in biochemical assays into cellular activity. The prodrug strategy focussed specifically on the amino acid functionality of the bisubstrate inhibitor. The carboxylic acid was masked as an ester using a variety of alkyl and benzyl groups and the amine was masked using the trimethyl-lock technology, in which the amine is masked as an amide, but can be released by an esterase. The different combination of prodrugs led to the selection of the isopropyl ester and the isopropyl ester/TML dual prodrug as the compounds with the most promising profile in terms of stability and cellular activity. The prodrugs were found to have little impact on cell viability. However, when evaluated in an assay allowing for the direct quantification of cellular MNA production, a clear dose-dependent effect was observed. The data presented here demonstrate the suitability of a prodrug strategy for the delivery of polar NNMT inhibitors into cells. Ongoing research is focussing on the effect of NNMT inhibition in a range of cell systems covering oncology as well as metabolic disorders.

4. Experimental procedures

All reagents employed were of American Chemical Society grade or finer and were used without further purification unless otherwise stated. For compound characterization, ^1H NMR spectra were recorded at 400, 500 MHz with chemical shifts reported in parts per million downfield relative to CHCl_3 (7.26) or CH_3OH (δ 3.31). ^1H NMR data are reported in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; and m, multiplet), coupling constant (J) in hertz (Hz), and the number of protons. Where appropriate, the multiplicity is preceded by br, indicating that the signal was broad. ^{13}C NMR spectra were recorded at 101 or 126 MHz with chemical shifts reported relative to CHCl_3 (77.16) or CH_3OH (δ 49.00). High-resolution mass spectrometry (HRMS) analysis was performed using a Q-TOF instrument. Purity was confirmed to be $\geq 95\%$ by LCMS performed on a Shimadzu LC-20AD system with a Shimadzu Shim-Pack GISS-HP C18 column (3.0 x 150 mm, 3 μm) at 30 $^\circ\text{C}$ and equipped with a UV detector monitoring at 214 and 254 nm. The following solvent system, at a flow rate of 0.5 mL/min, was used: solvent A, 0.1 % formic acid in water; solvent B, acetonitrile. Gradient elution was as follows: 95:5 (A/B) for 2 min, 95:5 to 0:100 (A/B) over 13 min, 0:100 (A/B) for 2 min, then reversion back to 95:5 (A/B) over 1 min, 95:5 (A/B) for 2 min. This system was connected to a Shimadzu 8040 triple quadrupole mass spectrometer (ESI ionization). The compounds were purified via preparative HPLC performed on a BESTA-Technik system with a Dr. Maisch Reprosil Gold 120 C18 column (25 x 250 mm, 10 μm) and equipped with an ECOM Flash UV detector monitoring at 214 nm.

The following solvent system, at a flow rate of 12 mL/min, was used: solvent A: 0.1 % TFA in water/acetonitrile 95/5; solvent B: 0.1 % TFA in water/acetonitrile 5/95. Gradient elution was as follows: 95:5 (A/B) for 5 min, 95:5 to 0:100 (A/B) over 40 min, 0:100 (A/B) for 5 min, then reversion back to 95:5 (A/B) over 2 min, 95:5 (A/B) for 8 min. HRMS analyses were performed on a Shimadzu Nexera X2 UHPLC system with a Waters Acquity HSS C18 column (2.1 × 100 mm, 1.8 μm) at 30°C and equipped with a diode array detector. The following solvent system, at a flow rate of 0.5 mL/min, was used: solvent A, 0.1 % formic acid in water; solvent B, 0.1 % formic acid in acetonitrile. Gradient elution was as follows: 95:5 (A/B) for 1 min, 95:5 to 15:85 (A/B) over 6 min, 15:85 to 0:100 (A/B) over 1 min, 0:100 (A/B) for 3 min, then reversion back to 95:5 (A/B) for 3 min. This system was connected to a Shimadzu 9030 QTOF mass spectrometer (ESI ionization) calibrated internally with Agilent's API-TOF reference mass solution kit (5.0 mM purine, 100.0 mM ammonium trifluoroacetate and 2.5 mM hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine) diluted to achieve a mass count of 10000.

Compounds **2c**²⁷, **2d**²⁸, **2e**²⁹, **3c**³⁰, **3d**²⁸, **4a**¹⁵, **4b**³¹, **4f**³², **5a**¹⁷ were prepared as previously described and had NMR spectra and mass spectra consistent with the assigned structures.

4.1 Synthetic procedures

(S)-3-((tert-butoxycarbonyl)amino)-4-isopropoxy-4-oxobutanoic acid (3e). To a stirred suspension of 4-benzyl 1-isopropyl (tert-butoxycarbonyl)-L-aspartate (**2e**) (1200 mg, 3.3 mmol), 10% Pd-C (120 mg) under H₂ atmosphere. After completion of reaction (TLC), the mixture was filtered through celite, and the filtrate was concentrated under vacuum to get **3e** (900 mg, 99% yield) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 5.55 (d, *J* = 8.4 Hz, 1H), 5.11 – 5.03 (m, 1H), 4.54 – 4.50 (m, 1H), 3.05 (dd, *J* = 17.4, 4.5 Hz, 1H), 2.99 (s, 2H), 2.91 (s, 2H), 2.85 (dd, *J* = 17.3, 4.3 Hz, 1H), 1.46 (s, 9H), 1.25 (dd, *J* = 10.2, 6.3 Hz, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 176.1, 170.5, 155.6, 80.3, 69.7, 50.0, 36.7, 28.3, 21.6. HRMS (ESI): calculated for C₁₂H₂₂NO₆ [M+H]⁺ 276.1447, found 276.1450.

ethyl N²-(tert-butoxycarbonyl)-N⁴-methoxy-N⁴-methyl-L-asparaginate (4c). To a stirred suspension of **3c** (100 mg, 0.4 mmol) in 10 mL CH₂Cl₂, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) (194 mg, 0.44 mmol) and 0.1 mL Et₃N were added and after 10 mins *N,O*-dimethylhydroxylamine hydrogen chloride (43 mg, 0.44) was added followed by another 0.1 mL Et₃N. The resulting mixture was stirred at room temperature for 2 hours, 10 mL water was added to quench the reaction, the product extracted with CH₂Cl₂ (10 mL × 3), the organic layer washed with water, brine, dried over NaSO₄. Solvent was removed and the crude compound purified by column chromatography to get compound **4c** as a

colorless oil (960 mg, 80% yield). ^1H NMR (400 MHz, CDCl_3) δ 5.73 (d, J = 8.8 Hz, 1H), 4.59 – 4.55 (m, 1H), 4.24 – 4.19 (m, 2H), 3.70 (s, 3H), 3.18 (s, 3H), 2.97 – 2.91 (m, 1H), 1.46 (s, 9H), 1.28 (t, J = 7.1 Hz, 3H). LRMS (ESI): calculated for $\text{C}_{13}\text{H}_{25}\text{N}_2\text{O}_6$ $[\text{M}+\text{H}]^+$ 305.17, found 305.19.

propyl *N*²-(*tert*-butoxycarbonyl)-*N*⁴-methoxy-*N*⁴-methyl-L-asparaginate (4d). Following the procedure described for compound **4c**, coupling compound **3d** (730 mg, 2.6 mmol) with *N*,*O*-dimethylhydroxylamine hydrogen chloride (284 mg, 2.9 mmol) yielded compound **4d** as a colourless oil (708 mg, 84% yield). ^1H NMR (400 MHz, CDCl_3) δ 5.70 (d, J = 8.9 Hz, 1H), 4.53 (dt, J = 9.3, 4.4 Hz, 1H), 4.08 – 4.01 (m, 2H), 3.66 (s, 3H), 3.21 – 3.15 (m, 1H), 3.12 (s, 3H), 2.91 – 2.86 (br m, 1H), 1.67 – 1.56 (m, 2H), 1.41 (s, 9H), 0.90 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 171.8, 155.8, 80.6, 67.1, 62.1, 49.1, 34.7, 32.4, 21.9, 4.8. HRMS (ESI): calculated for $\text{C}_{14}\text{H}_{27}\text{N}_2\text{O}_6$ $[\text{M}+\text{H}]^+$ 319.1869, found 318.1873.

isopropyl *N*²-(*tert*-butoxycarbonyl)-*N*⁴-methoxy-*N*⁴-methyl-L-asparaginate (4e). Following the procedure described for compound **4c**, coupling compound **3e** (800 mg, 2.9 mmol) with *N*,*O*-dimethylhydroxylamine hydrogen chloride (312 mg, 3.2 mmol) yielded compound **4e** as a colourless oil (760 mg, 82% yield). ^1H NMR (400 MHz, CDCl_3) δ 5.71 (d, J = 8.8 Hz, 1H), 5.11 – 5.01 (m, 1H), 4.55 – 4.50 (m, 1H), 3.70 (s, 3H), 3.25 – 3.18 (m, 1H), 3.17 (s, 3H), 2.95 – 2.89 (br m, 1H), 1.46 (s, 10H), 1.25 (dd, J = 13.5, 6.3 Hz, 6H). ^{13}C NMR (101 MHz, CDCl_3) δ 171.1, 155.8, 69.1, 61.3, 50.0, 34.7, 32.0, 28.4, 21.7. HRMS (ESI): calculated for $\text{C}_{14}\text{H}_{27}\text{N}_2\text{O}_6$ $[\text{M}+\text{H}]^+$ 319.1869, found 318.1872.

methyl (*S*)-2-((*tert*-butoxycarbonyl)amino)-4-oxobutanoate (5b). To a solution of methyl *N*²-(*tert*-butoxycarbonyl)-*N*⁴-methoxy-*N*⁴-methyl-L-asparaginate **4b** (1000 mg, 4.1 mmol) in CH_2Cl_2 (20 mL) at -78°C was added DIBAL-H (1M in hexane, 6.0 mL) and the resulting mixture was stirred at -78°C for 2 hours. 10 mL water was added to quench the reaction, 10 mL 1M HCl (aq) was added to the solution, the product was extracted with Et_2O . Combined organic layer wash with H_2O , brine, dried over Na_2SO_4 . The solvent was removed to yield compound **5b** as a colorless oil used in the next step without further purification.

ethyl (*S*)-2-((*tert*-butoxycarbonyl)amino)-4-oxobutanoate (5c). Following the procedure described for compound **5a**, compound ethyl *N*²-(*tert*-butoxycarbonyl)-*N*⁴-methoxy-*N*⁴-methyl-L-asparaginate **4c** (560 mg, 1.84 mmol) was reduced using DIBAL-H (1M in hexane, 3 mL) to yield compound **5c**, which was used in the next step without further purification.

propyl (*S*)-2-((*tert*-butoxycarbonyl)amino)-4-oxobutanoate (5d). Following the procedure described for compound **5a**, compound propyl *N*²-(*tert*-butoxycarbonyl)-*N*⁴-methoxy-*N*⁴-methyl-L-asparaginate **4d** (400 mg, 1.3 mmol) was reduced by DIBAL-H (1M in hexane, 2 mL) to yield compound **5d** which was used in the next step without further purification.

isopropyl (*S*)-2-((*tert*-butoxycarbonyl)amino)-4-oxobutanoate (5e). Following the procedure described for compound **5a**, compound isopropyl *N*²-(*tert*-butoxycarbonyl)-*N*⁴-methoxy-*N*⁴-methyl-*L*-asparaginate **4e** (450 mg, 1.4 mmol) was reduced by DIBAL-H (1M in hexane, 2 mL) to yield compound **5e** which was used in the next step without further purification.

benzyl (*S*)-2-((*tert*-butoxycarbonyl)amino)-4-oxobutanoate (5f). Following the procedure described for compound **5a**, compound benzyl *N*²-(*tert*-butoxycarbonyl)-*N*⁴-methoxy-*N*⁴-methyl-*L*-asparaginate **4f** (370 mg, 1.0mmol) was reduced by DIBAL-H (1M in hexane, 1.2 mL) to yield compound **5f** which was used in the next step without further purification.

***tert*-butyl *N*²-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanoyl)-*N*⁴-methoxy-*N*⁴-methyl-*L*-asparaginate (8a).** *tert*-butyl *N*²-(*tert*-butoxycarbonyl)-*N*⁴-methoxy-*N*⁴-methyl-*L*-asparaginate **4a** (300 mg, 0.9 mmol) in dioxane (5 mL) is selectively deprotected using 4N HCl in dioxanes (10 mL) while stirring for 1 hour at 0 °C and 75 minutes at room temperature. The mixture is concentrated and added to a mixture of TML acid **7** (220 mg, 0.83 mmol, 0.9 eq), Et₃N (330 µL, 2.4 mmol, 2.8 eq) and BOP (450 mg, 1 mmol, 1.1 eq) in CH₂Cl₂ (10 mL). The mixture was stirred overnight, diluted with CH₂Cl₂ to 100 mL, washed with saturated NaHCO₃, water and Brine, dried over sodium sulfate and concentrated. The crude product was purified by column chromatography (30% EtOAc in petroleum ether, followed by flushing with 20% MeOH in EtOAc) yielding compound **8a** (350 mg, 88%) of an off-white powder. ¹H NMR (300 MHz, CDCl₃) δ 6.77 (d, *J* = 2.0 Hz, 1H), 6.56 (d, *J* = 2.1 Hz, 1H), 6.51 (d, *J* = 8.4 Hz, 1H), 4.66 (dt, *J* = 8.4, 4.2 Hz, 1H), 3.62 (s, 3H), 3.11 (s, 3H), 2.98 (dd, *J* = 17.3, 4.2 Hz, 1H), 2.78 (d, *J* = 13.8 Hz, 1H), 2.64 – 2.41 (m, 5H), 2.30 (s, 3H), 2.19 (s, 3H), 1.61 (s, 3H), 1.54 (s, 3H), 1.40 (s, 9H). ¹³C NMR (75 MHz, CDCl₃) δ 170.66, 170.19, 170.08, 149.60, 138.14, 136.15, 133.61, 132.47, 123.21, 81.76, 61.15, 49.28, 48.51, 39.54, 34.32, 31.97, 31.64, 27.88, 25.45, 21.92, 20.24. LRMS (ESI): calculated for C₂₅H₃₉N₂O₇ [M+H]⁺ 479.28, found 479.35.

***tert*-butyl (*S*)-2-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamido)-4-oxobutanoate (9a).** Following the procedure described for compound **5a**, compound **8a** (110 mg, 0.23 mmol) was reduced by DIBAL-H (1M in hexane, 0.4 mL) to yield compound **9a** which was used in the next step without further purification.

methyl (*S*)-4-(((3*aR*,4*R*,6*R*,6*aR*)-6-(6-amino-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)-2-((*tert*-butoxycarbonyl)amino)butanoate (11b). 4-((*E*)-3-(((3*aR*,4*R*,6*R*,6*aR*)-6-(6-amino-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methyl)amino)prop-1-en-1-yl)benzonitrile **10** (50 mg, 0.11 mmol), **5b** (30, 0.13 mmol), NaBH(OAc)₃ (36 mg, 0.17 mmol) and AcOH (one drop) were dissolved in 1,2- dichloroethane (DCE, 10 mL) and stirred at room temperature under

a N₂ atmosphere overnight. The reaction was quenched by adding 1 N NaOH (10 mL), and the product was extracted with CH₂Cl₂. The combined organic layers were washed with brine and dried over Na₂SO₄. The solvent was evaporated, and the crude product was purified by column chromatography (5% MeOH in CH₂Cl₂) to give compound **11b** as a white powder (47 mg, 65% yield). ¹H NMR (400 MHz, CDCl₃) δ 8.21 (s, 1H), 7.94 (s, 1H), 7.49 (d, *J* = 8.3 Hz, 2H), 7.27 (d, *J* = 8.3 Hz, 2H), 6.60 (s, 2H), 6.35 (d, *J* = 16.0 Hz, 1H), 6.27 – 6.22 (m, 1H), 6.06 (s, 1H), 5.94 (d, *J* = 8.1 Hz, 1H), 5.45 (d, *J* = 6.2 Hz, 1H), 5.03 – 4.95 (m, 1H), 4.41 – 4.30 (m, 2H), 3.64 (s, 3H), 3.23 (d, *J* = 6.0 Hz, 2H), 2.79 – 2.69 (m, 2H), 2.58 – 2.54 (m, 2H), 2.00 – 1.89 (m, 1H), 1.82–1.77 (br m, 1H), 1.58 (s, 3H), 1.38 (br s, 12H). ¹³C NMR (101 MHz, CDCl₃) δ 173.3, 155.9, 155.6, 153.0, 149.0, 141.3, 140.1, 132.3, 131.0, 126.7, 120.2, 119.0, 114.4, 110.5, 90.7, 85.7, 83.9, 83.3, 56.6, 56.1, 53.6, 52.2, 50.6, 44.8, 29.2, 28.4, 27.2, 25.4. HRMS (ESI): calculated for C₃₃H₄₃N₈O₇ [M+H]⁺ 663.3255, found 663.3262.

ethyl (S)-4-(((3aR,4R,6R,6aR)-6-(6-amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl)((E)-3-(4-cyanophenyl)allyl)amino)-2-((tert-butoxycarbonyl)amino)butanoate (11c). Following the procedure described for compound **11b**, coupling compound **10** (50 mg, 0.11 mmol) with **5c** (32 mg, 0.13 mmol) afforded compound **11c** as a white powder (53 mg, 72% yield). ¹H NMR (400 MHz, CDCl₃) δ 8.15 (s, 1H), 7.91 (s, 1H), 7.46 (d, *J* = 8.3 Hz, 2H), 7.24 (d, *J* = 8.3 Hz, 2H), 6.58 (s, 2H), 6.32 (d, *J* = 16.0 Hz, 1H), 6.26 – 6.16 (br, 1H), 6.01 (s, 1H), 5.85 (d, *J* = 8.0 Hz, 1H), 5.39 (t, *J* = 8.2 Hz, 2H), 4.95–4.89 (m, 2H), 4.36 – 4.17 (m, 2H), 3.74 – 3.51 (m, 6H), 3.43 (s, 4H), 3.29 – 3.03 (m, 4H), 2.80 – 2.52 (m, 6H), 2.49 (s, 5H), 1.83–1.64 (m, 3H), 1.53 (s, 3H), 1.34 (br d, *J* = 7.8 Hz, 15H). ¹³C NMR (101 MHz, CDCl₃) δ 172.2, 156.1, 155.8, 153.0, 148.9, 141.3, 132.3, 126.7, 120.2, 114.4, 110.4, 90.6, 85.6, 83.9, 83.2, 68.8, 65.0, 59.7, 56.8, 56.0, 52.4, 51.0, 44.8, 29.3, 28.3, 25.4, 21.7. HRMS (ESI): calculated for C₃₃H₄₃N₈O₇ [M+H]⁺ 677.3411, found 677.3420.

propyl (S)-4-(((3aR,4R,6R,6aR)-6-(6-amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl)((E)-3-(4-cyanophenyl)allyl)amino)-2-((tert-butoxycarbonyl)amino)butanoate (11d). Following the procedure described for compound **11b**, coupling compound **10** (50 mg, 0.11 mmol) with **5d** (34 mg, 0.13 mmol) afforded compound **11d** as a white powder (46 mg, 60% yield). ¹H NMR (400 MHz, CDCl₃) δ 8.18 (s, 1H), 7.93 (s, 1H), 7.48 (d, *J* = 8.3 Hz, 2H), 7.26 (d, *J* = 8.3 Hz, 2H), 6.55 (s, 2H), 6.34 (d, *J* = 15.9 Hz, 1H), 6.27 – 6.17 (m, 1H), 6.03 (s, 1H), 5.88 (d, *J* = 8.1 Hz, 1H), 5.41 (d, *J* = 5.6 Hz, 2H), 4.96 (dd, *J* = 6.3, 3.7 Hz, 1H), 4.33 (t, *J* = 8.1 Hz, 2H), 3.98 (t, *J* = 6.7 Hz, 2H), 3.64 (d, *J* = 2.7 Hz, 1H), 3.59 (d, *J* = 3.1 Hz, 1H), 3.26 – 3.19 (m, 2H), 3.11 (d, *J* = 6.8 Hz, 1H), 2.79 – 2.50 (m, 8H), 2.02 – 1.65 (m, 4H), 1.62–1.52 (m, 6H), 1.39 – 1.34 (m, 19H). ¹³C NMR (101 MHz, CDCl₃) δ 172.8, 155.9,

153.0, 149.0, 141.3, 132.3, 130.9, 126.7, 120.1, 119.0, 114.4, 110.5, 90.6, 85.6, 83.9, 79.6, 66.6, 65.1, 59.6, 52.31, 44.8, 28.4, 25.4, 21.8. HRMS (ESI): calculated for C₃₃H₄₃N₈O₇ [M+H]⁺ 691.3568, found 691.3573.

isopropyl **(S)-4-((((3aR,4R,6R,6aR)-6-(6-amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl)((E)-3-(4-cyanophenyl)allyl)amino)-2-((tert-butoxycarbonyl)amino)butanoate (11e).** Following the procedure described for compound **11b**, coupling compound **10** (50 mg, 0.11 mmol) with **5e** (34 mg, 0.13 mmol) afforded compound **11e** as a white powder (47 mg, 62% yield). ¹H NMR (400 MHz, CDCl₃) δ 8.16 (s, 1H), 7.92 (s, 1H), 7.45 (d, *J* = 8.3 Hz, 2H), 7.24 (d, *J* = 8.4 Hz, 2H), 6.62 (br s, 2H), 6.32 (br s, 1H), 6.24-6.16 (m, 1H), 6.04 – 5.99 (m, 1H), 5.91 (d, *J* = 8.1 Hz, 1H), 5.40 (t, *J* = 8.1 Hz, 2H), 4.33-4.25 (m, 2H), 3.61 (d, *J* = 2.3 Hz, 1H), 3.56 (d, *J* = 3.6 Hz, 2H), 3.44 (s, 3H), 3.20 (t, *J* = 5.6 Hz, 2H), 3.09 (d, *J* = 4.8 Hz, 1H), 2.78 – 2.52 (m, 6H), 2.49 (s, 3H), 1.85 – 1.62 (m, 3H), 1.53 (s, 3H), 1.34 (br s, 20H). ¹³C NMR (101 MHz, CDCl₃) δ 172.7, 156.1, 153.0, , 132.29, 141.3, 132.3, 130.9, 126.6, 119.0, 114.3, 110.4, 90.6, 85.6, 83.9, 83.2, 59.7, 52.3, 51.0, 44.8, 29.2, 28.3, 27.1, 25.4. HRMS (ESI): calculated for C₃₃H₄₃N₈O₇ [M+H]⁺ 691.3568, found 691.3577.

benzyl **(S)-4-((((3aR,4R,6R,6aR)-6-(6-amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl)((E)-3-(4-cyanophenyl)allyl)amino)-2-((tert-butoxycarbonyl)amino)butanoate (11f).** Following the procedure described for compound **11b**, coupling compound **5f** (50 mg, 0.11 mmol) with compound **10** (40 mg, 0.13 mmol) afforded compound **11f** as a white powder (54 mg, 66% yield). ¹H NMR (400 MHz, CDCl₃) δ 8.18 (s, 1H), 7.91 (s, 1H), 7.48 (d, *J* = 8.4 Hz, 2H), 7.25 (s, 7H), 6.48 (s, 2H), 6.02 (s, 1H), 5.92 (d, *J* = 8.1 Hz, 1H), 5.38 (d, *J* = 6.4 Hz, 2H), 5.19 – 4.99 (m, 2H), 4.98 – 4.90 (m, 1H), 4.38 – 4.31 (br m, 2H), 3.78 – 3.68 (m, 2H), 3.66 (s, 1H), 3.60 (d, *J* = 3.9 Hz, 3H), 3.48 (s, 5H), 3.23-3.17 (m, 2H), 2.79 – 2.59 (m, 6H), 1.88 – 1.82 (m, 2H), 1.74-1.69 (m, 2H), 1.56 (s, 3H), 1.38 (br, 12H). ¹³C NMR (101 MHz, CDCl₃) δ 172.6, 156.1, 155.8, 153.0, 149.0, 141.2, 135.5, 132.3, 128.2, 126.7, 120.0, 90.6, 85.6, 83.9, 83.3, 66.9, 65.1, 59.8, 56.6, 56.1, 52.4, 44.8, 28.4, 27.2, 25.4. HRMS (ESI): calculated for C₃₃H₄₃N₈O₇ [M+H]⁺ 739.3568, found 739.3571.

methyl **(S)-2-amino-4-((((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)((E)-3-(4-cyanophenyl)allyl)amino)butanoate (12b).** To a solution of compound **11b** (30 mg, 0.045 mmol) in 1 mL of CH₂Cl₂ was added a mixture of 9 mL TFA and 1 mL H₂O, and the solution was stirred for 2 h at room temperature. The mixture was concentrated, and the crude product was purified by preparative HPLC affording compound **12b** as a white powder (24mg, 93% yield). ¹H NMR (400 MHz, CD₃OD) δ 8.47 (s, 1H), 8.33 (s, 1H), 7.69 (d, *J* = 8.4 Hz, 2H), 7.51 (d, *J* = 8.5 Hz, 2H), 6.84 (d, *J* = 15.8 Hz, 1H), 6.49 (dt, *J* = 15.8, 7.2 Hz,

1H), 6.17 (d, $J = 3.4$ Hz, 1H), 4.70 (dd, $J = 4.8, 3.4$ Hz, 1H), 4.60 – 4.53 (m, 2H), 4.25 (dd, $J = 7.4, 5.7$ Hz, 1H), 4.14 (d, $J = 7.3$ Hz, 2H), 3.92 – 3.85 (m, 1H), 3.84 (s, 3H), 3.74 – 3.66 (m, 1H), 3.64 – 3.47 (m, 2H), 2.58 – 2.47 (m, 1H), 2.46 – 2.34 (m, 1H). ^{13}C NMR (101 MHz, CD_3OD) δ 168.3, 161.84, 161.5, 151.6, 148.2, 139.75, 145.5, 143.0, 139.8, 138.4, 132.3, 127.2, 119.8, 118.191.2, 78.7, 73.5, 72.2, 55.5, 54.9, 52.8, 50.2, 49.8, 24.9. HRMS (ESI): calculated for $\text{C}_{25}\text{H}_{31}\text{N}_8\text{O}_5$ $[\text{M}+\text{H}]^+$ 523.2417, found 523.2422.

ethyl **(*S*)-2-amino-4-((((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoate** (12c).

Following the procedure described for compound **12b**, compound **11c** (30 mg, 0.044 mmol) was deprotected and purified, affording compound **12c** as a white powder (19 mg, 67% yield). ^1H NMR (400 MHz, CD_3OD) δ 8.42 (s, 1H), 8.30 (s, 1H), 7.70 (d, $J = 8.4$ Hz, 2H), 7.50 (d, $J = 8.4$ Hz, 2H), 6.84 (br d, $J = 15.9$ Hz, 1H), 6.53 – 6.42 (m, 1H), 6.15 (d, $J = 3.4$ Hz, 1H), 4.69 (dd, $J = 5.1, 3.5$ Hz, 1H), 4.59 – 4.48 (m, 2H), 4.33 – 4.25 (m, 2H), 4.21 (t, $J = 6.6$ Hz, 1H), 4.09 (t, $J = 7.2$ Hz, 2H), 3.80 (dd, $J = 13.9, 9.8$ Hz, 1H), 3.65 – 3.61 (dd, $J = 13.2, 5.8$ Hz, 1H), 3.56 – 3.44 (m, 2H), 2.52 – 2.44 (m, 1H), 2.41 – 2.30 (m, 1H), 1.30 (t, $J = 7.1$ Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 167.9, 161.6, 152.5, 148.3, 139.8, 138.0, 132.3, 127.2, 121.0, 119.7, 118.1, 111.7, 91.0, 78.8, 73.4, 72.2, 62.9, 55.5, 55.0, 50.4, 49.9, 25.0. HRMS (ESI): calculated for $\text{C}_{26}\text{H}_{33}\text{N}_8\text{O}_5$ $[\text{M}+\text{H}]^+$ 537.2574, found 537.2579.

propyl **(*S*)-2-amino-4-((((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoate** (12d).

Following the procedure described for compound **12b**, compound **11d** (30 mg, 0.043 mmol) was deprotected and purified, affording compound **12d** as a white powder (20 mg, 69% yield). ^1H NMR (400 MHz, CD_3OD) δ 8.46 (s, 1H), 8.33 (s, 1H), 7.69 (d, $J = 8.3$ Hz, 2H), 7.51 (d, $J = 8.3$ Hz, 2H), 6.84 (d, $J = 15.8$ Hz, 1H), 6.55–6.45 (m, 1H), 6.16 (d, $J = 3.4$ Hz, 1H), 4.75 – 4.67 (m, 1H), 4.59 – 4.53 (m, 2H), 4.28 – 4.09 (m, 5H), 3.90 – 3.77 (m, 1H), 3.69 (d, $J = 13.4$ Hz, 1H), 3.63 – 3.47 (m, 2H), 2.57 – 2.33 (m, 2H), 1.73–1.64 (m, 2H), 1.24 (s, 6H), 0.94 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 168.0, 152.0, 148.3, 146.1, 142.8, 139.81, 120.6, 118.1, 111.8, 91.1, 78.8, 73.5, 72.2, 68.3, 55.6, 55.0, 29.8, 25.0, 21.4. HRMS (ESI): calculated for $\text{C}_{27}\text{H}_{35}\text{N}_8\text{O}_5$ $[\text{M}+\text{H}]^+$ 551.2730, found 551.2732.

isopropyl **(*S*)-2-amino-4-((((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoate** (12e).

Following the procedure described for compound **12b**, compound **11e** (30 mg, 0.043 mmol) was deprotected and purified, affording compound **12e** as a white powder (21 mg, 73% yield). ^1H NMR (400 MHz, CD_3OD) δ 8.41 (s, 1H), 8.30 (s, 1H), 7.70 (d, $J = 8.4$ Hz, 2H), 7.50 (d, $J = 8.3$ Hz, 2H),

6.84 (br d, $J = 15.8$ Hz, 1H), 6.53 – 6.42 (m, 1H), 6.14 (d, $J = 3.4$ Hz, 1H), 5.14 – 5.07 (m, 1H), 4.70 (dd, $J = 5.1, 3.5$ Hz, 1H), 4.59 – 4.54 (m, 1H), 4.54 – 4.48 (m, 1H), 4.18 (t, $J = 6.6$ Hz, 1H), 4.07 (d, $J = 7.1$ Hz, 2H), 2.51 – 2.29 (m, 2H), 1.29 (t, $J = 6.1$ Hz, 6H). ^{13}C NMR (101 MHz, CD_3OD) δ 167.5, 152.7, 139.8, 137.8, 132.3, 127.2, 119.7, 118.1, 111.7, 91.0, 78.9, 73.4, 72.2, 71.4, 55.1, 50.6, 50.0, 25.1, 20.3. HRMS (ESI): calculated for $\text{C}_{27}\text{H}_{35}\text{N}_8\text{O}_5$ $[\text{M}+\text{H}]^+$ 551.2730, found 551.2734.

benzyl **(*S*)-2-amino-4-((((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoate (12f).**

Following the procedure described for compound **12b**, compound **11f** (30 mg, 0.041 mmol) was deprotected and purified, affording compound **12f** as a white powder (22 mg, 77% yield). ^1H NMR (400 MHz, CD_3OD) δ 8.41 (s, 1H), 8.29 (s, 1H), 7.48 (s, 1H), 7.46 (s, 1H), 7.43 – 7.36 (m, 5H), 6.79 (br d, $J = 15.9$ Hz, 1H), 6.47 – 6.37 (m, 1H), 6.13 (d, $J = 3.4$ Hz, 1H), 5.29 (d, $J = 2.8$ Hz, 2H), 4.67 (dd, $J = 5.1, 3.4$ Hz, 1H), 4.57 – 4.45 (m, 2H), 4.28 (t, $J = 6.6$ Hz, 1H), 4.03 (d, $J = 7.2$ Hz, 2H), 3.76 (dd, $J = 13.9, 9.9$ Hz, 1H), 3.60 (br d, $J = 12.8$ Hz, 1H), 3.53 – 3.40 (m, 2H), 2.54 – 2.33 (m, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 167.9, 152.1, 148.2, 134.7, 132.3, 119.7, 118.1, 111.78, 91.0, 78.8, 73.4, 72.2, 68.2, 55.5, 54.9, 50.4, 49.9, 25.0. HRMS (ESI): calculated for $\text{C}_{31}\text{H}_{34}\text{N}_8\text{O}_5$ $[\text{M}+\text{H}]^+$ 598.2652, found 598.2656.

***tert*-butyl** **(*S*)-2-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamido)-4-((((3*aR*,4*R*,6*R*,6*aR*)-6-(6-amino-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoate (13a).**

Following the procedure described for compound **11b**, coupling compound **10** (95 mg, 0.21 mmol, 1.1 eq) with compound **9a** (80 mg, 0.19 mmol, 1.0 eq) afforded compound **13a** as a yellowish oil (55 mg, 34% yield). ^1H NMR (400 MHz, CDCl_3) δ 8.24 (s, 1H), 7.90 (s, 1H), 7.55 (d, $J = 8.4$ Hz, 1H), 7.31 (d, $J = 8.4$ Hz, 2H), 6.79 (d, $J = 2.1$ Hz, 1H), 6.58 (d, $J = 2.1$ Hz, 1H), 6.43 – 6.19 (m, 3H), 6.14 (s, 2H), 6.04 (d, $J = 2.0$ Hz, 1H), 5.44 (dd, $J = 6.4, 2.0$ Hz, 1H), 4.98 (dd, $J = 6.4, 3.6$ Hz, 1H), 4.38 – 4.27 (m, 2H), 3.21 (d, $J = 6.3$ Hz, 2H), 2.77 – 2.66 (m, 2H), 2.57 (q, $J = 13.5$ Hz, 2H), 2.49 (s, 3H), 2.31 (s, 3H), 2.20 (s, 3H), 1.85 – 1.73 (m, 1H), 1.67 – 1.56 (m, 11H), 1.52 – 1.38 (m, 16H), 1.26 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 171.15, 170.94, 170.76, 155.65, 153.02, 149.87, 149.12, 141.25, 140.14, 138.46, 136.51, 133.68, 132.73, 132.39, 130.90, 126.69, 124.82, 123.39, 119.05, 114.43, 114.13, 110.59, 90.80, 85.71, 83.96, 83.25, 81.71, 56.83, 56.01, 51.56, 50.84, 49.26, 43.53, 39.80, 31.97, 31.81, 31.69, 30.35, 29.74, 29.55, 29.46, 27.96, 27.94, 27.20, 25.53, 25.43, 21.97, 20.27. HRMS (ESI): calculated for $\text{C}_{46}\text{H}_{59}\text{N}_8\text{O}_8$ $[\text{M}+\text{H}]^+$ 851.4450, found 851.4456.

methyl **(*S*)-2-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamido)-4-((((3*aR*,4*R*,6*R*,6*aR*)-6-(6-amino-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-**

4-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoate (**13b**). To a solution of compound **11b** (30 mg, 0.045 mmol) in 9 mL dry of CH₂Cl₂ was added a 1 mL TFA and the solution was stirred for 1 h at room temperature. The mixture was concentrated. 5 mL CH₂Cl₂ was added to the mixture, followed by adding benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP, 20 mg), 3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanoic acid **7** (TML acid, 0.045 mmol, 12 mg) and 0.5 ml Et₃N. The reaction mixture stirred 2 hours at r.t. 10 mL water was added, then extracted with CH₂Cl₂ (10 ml × 3), the combined organic phase dried over Na₂SO₄. The solvent was evaporated, and the crude product was purified by column chromatography (100% EtOAc) to give compound **13b** as a white powder (15 mg, 42% yield over 2 steps). ¹H NMR (400 MHz, CDCl₃) δ 8.20 (s, 1H), 7.91 (s, 1H), 7.54 (d, *J* = 8.3 Hz, 2H), 7.30 (d, *J* = 8.5 Hz, 2H), 6.79 (d, *J* = 1.5 Hz, 1H), 6.58 (d, *J* = 7.3 Hz, 3H), 6.43 – 6.32 (m, 2H), 6.27 – 6.17 (m, 1H), 6.04 (d, *J* = 2.0 Hz, 1H), 5.43 (dd, *J* = 6.4, 2.0 Hz, 1H), 4.97 (dd, *J* = 6.4, 3.4 Hz, 1H), 4.48-4.43 (m, 1H), 4.37-4.32 (m, 1H), 3.62 (s, 3H), 3.19 (d, *J* = 6.2 Hz, 2H), 2.69 (d, *J* = 6.8 Hz, 2H), 2.55 (d, *J* = 4.8 Hz, 2H), 2.48 (s, 3H), 2.41 – 2.32 (m, 1H), 2.30 (s, 3H), 2.19 (s, 3H), 2.12 (s, 1H), 1.81-1.74 (m, 1H), 1.69 – 1.55 (m, 9H), 1.38 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 172.5, 171.1, 155.8, 152.7, 149.9, 141.2, 140.1, 138.5, 136.6, 132.7, 133.6, 132.4, 131.0, 130.9, 126.7, 123.4, 114.4, 110.6, 90.9, 85.6, 83.9, 83.2, 56.4, 55.9, 52.2, 50.9, 49.2, 39.8, 31.7, 29.1, 27.2, 25.5, 25.3, 22.0, 20.2. HRMS (ESI): calculated for C₄₃H₅₃N₈O₈ [M+H]⁺ 809.3986, found 809.3991.

ethyl (S)-2-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamido)-4-(((3*aR*,4*R*,6*R*,6*aR*)-6-(6-amino-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoate (**13c**). Following the procedure described for compound **13b**, compound **11c** (85 mg, 0.126 mmol) was selectively deprotected and coupled with TML acid **7** (40 mg, 0.15 mmol), affording crude **13c** which was used in the next step without further purification.

propyl (S)-2-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamido)-4-(((3*aR*,4*R*,6*R*,6*aR*)-6-(6-amino-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoate (**13d**). Following the procedure described for compound **13b**, compound **11d** (31 mg, 0.045 mmol) was selectively deprotected and coupled with TML acid **7** (12 mg, 0.045 mmol), affording crude **13d** which was used in the next step without further purification.

isopropyl (S)-2-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamido)-4-(((3*aR*,4*R*,6*R*,6*aR*)-6-(6-amino-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoate (**13e**). Following the procedure described for compound **13b**, compound **11e** (31 mg, 0.045 mmol) was selectively deprotected and

coupled with TML acid **7** (15 mg, 0.045 mmol), affording compound **13e** as a white powder (18 mg, 39% yield over 2 steps). ¹H NMR (400 MHz, CDCl₃) δ 8.22 (s, 1H), 7.90 (s, 1H), 7.54 (d, *J* = 8.3 Hz, 2H), 7.30 (d, *J* = 6.4 Hz, 2H), 6.79 (d, *J* = 1.6 Hz, 1H), 6.59 (d, *J* = 1.6 Hz, 1H), 6.43 – 6.32 (m, 4H), 6.23 (dt, *J* = 15.9, 6.4 Hz, 1H), 6.04 (d, *J* = 2.0 Hz, 1H), 5.44 (dd, *J* = 6.4, 2.0 Hz, 1H), 5.01 – 4.92 (m, 2H), 4.46 – 4.30 (m, 2H), 3.20 (d, *J* = 6.2 Hz, 2H), 2.70 (d, *J* = 7.9 Hz, 2H), 2.64 – 2.51 (m, 3H), 2.49 (s, 3H), 2.41 – 2.36 (m, 2H), 2.30 (s, 3H), 2.20 (s, 3H), 1.83 – 1.78 (m, 1H), 1.67 – 1.57 (m, 11H), 1.38 (s, 3H), 1.26 (s, 4H), 1.20 (d, *J* = 6.3 Hz, 3H), 1.15 (d, *J* = 6.2 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 171.6, 171.0, 170.8, 155.7, 152.9, 149.9, 141.2, 140.1, 138.6, 132.7, 132.4, 123.42, 120.2, 119.0, 114.4, 109.8, 90.9, 85.7, 84.0, 83.2, 68.9, 56.7, 56.0, 51.1, 50.8, 49.2, 39.8, 31.7, 29.7, 27.2, 25.5, 25.4, 22.0, 21.7, 20.3. HRMS (ESI): calculated for C₄₅H₅₇N₈O₈ [M+H]⁺ 837.4299, found 837.4303.

benzyl *(S)*-2-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamido)-4-(((3*aR*,4*aR*,6*aR*)-6-(6-amino-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoate (**13f**). Following the procedure described for compound **13b**, compound **11f** (82 mg, 0.13 mmol) was selectively deprotected and coupled with TML acid **7** (34 mg, 0.13 mmol), affording crude **13f** which was used in the next step without further purification.

(S)-2-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamido)-4-(((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoic acid (**14a**). Following the procedure described for compound **12a**, compound **13a** (55 mg, 0.065 mmol) was deprotected and purified, affording compound **14a** as a white powder (28 mg, 54% yield). ¹H NMR (400 MHz, CD₃OD) δ 8.47 (s, 1H), 8.35 (s, 1H), 7.72 (d, *J* = 8.1 Hz, 2H), 7.54 (d, *J* = 8.1 Hz, 2H), 6.85 – 6.75 (m, 2H), 6.61 (d, *J* = 2.1 Hz, 1H), 6.44 – 6.34 (m, 1H), 6.15 (d, *J* = 3.5 Hz, 1H), 4.54 – 4.47 (m, 2H), 4.32 (dd, *J* = 8.6, 4.8 Hz, 1H), 3.93 (d, *J* = 7.5 Hz, 2H), 3.84 – 3.76 (m, 1H), 3.62 (d, *J* = 13.8 Hz, 1H), 3.27 – 3.16 (m, 2H), 2.76 (d, *J* = 14.9 Hz, 1H), 2.69 – 2.60 (m, 1H), 2.55 (s, 3H), 2.33 (s, 3H), 2.16 (s, 3H), 2.08 – 1.96 (m, 1H), 1.60 (s, 3H), 1.56 (s, 3H). ¹³C NMR (101 MHz, CD₃OD) δ 175.2, 171.7, 162.9, 157.1, 150.4, 147.3, 139.6, 138.7, 138.3, 136.0, 134.4, 133.0, 132.3, 131.9, 127.3, 123.0, 119.8, 118.1, 117.7, 114.8, 112.4, 91.1, 77.0, 73.4, 71.7, 54.9, 50.9, 50.5, 36.9, 31.0, 29.7, 26.3, 20.5, 18.5. HRMS (ESI): calculated for C₃₉H₄₇N₈O₈ [M+H]⁺ 755.3511, found 755.3508.

methyl *(S)*-2-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamido)-4-(((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoate (**14b**). Following the procedure described for compound **12a**, compound **13b** (10 mg, 0.012 mmol) was deprotected and purified, affording compound **14b**

as a white powder (5 mg, 48% yield). ^1H NMR (400 MHz, CD_3OD) δ 8.48 (s, 1H), 8.36 (s, 1H), 7.70 (d, J = 8.3 Hz, 2H), 7.53 (d, J = 8.1 Hz, 2H), 6.87 – 6.77 (m, 2H), 6.61 (d, J = 1.5 Hz, 1H), 6.42 – 6.35 (m, 1H), 6.17 (d, J = 3.5 Hz, 1H), 4.69 (t, J = 3.8 Hz, 1H), 4.51 (d, J = 6.6 Hz, 2H), 4.40 (dd, J = 8.7, 5.0 Hz, 1H), 3.95 (br s, 2H), 3.82 – 3.74 (m, 1H), 3.69 (s, 3H), 3.62 (br d, J = 13.6 Hz, 1H), 3.25 – 3.18 (m, 2H), 2.76 (d, J = 14.9 Hz, 1H), 2.64 (br d, J = 14.8 Hz, 1H), 2.54 (s, 3H), 2.16 (s, 3H), 2.00 (s, 1H), 1.61 (s, 3H), 1.55 (s, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 172.9, 170.9, 160.96, 161.3, 161.0, 160.6, 160.2, 151.1, 149.7, 148.1, 139.66, 138.7, 136.0, 133.6, 132.3, 131.9, 127.3, 123.0, 119.9, 118.1, 117.7, 114.8, 111.9, 91.1, 78.5, 73.4, 72.2, 54.3, 51.8, 49.5, 39.1, 31.1, 30.8, 26.1, 24.4, 20.6, 18.9. HRMS (ESI): calculated for $\text{C}_{40}\text{H}_{49}\text{N}_8\text{O}_8$ $[\text{M}+\text{H}]^+$ 769.3673, found 769.3681.

ethyl (*S*)-2-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamido)-4-((((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoate (**14c**). Following the procedure described for compound **12a**, compound **13c** (10 mg, 0.012 mmol) was deprotected and purified, affording compound **14c** as a white powder (6 mg, 56% yield). ^1H NMR (400 MHz, Methanol- d_4) δ 8.46 (s, 1H), 8.34 (s, 1H), 7.72 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.2 Hz, 2H), 6.86 – 6.78 (m, 2H), 6.61 (d, J = 2.1 Hz, 1H), 6.42 – 6.31 (m, 1H), 6.16 (d, J = 3.6 Hz, 1H), 4.70 (s, 1H), 4.55 – 4.46 (m, 2H), 4.38 (dd, J = 8.8, 5.0 Hz, 1H), 4.18 – 4.13 (m, 2H), 3.93 (s, 2H), 3.81 – 3.75 (m, 1H), 3.61 (br d, J = 14.9 Hz, 1H), 3.26 – 3.15 (m, 2H), 2.55 (s, 3H), 2.34 (s, 3H), 2.16 (s, 3H), 1.62 (s, 3H), 1.56 (s, 3H), 1.24 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Methanol- d_4) δ 172.9, 171.0, 151.6, 149.7, 147.1, 139.6, 138.7, 138.3, 136.0, 133.6, 132.7, 131.9, 128.1, 123.6, 119.8, 118.9, 112.0, 91.0, 78.5, 78.5, 73.4, 72.2, 61.6, 55.5, 50.9, 49.6, 39.1, 29.9, 26.1, 24.4, 20.6, 18.8. HRMS (ESI): calculated for $\text{C}_{41}\text{H}_{51}\text{N}_8\text{O}_8$ $[\text{M}+\text{H}]^+$ 783.3830, found 783.3835.

propyl (*S*)-2-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamido)-4-((((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)((*E*)-3-(4-cyanophenyl)allyl)amino)butanoate (**14d**). Following the procedure described for compound **12a**, compound **13d** (10 mg, 0.012 mmol) was deprotected and purified, affording compound **14d** as a white powder (4 mg, 42% yield). ^1H NMR (500 MHz, CD_3OD) δ 8.45 (s, 1H), 8.34 (s, 1H), 7.70 (d, J = 8.3 Hz, 2H), 7.51 (d, J = 8.1 Hz, 2H), 6.85 – 6.78 (m, 2H), 6.60 (d, J = 1.8 Hz, 1H), 6.40 – 6.34 (m, 1H), 6.15 (d, J = 3.5 Hz, 1H), 4.68 (t, J = 4.0 Hz, 1H), 4.52 – 4.47 (m, 2H), 4.37 (dd, J = 8.6, 5.1 Hz, 1H), 4.04 (t, J = 6.7 Hz, 2H), 3.97 – 3.89 (m, 2H), 3.79 – 3.74 (m, 1H), 3.64 – 3.57 (m, 1H), 3.24 – 3.17 (m, 2H), 2.79 – 2.70 (m, 2H), 2.67 (d, J = 4.2 Hz, 1H), 2.64 (s, 1H), 2.53 (s, 3H), 2.32 (s, 3H), 2.15 (s, 3H), 2.04 – 1.94 (m, 1H), 1.67 – 1.58 (m, 6H), 1.54 (s, 3H), 0.91 (t, J = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, CD_3OD) δ 172.9, 171.1, 170.6, 149.8, 139.7, 138.4,

136.1, 133.7, 134.4, 132.0, 127.4, 123.1, 119.9, 118.1, 112.0, 91.1, 78.6, 73.5, 72.3, 67.1, 54.5, 50.9, 49.7, 39.2, 31.1, 30.9, 24.5, 21.6, 20.7, 18.9. HRMS (ESI): calculated for C₄₂H₅₃N₈O₈ [M+H]⁺ 783.3986, found 797.3991.

isopropyl (S)-2-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamido)-4-(((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)((E)-3-(4-cyanophenyl)allyl)amino)butanoate (14e). Following the procedure described for compound **12a**, compound **13e** (10 mg, 0.012 mmol) was deprotected and purified, affording compound **14e** as a white powder (5.3 mg, 49% yield). ¹H NMR (400 MHz, CD₃OD) δ 8.47 (s, 1H), 8.35 (s, 1H), 7.71 (d, *J* = 8.1 Hz, 2H), 7.53 (d, *J* = 8.1 Hz, 2H), 6.86 – 6.77 (m, 2H), 6.61 (d, *J* = 2.1 Hz, 1H), 6.42 – 6.35 (m, 1H), 6.17 (d, *J* = 3.5 Hz, 1H), 4.72 – 4.66 (m, 1H), 4.54 – 4.49 (m, 2H), 4.33 (dd, *J* = 8.8, 5.0 Hz, 1H), 3.93 (d, *J* = 7.5 Hz, 2H), 3.78 (dd, *J* = 14.0, 9.6 Hz, 1H), 3.62 (d, *J* = 13.8 Hz, 1H), 3.27 – 3.17 (m, 2H), 2.77 (d, *J* = 14.9 Hz, 1H), 2.64 (br d, *J* = 14.9 Hz, 1H), 2.55 (s, 3H), 2.33 (s, 3H), 2.16 (s, 3H), 2.07 – 1.93 (m, 1H), 1.61 (s, 3H), 1.56 (s, 3H), 1.22 (dd, *J* = 6.3, 1.8 Hz, 6H). ¹³C NMR (101 MHz, CD₃OD) δ 175.2, 171.7, 162.9, 157.1, 150.4, 147.3, 139.6, 138.7, 138.3, 136.0, 134.4, 133.0, 132.3, 131.9, 127.3, 123.0, 119.8, 118.1, 117.7, 114.8, 112.4, 91.1, 77.0, 73.4, 71.7, 69.2, 54.9, 50.9, 50.5, 36.9, 31.0, 29.7, 26.3, 24.4, 20.5, 18.5. HRMS (ESI): calculated for C₄₂H₅₃N₈O₈ [M+H]⁺ 783.3986, found 797.3994.

benzyl (S)-2-(3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamido)-4-(((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)((E)-3-(4-cyanophenyl)allyl)amino)butanoate (14f). compound **13f** (13mg, 0.015 mmol) was deprotected and purified, affording compound **14f** as a white powder (5 mg, 39%). ¹H NMR (500 MHz, CD₃OD) δ 8.41 (s, 1H), 8.31 (s, 1H), 7.69 (d, *J* = 6.3 Hz, 2H), 7.49 (d, *J* = 8.1 Hz, 2H), 7.33 (d, *J* = 9.1 Hz, 5H), 6.82 – 6.75 (m, 2H), 6.57 (d, *J* = 2.1 Hz, 1H), 6.36 – 6.27 (m, 1H), 6.12 (d, *J* = 3.5 Hz, 1H), 5.12 (d, *J* = 2.9 Hz, 2H), 4.65 (t, *J* = 4.2 Hz, 1H), 4.50 – 4.38 (m, 3H), 3.89 (d, *J* = 11.8 Hz, 2H), 3.76 – 3.70 (m, 1H), 3.56 (br d, *J* = 14.6 Hz, 1H), 3.35 (s, 1H), 3.23 – 3.11 (m, 2H), 2.28 (s, 3H), 2.12 (s, 3H), 2.01 – 1.93 (m, 1H), 1.56 (s, 3H), 1.49 (s, 3H). ¹³C NMR (126 MHz, CD₃OD) δ 171.1, 149.8, 139.6, 138.4, 136.1, 133.7, 1132.4, 128.3, 128.1, 123.1, 91.1, 72.3, 67.1, 42.4, 31.7, 24.4, 20.6, 18.9. HRMS (ESI): calculated for C₄₆H₅₂N₈O₈ [M+H]⁺ 844.3908, found 844.3911.

4.2 Ester stability assay

The prodrugs were evaluated for their stability in both PBS buffer at pH 7.4 and Tris buffer at pH 8.4. The compounds were dissolved in DMSO at a concentration of 40 mM and diluted with the respective buffer to a final concentration of 1 mM. Compounds were tested directly (to) and

subsequently every 2 hours over a time period of 16 hours by HPLC. Compounds were eluted from a Dr. Maisch repositil-pur C18 column (4.6 x 250 mm, 10 μ m) with the following solvent system at a flow rate of 0.5 mL/min: solvent A, 0.1 % trifluoroacetic acid in water/acetonitrile (95:5) ; solvent B, 0.1 % trifluoroacetic acid in water/acetonitrile (5:95). Gradient elution was as follows: 100:0 (A/B) to 0:100 (A/B) over 8 min, 0:100 (A/B) for 1 min, then reversion back to 100:0 (A/B) over 1 min, 100:0 (A/B) for 2 min. The formation of the parent compound was evaluated and normalized by measuring the peak area at 214 nm and comparing it to the initial timepoint.

4.3 Esterase-mediated hydrolysis

The conversion of the prodrugs to the parent compound by esterases was evaluated using pig liver esterase (PLE, 18 U/mg, Sigma-Aldrich) in PBS at pH 7.4. Compounds were dissolved in DMSO at 40 mM, diluted to a final concentration of 2 mM with PBS and added to an equal volume of a 10 mg/mL solution of PLE in PBS (pH 7.4), resulting in final concentrations of 2.5% DMSO, 1 mM compound and 5 mg/mL PLE. At different time-points 50 μ L aliquots were taken, added to 100 μ L acetonitrile to precipitate the proteins and centrifuged for 5 minutes at 10,000 rpm. The supernatant was subsequently analyzed by HPLC as described in section 4.2 above.

4.4 Neon electroporation assay

4.4.1. Cell culture

A549 lung cancer cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and seeded at 50,000 cells per well in a 48 well plate. After addition of compound (1, 10 or 100 μ M) or DMSO (0.5%), the cells were electroporated in 10 μ L Neon transfection tips with 2 pulses with a pulse voltage of 1700 mV and a pulse width of 20ms. Cells were resuspended in 250 mM sucrose buffer with Mg²⁺ and transferred to 96 well plates (100 μ L, 15,000 cells/well) and incubated for 24, 48 or 72 hours at 37 °C with 5% CO₂. The cells were subsequently checked for their viability using the MTS cell viability assay and compared to both non-treated cells (DMSO) and non-electroporated cells. All experiments were performed in triplicate.

4.4.2. MTS cell viability assay

Cell proliferation was determined using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). The MTS assay measures the conversion of MTS to a water-soluble formazan product by dehydrogenase enzymes of the intact mitochondria of living cells. Cell proliferation was evaluated by measuring the absorbance at 490 nm upon treatment with compounds or DMSO only for 24, 48 and 72 hours. Briefly, medium was aspirated from the cells and MTS in DMEM/FBS medium was added. The

cells were incubated for 1 hour at 37°C. The reaction product was quantified by measuring the absorbance at 490 nm using a plate reader. Experiments were repeated three times. Results were expressed as percentage of the control (control equals 100% and corresponds to the absorbance value of each sample at time zero) and presented as mean values $\pm$ standard deviation of three independent experiments performed in triplicate.

4.5 Cell proliferation assay

4.5.1. Cell culture

HSC-2 human oral cancer cell line, T24 human bladder cancer cell line and A549 human lung cancer line were cultured in DMEM/F12 medium, supplemented with 10% fetal bovine serum and 50 μ g/ml gentamicin, at 37 °C in a humidified 5% CO₂ incubator. For each compound tested, powder was dissolved in DMSO at 100mM concentration. This stock solution was then diluted in culture medium to final concentration values ranging between 1 μ M and 100 μ M. For each sample, DMSO was kept constant at 0.1% final concentration. The day before starting treatment, cells were seeded in 96-well plates, at a density of 2,000 cells/well. Cells were allowed to attach overnight and then incubated with compounds at different concentrations, or with DMSO only, for 24, 48 and 72 hours. All experiments were performed in triplicate.

4.5.2. MTT cell viability assay

Cell proliferation was determined using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The MTT assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzymes of the intact mitochondria of living cells. Cell proliferation was evaluated by measuring the conversion of the tetrazolium salt MTT to formazan crystals upon treatment with compounds or DMSO only for 24, 48 and 72 hours. Briefly, cells were incubated for 2 hours at 37 °C with 100 μ l fresh culture medium containing 5 μ l of MTT reagent (5mg/ml in PBS). The medium was removed and 200 μ l isopropanol were added. The amount of formazan crystals formed correlated directly with the number of viable cells. The reaction product was quantified by measuring the absorbance at 540nm using a plate reader. Experiments were repeated three times. Results were expressed as percentage of the control (control equals 100% and corresponds to the absorbance value of each sample at time zero) and presented as mean values $\pm$ standard deviation of three independent experiments performed in triplicate.

4.6. 1-methylnicotinamide (MNA) quantification in A549 cells

4.6.1. Cell culture

Human lung adenocarcinoma line (A549, ATCC, VA, USA) was cultured according to the provider's indications and seeded in 6-well format. After 24h stabilization, when cells reached $\approx 100\%$ confluence, A549 line was preincubated with normal Hank's buffer (HBSS), then treated with NNMT peptide inhibitors (1 or 10 μM), applied for 1h-incubation in a presence of nicotinamide 100 μM and S-adenosyl-methionine 10 μM (Sigma Aldrich, MO, USA). After incubation, effluent samples were taken and frozen ($-80\text{ }^{\circ}\text{C}$) for further MNA measurement. Cells also were collected, centrifuged ($2 \times 500\text{ G}/5\text{ min.}$) and frozen both for the measurement of intracellular MNA and for BCA protein assay.

4.6.2. Quantification of 1-methylnicotinamide (MNA)

The quantification of 1-methylnicotinamide (MNA), nicotinamide (NAM), nicotinic acid (NicA), 1-methyl-2-pyridone-5-carboxamide (Met-2Pyr) and 1-methyl-4-pyridone-5-carboxamide (Met-4Pyr) was performed applying ultra-high pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS) according to the methodology previously described with minor modifications. An UPLC-MS system comprised of an UPLC Ultimate 3000 (Dionex, Thermo Scientific, USA) connected to a TSQ Quantum Ultra mass spectrometer (Thermo Scientific, USA) equipped with a heated electrospray ionization interface (HESI-II Probe) was used. Chromatographic separation of analytes was carried out on an Aquasil C18 analytical column ($4.6\text{ mm} \times 150\text{ mm}$, 5 mm ; Thermo Scientific) under isocratic elution using acetonitrile with 0.1% of formic acid (A) and 5 mM ammonium formate in water (B) as mobile phases delivered at the flow rate of 0.8 ml/min (A:B, 80:20, v/v). The cell pellet was resuspended in 60 μL of deionized water and 50 μL of suspension was transferred to a fresh test tube. 50 μL of effluent sample was used for the measurement of extracellular MNA. The internal standard (IS) containing MNA- d_3 was added to each sample (5 μL) obtaining the final concentration of 500 ng/mL. After the sample mixing, the proteins were precipitated using 100 μL of acidified acetonitrile (0.1% of formic acid), and samples were mixed (10 min), cooled at $4\text{ }^{\circ}\text{C}$ (15 min) and finally centrifuged ($15\text{ }000 \times g$, 15 min, $4\text{ }^{\circ}\text{C}$). A clear supernatant was transferred to a chromatographic vial and directly injected (5 μL) into UPLC-MS system. The mass spectrometer was operating in the positive ionisation mode using selected reactions monitoring (SRM) mode monitoring the following ion transitions for analysed metabolite: $m/z\ 137 \rightarrow 94$ for MNA and $140 \rightarrow 97$ for MNA- d_3 . The concentration of MNA was calculated based on the calibration curve plotted for the analyte as the relationship between the peak area ratios of analyte/IS to the nominal concentration of the analyte. The concentration of analytes was normalized to milligram of proteins, which was assessed using PierceTM BCA Protein Assay Kit (Thermo Fisher, Waltham, MA, USA) and a Synergy4 multiplate reader (BioTek, Winooski, VT, USA). MNA was obtained from Sigma-Aldrich, and the deuterated internal standard MNA- d_3 was

synthesized by Dr. Adamus (Technical University, Lodz, Poland). LC-MS–grade acetonitrile, ammonium formate and formic acid were purchased from Sigma-Aldrich. Ultrapure water was obtained from a Millipore system (Direct-Q 3UV).

4.7 Statistical Analysis

Data were analysed using GraphPad Prism software for Windows (GraphPad Software, San Diego, CA). Significant differences between groups were determined using the one-way analysis of variance (ANOVA). A p value <0.05 was considered as statistically significant.

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