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**Publication Date** 08 Feb. 2024

**Article Type** Full Research Paper

**Supporting Information File 1** Experimental\_part\_submission.pdf; 1.2 MB

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## Synthesis of 1,4-azaphosphinine pyrimidine nucleosides and their evaluation as inhibitors of human cytidine deaminase and APOBEC3A

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**Keywords:** APOBEC3, cytidine deaminase, zebularine, enzyme activity, inhibitor, transition state analogue, nucleoside, nucleotide

### Abstract:

Nucleoside and polynucleotide cytidine deaminases (CDA and APOBEC3) share similar mechanism of cytosine to uracil conversion. In 1984 phosphapyrimidine riboside was characterised as the most potent inhibitor of human CDA but its quick degradation in water limited its applicability as a potential therapeutic. To improve stability in water, we synthesised a derivative **dPC** of phosphapyrimidine nucleoside having CH<sub>2</sub> group instead of the N3 atom in the nucleobase. A charge-neutral phosphinamide **dPC-NH<sub>2</sub>** and a negatively-charged phosphinic acid derivative **dPC-OH** had excellent stability in water at pH 7.4 but only the charge-neutral **dPC-NH<sub>2</sub>** inhibited human CDA similar to previously described 2'-deoxyzebularine ( $K_i = 8.0 \pm 1.9$  and  $10.7 \pm 0.5$   $\mu$ M, respectively). However, at basic conditions the charge-neutral phosphinamide **dPC-NH<sub>2</sub>** was unstable which prevented its incorporation into DNA using conventional DNA chemistry. In contrast, the negatively charged phosphinic acid derivative **dPC-OH** was incorporated into DNA instead of the target dC using an automated DNA synthesiser but no inhibition of APOBEC3A was observed for modified DNAs. Although this shows that negative charge is poorly accommodated in the active site of CDA and

APOBEC3, the synthetic route reported here provides opportunities for the synthesis of other derivatives of phosphapyrimidine riboside for potential development of more potent CDA and APOBEC3 inhibitors.

## 1. Introduction

Spontaneous hydrolytic deamination of cytosine to uracil (**Fig. 1A**) is very slow under ambient conditions[1] but it is greatly accelerated by enzymes. These enzymes share a similar mechanism of cytosine deamination and a similar tertiary structure. Despite this similarity, individual enzymes are selective for their corresponding cytosine-containing substrates with little or no cross-reactivity.

Cytosine deaminase which is present in bacteria and fungi but not in mammalian cells acts only on cytosine. Cytidine deaminase (CDA) as a key enzyme in the pyrimidine salvage pathway in mammals deaminates both cytidine and 2'-deoxycytidine. Members of the APOBEC family (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like), such as activation-induced deaminase (AID) and APOBEC3, act preferentially on single-stranded DNA containing one or multiple cytosines. Although some activity was detected on RNA, none was observed on cytidine or cytosine alone.

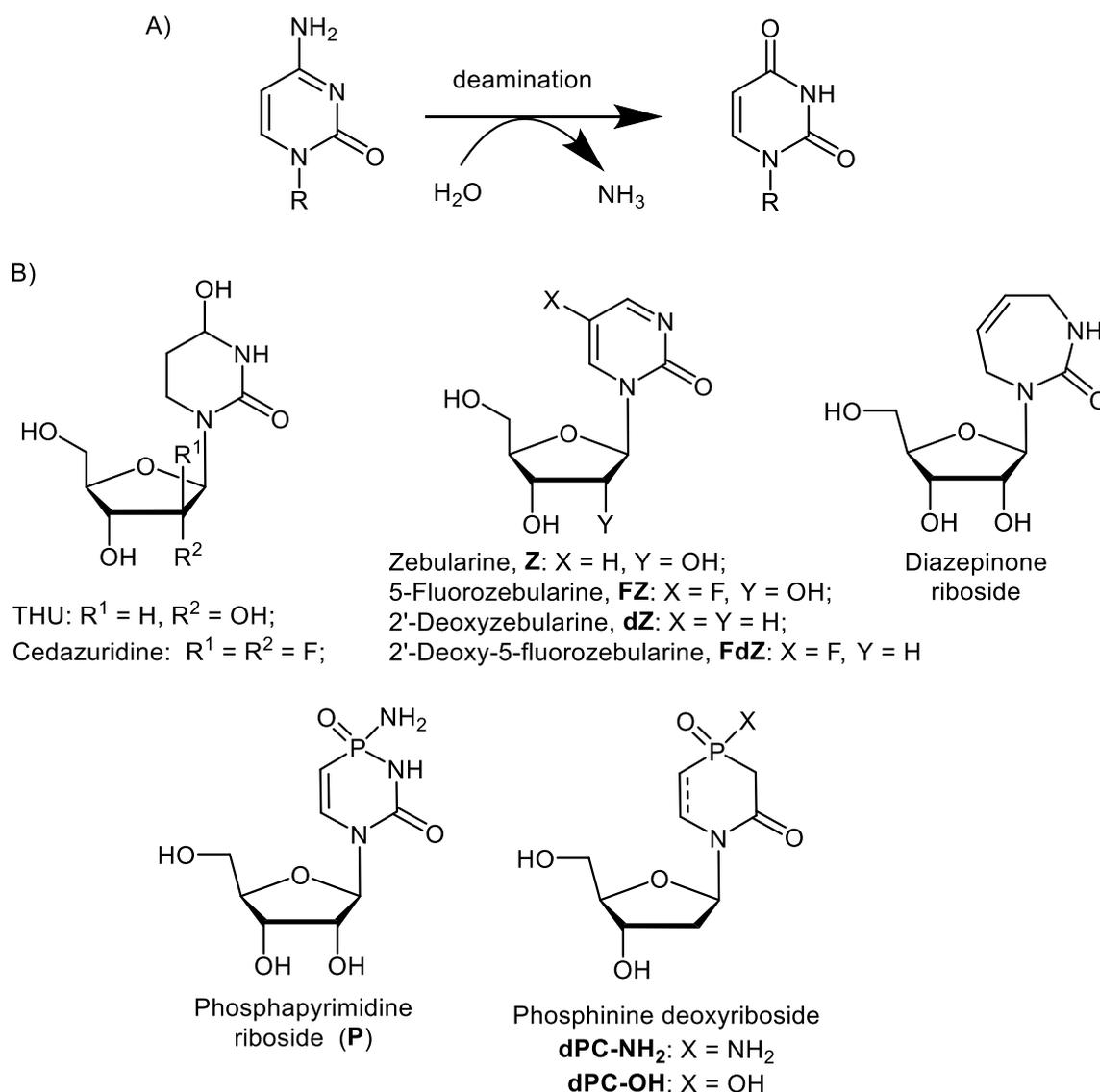
Each cytosine deaminase has an important biological function in an organism, but their activities can also be detrimental. CDA is highly active in liver and spleen which results in deamination and consequent deactivation of several chemotherapeutic agents, including the anti-cancer agents cytarabine, gemcitabine and decitabine.[2-5] Full inhibition of CDA leads to accumulation of toxic pyrimidine catabolism intermediates.[6, 7] However, local and temporary inhibition of CDA in the liver provides a therapeutic benefit by allowing cytosine-like containing drugs to by-pass liver with an intact nucleobase. Recently, a combination of a CDA inhibitor cedazauridine, (4R)-2'-deoxy-2',2'-difluoro-3,4,5,6-tetrahydrouridine (**Fig. 1B**), with an anticancer drug decitabine was approved as an oral pill (C-DEC or ASTX727) for the treatment of patients with intermediate/high-risk myelodysplastic syndromes MDS and chronic myelomonocytic leukaemia (CMML).[8]

In normal human cells the enzyme family APOBEC3 (A3)[9-12] disables pathogens by scrambling single-stranded DNA (ssDNA) by cytidine to uridine mutation (**Fig. 1A**).[9, 13-15] However, several enzymes, particularly A3A, A3B, A3H and A3G, deaminate cytosine in human nuclear and mitochondrial genomes.[16] This A3-induced mutational activity is used by viruses and cancer cells to increase the rates of mutagenesis, which allows them to escape adaptive immune responses, and become drug resistant[17-21] leading to poor clinical outcomes. A range of genetic, biochemical and structural studies support a model in which this A3-mediated mutagenesis promotes tumour evolution and strongly influences disease trajectories, including the development of drug resistance and metastasis.[19-24] Of the seven A3 enzymes, three (A3A, A3B and A3H) are at least partially localised in the nucleus of cells and are genotoxic.[25] A3A and A3H are single-domain enzymes, whereas A3B is a double-domain enzyme, in which only the C-terminal domain (CTD) has catalytic activity and the N-terminal domain (NTD) is responsible for binding of DNA and for nuclear localisation.

Initially, A3B had been identified as the primary source of genetic mutations in breast[19-24, 26, 27] and other cancers.[28, 29] The breast cancers with high expression of A3B show a two-fold increase in overall mutational load. Elevated A3B expression correlates with reduced tamoxifen sensitivity of tumours in those patients [20] and poor survival rates for estrogen receptor-positive (ER+) breast cancer patients.[22, 30] In line with these observations, A3B overexpression accelerates the development of tamoxifen resistance in murine xenograft with ER+ breast cancer. In contrast, knockdown of A3B results in prolonged tamoxifen responses and leads to the survival of mice during the experimental time (1 year).[20] More recent research points also to a prominent role of A3A in breast[31] and other cancers.[31-34] Overexpression of A3A and A3B leads to tumourigenesis in transgenic mouse models.[25, 29, 35, 36] High levels of A3A and A3B mRNA are linked also to the more aggressive breast cancers including triple negative cancers.[37] Since A3B is not essential for humans[38] and A3A does not take part in primary metabolism, inhibition of A3A and A3B offers a potent strategy to suppress cancer evolution and prolong efficacy of existing anticancer therapies.[20, 39, 40]

Despite of low sequence identity, cytidine deaminase (CDA) and A3 share a similar overall structural topology and close structural homology for the  $Zn^{2+}$ -containing active site. Since cytosine deamination involves a nucleophilic attack at the C4 position by a  $Zn^{2+}$ -activated water molecule,[41-43] it was proposed to employ transition state analogues and mimetics of the tetrahedral intermediate formed as inhibitors of these enzymes.[44-48] More than 30 compounds have been synthesised in the past and evaluated as inhibitors targeting the active site of CDA.

Tetrahydrouridine (THU),[46, 49] diazepam riboside,[43-45, 50] zebularine[48, 51, 52] and 5-fluorozebularine[48, 53] were among the most potent compounds (**Fig. 1B**). THU quickly converts into inactive  $\beta$ -ribopyranosyl form in solution, but substituting hydrogen atoms with fluorine atoms in the 2'-position leads to cedazuridine, which is stable[54] and used now in clinics as a CDA inhibitor in the liver extending the life-time of co-administered decitabine (5-aza-2'-deoxycytidine).[8]



**Figure 1.** A) Cytosine deamination,  $R = H$  (cytosine) or 1- $\beta$ -deoxyribofuranosyl (dC) and 1- $\beta$ -ribofuranosyl (C) as individual nucleosides or as a part of a polynucleotide chain; B) Previously described CDA inhibitors and a structure of proposed inhibitors **dPC**.

We have recently developed the first rationally designed competitive inhibitors of A3 by incorporating 2-deoxy derivatives of zebularine (2'-deoxyzebularine, **dZ** and 5-fluoro-2'-deoxyzebularine, **FdZ**, **Fig. 1B**)[55] and diazepinone riboside[56] into DNA fragments. We demonstrated that **dZ** does not inhibit A3 enzymes as the free nucleoside but becomes a low  $\mu M$  inhibitor if it is used in single-stranded DNA (ssDNA) instead of the target dC in the recognition motifs of A3A/A3B and A3G.[55] This observation supports a mechanism in which ssDNA delivers **dZ** to the active site for inhibition. By changing the nucleotides around **dZ**, we obtained the first A3B-selective inhibitor.[57] By inserting the fluoro-substituted **FdZ** into ssDNA we observed three times better inhibition of A3Bctd and wild-type A3A in comparison with the **dZ**-containing DNA,[58] which

correlates with the trend reported for CDA inhibitors.[48, 53] We also demonstrated that **dZ**- and **FdZ**-containing DNAs also inhibit full-length wild type A3G with similar efficiency to that for the single catalytically active C-terminal domain.[58, 59] Recently, analysis of crystal structures revealed that both **dZ** and **FdZ** form tetrahedral intermediates after hydrolysis of N3-C4 double bond in the active sites of A3Gctd and A3A.[60, 61] The intermediates formed had the same *R*-stereochemistry at C4 atom of the nucleobase as previously observed for CDA thus confirming the general mechanism of cytosine deamination for A3 and CDA.[52, 60-65]

The fact that **dZ**, **FdZ** and diazepinone deoxynucleoside used in the same DNA sequence had different inhibitory effect on individual A3 under identical conditions means that structure of the cytidine analogue determines the inhibitory potential of the inhibitor-containing oligo.[56, 58] This also supports our strategy of using more potent CDA inhibitors in DNA sequences for development of more powerful A3 inhibitors. The most potent inhibitor of CDA reported so far is phosphapyrimidine riboside (**P**,  $K_i = 0.9$  nM, **Fig. 1B**).[46] However, it is unstable in solution and thus cannot be used as CDA inhibitor and cannot be incorporated into ssDNA and evaluated as A3 inhibitor. Here, we report the synthesis of novel inhibitors of CDA and A3 based on the 1,4-azaphosphinine scaffold, compounds **dPC** (**Fig. 1B**), in which N3 atom present in the nucleobase of **P** is replaced with CH<sub>2</sub>. We assumed that these changes should not significantly affect their inhibitory potential but should increase stability of the target nucleosides in water and allow their chemical incorporation into ssDNA.

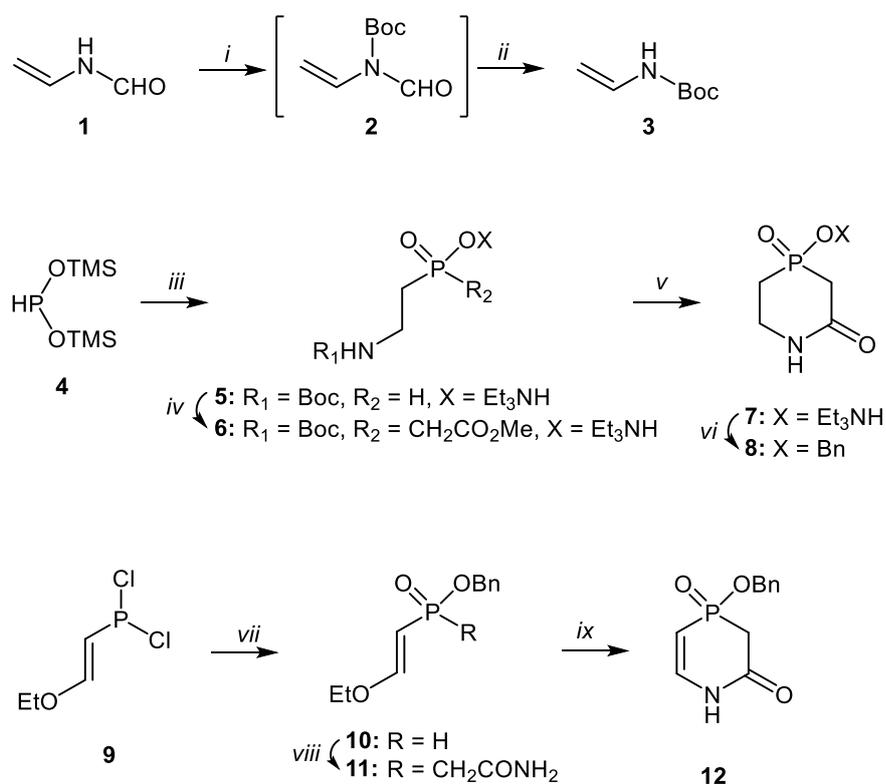
## 2. Results

### 2.1. Synthesis of nucleosides

It is more straightforward to start the synthesis of a modified nucleoside from assembly of a nucleobase that afterwards can be coupled to the sugar using the Hilbert-Johnson reaction or a silyl variation of it as described in the literature.[66] Scheme 1 shows the synthesis of the target nucleobases.

*N*-Boc-vinylamine **3** was synthesised from commercially available *N*-vinylformamide **1** as a stable source of vinylamine by treatment of **1** with Boc<sub>2</sub>O in THF in the presence of a catalytic amount of DMAP followed by cleavage of the formyl moiety in basic conditions. Compound **3** was obtained in nearly 20 g scale in 89% yield after its purification by sublimation *in vacuo*. In the presence of catalytic amount of AIBN compound **3** reacted with bis-(trimethylsilyloxy)phosphine (**4**) that was prepared *in situ*. [67] Treatment of the reaction mixture with MeOH/Et<sub>3</sub>N followed by silica gel

column chromatography led to triethylammonium salt of 2-*N*-*tert*-butoxycarbonyl-aminoethylphosphinic acid **5** in 50 % yield. Alkylation of acid **5** with methyl chloroacetate in the presence of TMSCl and Et<sub>3</sub>N took five days at room temperature and compound **6** as triethylammonium salt was obtained in 84% yield after silica gel purification. Removal of Boc-protection from **6** in the presence of trifluoroacetic acid in DCM at room temperature overnight followed by cyclisation in boiling pyridine/triethylamine led to 4-hydroxy-1,4-azaphosphinan-2,4-dione (**7**) in 84% yield. The free phosphinic acid **7** was further protected with benzyl alcohol by a procedure adopted from reference[68] using TBTU (*O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate) and Et<sub>3</sub>N in refluxing dichloroethane. Compound **8** was obtained in 65% yield after silica gel column chromatography.



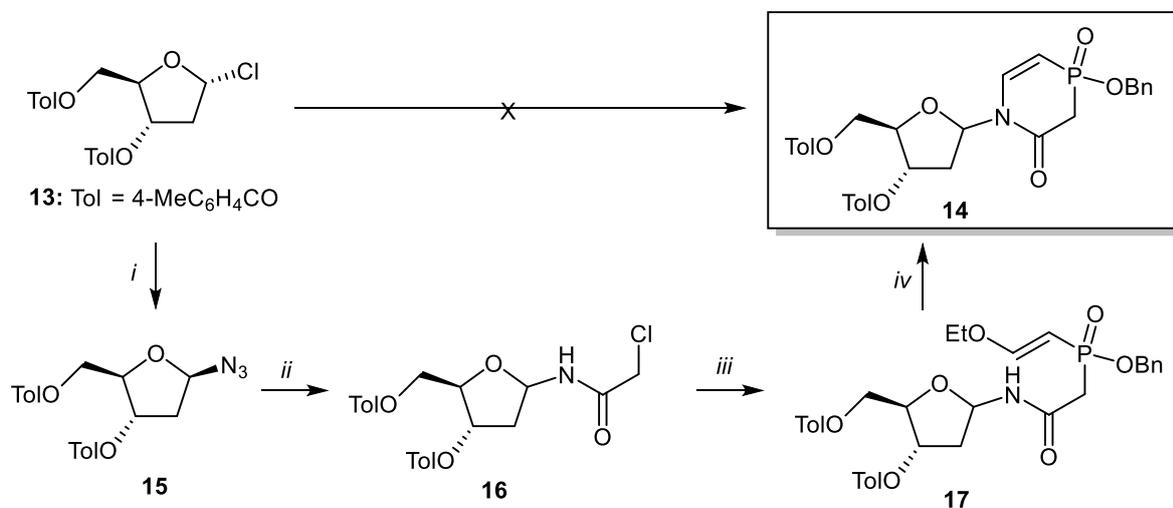
**Scheme 1.** *i*) Boc<sub>2</sub>O, DMAP, THF, r.t., overnight; *ii*) aq. 5M NaOH, r.t., 3h; *iii*) **3**, azobisisobutyronitrile (AIBN), ACN, r.t. then 80-90 °C overnight under Ar followed by Et<sub>3</sub>N/MeOH work-up, r.t.; *iv*) methyl chloroacetate, Et<sub>3</sub>N, TMSCl, CH<sub>2</sub>Cl<sub>2</sub>, r.t., 5d; *v*) trifluoroacetic acid, CH<sub>2</sub>Cl<sub>2</sub>, r.t., overnight then 5h reflux in pyridine/Et<sub>3</sub>N; *vi*) BnOH, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU), Et<sub>3</sub>N, DCE, reflux, 3h; *vii*) BnOH, abs. Et<sub>2</sub>O, pyridine, -78 → 0 °C; *viii*) chloroacetamide, hexamethyldisilazane (HMDS), ACN, 70 °C, 48h under argon; *ix*) trifluoroacetic acid, CH<sub>2</sub>Cl<sub>2</sub>, r.t., overnight.

To synthesise a nucleobase for nucleoside **dPC** we first obtained dichlorophosphane **9** from commercially available PCl<sub>3</sub> and ethylvinyl ether using previously published procedure.[69] Compound **9** reacted with 1 equivalent of benzyl alcohol in absolute Et<sub>2</sub>O and pyridine at -78 °C

followed by quenching of the reaction mixture with H<sub>2</sub>O. This procedure provided phosphinate **10** in quantitative yield with more than 90% purity according to <sup>31</sup>P NMR; compound **10** was used in the next step without further purification. A linear amide **11** was obtained in 47% yield by reacting phosphinate **10** with chloroacetamide in the presence of large excess of HMDS in acetonitrile at 70 °C for two days. A cyclisation of the linear amide **11** was performed in DCM using 10-fold excess of trifluoroacetic acid at room temperature providing 1,4-azaphosphinine **12** in 68% yield.

Various conditions used for coupling of nucleobase **8**, such as silylated (HMDS or BSA) derivative or as a salt obtained after treatment with the base (NaH, *t*-BuOK), with Hoffer's chlorosugar **13** in the presence or absence of Lewis acids (TMSOTf, SnCl<sub>4</sub>) did not result in formation of a reasonable amount of nucleoside **14**. Nucleobase **12** could not be converted to the corresponding silylated derivative by using HMDS, TMSCl or combination of both. Difficulties in the Hilbert-Johnson reaction and the low yield observed for nucleoside **14** prompted us to use an alternative option for the synthesis of the target nucleosides based on the assembly of a nucleobase on the 2-deoxyribofuranos-1-yl scaffold.

Hydrogenation of azide **15**[70] followed by treatment of 2-deoxyribofuranosyl amine formed in situ with chloroacetyl chloride and Et<sub>3</sub>N led to 2-deoxyribofuranosyl 2-chloroacetamide **16** in 38% with β/α ratio about 1:1 (Scheme 2). Phosphinate **10** was then alkylated with compound **16** in the presence of HMDS at elevated temperatures providing a linear nucleoside **17** as a mixture of two anomers which were successfully separated on a silica gel column. Finally, cyclisation of a linear nucleoside was accomplished in the presence of a catalytic amount of a Lewis acid (TMSOTf) in 64% yield. Unfortunately, cyclisation precedes by racemisation and nucleoside **14** with the same α/β ratio of 3:2 forms from either anomerically pure **17** or mixture of its anomers.

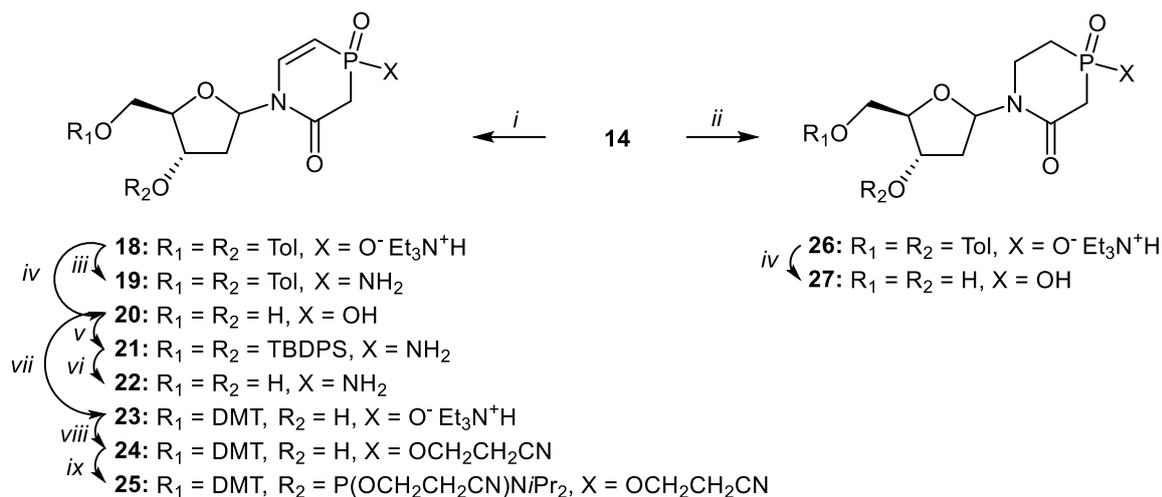


**Scheme 2.** *i*) NaN<sub>3</sub>, n-Bu<sub>4</sub>NHSO<sub>4</sub>, NaHCO<sub>3</sub>/CHCl<sub>3</sub> (1:1), r.t., 20 min; *ii*) a) H<sub>2</sub>, Pd/C, CH<sub>2</sub>Cl<sub>2</sub>, r.t.; 3h; b) chloroacetyl chloride, Et<sub>3</sub>N, 0 °C, overnight; *iii*) **10**, HMDS, DCE, 90 °C, 24h; *iv*) TMSOTf, ACN, 40 °C, 2.5h.

Catalytic hydrogenation is usually used for removal of benzyl protecting group. However, standard hydrogenation conditions using 10% Pd/C led to reduction of the C-C double bond in the nucleobase providing nucleoside **26** (Scheme 3). To circumvent this problem, we used poisoned Pd-catalyst (Lindlar's catalyst, 5%Pd/CaCO<sub>3</sub>/3%Pb) and obtained the desired nucleoside **18**. Individual anomers of nucleosides **18** and **26** were separated on a C18 column in a gradient of CH<sub>3</sub>CN in H<sub>2</sub>O. Removal of toluoyl groups was accomplished in aq. NH<sub>3</sub> providing pure  $\alpha$ - and  $\beta$ -nucleosides **20** and **27** carrying a negative charge on the phosphinic group. These compounds were found to be stable in sodium phosphate buffer at pH 7.0 as no decomposition was observed in NMR samples for several days.

To synthesise a charge-neutral nucleoside **22** (compound **dPC-NH<sub>2</sub>** as shown in **Figure 1**), a phosphinic acid **18** was converted to the phosphinic chloride followed by ammonolysis in CHCl<sub>3</sub> (Scheme 3). The resulting toluoyl-protected compound **19** was obtained in 46% yield but was found to be unstable in basic media required to remove toluoyl groups in the next step. This unfortunate instability of nucleoside **19** in basic media repelled us from the idea to introduce the charge-neutral compound **22** into DNA because basic conditions are used for DNA cleavage and deprotection. To obtain **22** for experiments with human CDA (hCDA), we used deprotected nucleoside **20** as a mixture of anomers and converted it to **22** through a four-step (silylation, treatment with oxalyl chloride, ammonolysis, removal of silyl groups) one-pot synthesis. Purified phosphinamide **22** was obtained as a mixture of anomers with  $\alpha/\beta$  ratio of 2:1 as estimated by <sup>1</sup>H and <sup>13</sup>C NMR spectra.

Deprotected nucleosides **20**, **22** but not **27** exhibited absorbance in UV-region with  $\epsilon_{258} = 4230 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$  and  $\epsilon_{262} = 4730 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ , respectively. This most likely a result of presence of a double bond next to the P=O in nucleosides **20** and **22**, whereas there is no double bond in the nucleobase of compound **27**.



**Scheme 3.** *i*) H<sub>2</sub>, 5%Pd/CaCO<sub>3</sub>/3%Pb, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>, r.t.; 1.5 h; *ii*) H<sub>2</sub>, 10%Pd/C, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>, r.t.; overnight; *iii*) oxalyl chloride, CHCl<sub>3</sub>, r.t., 15 min then satd. NH<sub>3</sub> in CHCl<sub>3</sub>, r.t., 10 min; *iv*) 28% aq. NH<sub>4</sub>OH, r.t., overnight; *v*) *tert*-butyldiphenylsilyl chloride (TBDPSCI), Et<sub>3</sub>N, CHCl<sub>3</sub>, r.t., 2h then oxalyl chloride, CHCl<sub>3</sub>, r.t., 15 min followed by satd. NH<sub>3</sub> in CHCl<sub>3</sub>, r.t., 10 min; *vi*) *n*-Bu<sub>4</sub>NF·3H<sub>2</sub>O, THF, r.t.; 1h; *vii*) 4,4'-dimethoxytrityl chloride (DMTCl), dry pyridine, 0 °C to rt, overnight; *viii*) 3-hydroxypropionitrile, TBTU, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>, reflux, 1h; *ix*) *N,N*-diisopropylamino-2-cyanoethoxychlorophosphine (CEP-Cl), Et<sub>3</sub>N, dry CH<sub>2</sub>Cl<sub>2</sub>, 0 °C to rt, 1h.

For incorporation of nucleoside **20** into DNA, we need to equip it with standard 5'-*O*-DMT and 3'-*O*-*N,N*-diisopropylamino-2-cyanoethoxyphosphanyl groups and also eliminate a negative charge on the nucleobase as it might interfere with automated DNA synthesis. Starting from compound **14** as a mixture of anomers, compound **20** was obtained using above-described steps and after installation of 5'-*O*-DMT group, individual anomers of **23** were isolated on reverse-phase column (C18 media). Then, α- or β-anomer of salt **23** was converted to 2-cyanoethoxy-derivative **24** using 3-hydroxypropionitrile and TBTU which was further transformed into the required phosphoramidite **25** as individual α- and β-anomers which were used in preparation of modified DNA sequences on DNA synthesiser.

## 2.2. Evaluation of 1,4-azaphosphinine derivatives as inhibitors of human CDA, engineered APOBEC3B and wild-type APOBEC3A

### 2.2.1. Evaluation of hCDA inhibition

We monitored human CDA (hCDA)-catalysed deamination of dC at 286 nm [71] and analysed kinetic profiles at various inhibitor concentrations using a global regression analysis of the kinetic data using Lambert's W function. [72] This method provides better estimates for *K<sub>m</sub>* and *V<sub>max</sub>* than non-linear regression analysis of initial rate or any of the known linearised transformations of the Michaelis-

Menten equation, such as Lineweaver-Burk, Hanes-Woolf and the Eadie-Hofstee transformations.[72] Then Michael-Menten constant ( $K_M$ ) for the substrate and the inhibition constant ( $K_i$ ) for each inhibitor were calculated (**Table 1**) assuming competitive nature of inhibitors.

**Table 1.**  $K_m$  of the substrate dC and  $K_i$  of dZ and 1,4-azaphosphinine nucleosides against hCDA.

Inhibitor	pH	$K_m$ of dC ( $\mu\text{M}$ ) <sup>a)</sup>	$K_i$ ( $\mu\text{M}$ )	$K_m/K_i$
<b>dZ</b>	7.4	260 $\pm$ 40	10.7 $\pm$ 0.5	24
$\beta$ -anomer of <b>22</b> <sup>b)</sup>	7.4	240 $\pm$ 150	8.0 $\pm$ 1.9	30
$\beta$ -anomer of <b>20</b>	7.4		No inhibition	
<b>dZ</b>	6.0	270 $\pm$ 60	49 $\pm$ 13	5.5
$\beta$ -anomer of <b>20</b>	6.0		No inhibition	
$\beta$ -anomer of <b>20</b>	4.7	90 $\pm$ 20	560 $\pm$ 100	

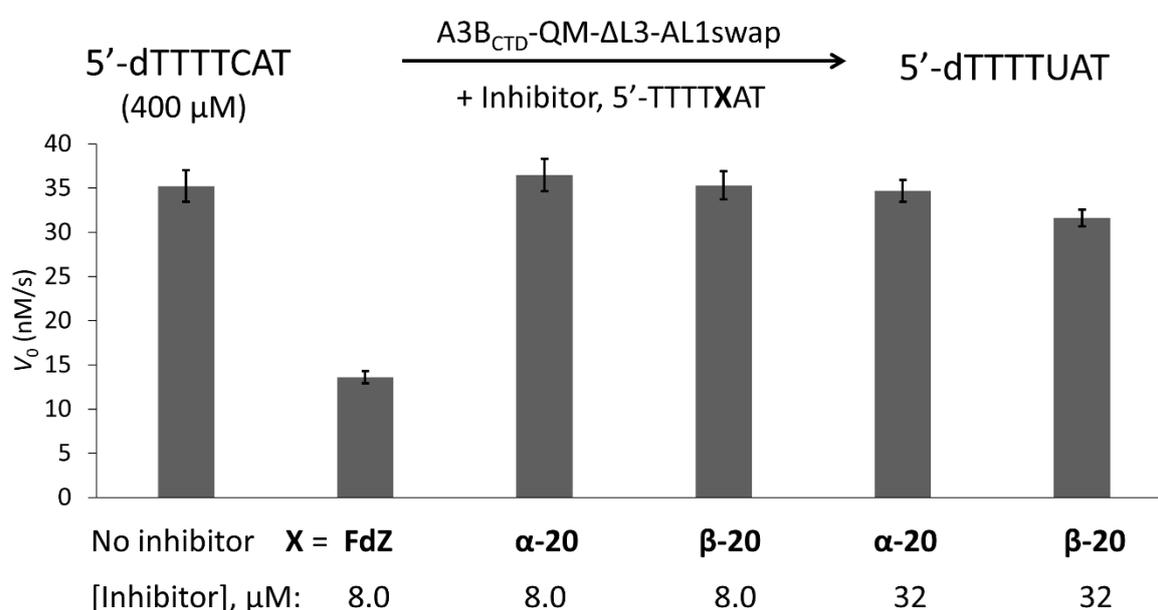
a)  $K_M$  was fitted in each experiment independently (see Supplementary Information). b) Concentration of  $\beta$ -anomers in solutions was determined by NMR (see Supplementary Information) and was used as inhibitor concentration assuming that  $\alpha$ -anomers were not inhibiting hCDA.

Initially, we performed this assay in 50 mM sodium phosphate buffer at pH 7.4 (25 °C) and observed that  $\beta$ -anomer of charge-neutral nucleoside **22** exhibited similar inhibition of hCDA as control **dZ**. Presence of a negative charge in nucleoside **20** led to lack of inhibition at pH 7.4. We assumed that protonation of **20** might result in some inhibition of hCDA. However,  $pK_a$  of **20** was estimated to be  $\leq 1.5$  (see Supplementary Information). This means that pH of the assay should be close to pH = 1.5 to see any meaningful effect of partially protonated compound **20** but hCDA will be denatured at this pH. By lowering pH to 6.0, **dZ** started to lose potency against hCDA (Table 1) which might be a result of protonation of the pyrimidine ring in **dZ**. Some inhibition of hCDA by  $\beta$ -anomer of **20** was observed at pH 4.7 with  $K_i$  value of 560  $\mu\text{M}$ . At this pH less than 1 in 1000 molecules of **20** might be protonated which could mean that protonated phosphinic acid **20** is a potent hCDA inhibitor.

### 2.2.2. Evaluation of inhibitors against engineered A3A-mimic and wild-type A3A by <sup>1</sup>H-NMR assay

We used the <sup>1</sup>H NMR assay to test our short oligodeoxynucleotides (ODNs), linear and hairpins, containing individual  $\alpha$ - and  $\beta$ -anomers of nucleoside **20** as inhibitors of A3. This real-time NMR assay is a direct assay; it uses only A3 enzymes and ODNs in a buffer, unlike many fluorescence-based assays where a secondary enzyme and a fluorescently modified oligo are used.[73] The NMR-

based assay provides the initial velocity of deamination of ssDNA substrates, including the modified ones,[57] in the presence of A3 enzymes. Consequently, the Michaelis–Menten kinetic model can be used to characterise substrates and inhibitors of A3. Both anomers of nucleoside **20** were individually incorporated instead of the target dC in the preferred DNA motif (TCA) of A3A and A3B on linear DNA. A previously described A3 inhibitor 5'-T<sub>4</sub>FdZAT, was used as a control.[55, 57, 58] The engineered A3A-mimic was used in our initial experiments. This is a well-characterised and active derivative of C-terminal domain of A3B (A3B<sub>CTD</sub>), originally called A3B<sub>CTD</sub>-QM-ΔL3-AL1swap[55], in which loop 3 is deleted and loop 1 is replaced with the corresponding loop 1 from A3A. The residual activity of A3A-mimic on the unmodified oligo (5'-T<sub>4</sub>CAT) as a substrate in the presence of a known concentration of inhibitors was measured using the NMR assay (**Fig. 2**).



**Figure 2.** Initial rate of A3A-mimic catalysed deamination of 5'-T<sub>4</sub>CAT in the absence (no inhibitor) and presence of inhibitors at concentrations indicated.

Conditions: 400 μM of 5'-T<sub>4</sub>CAT, 36 μM of seven-membered ring containing oligos and 8 μM of FdZ-containing ODN, 300 nM of A3A-mimic in a 50 mM sodium phosphate buffer (pH 6.0) containing 100 mM NaCl, 2.5 mM β-mercaptoethanol, 50 μM 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid (TSP) and 20% D<sub>2</sub>O at 25 °C. Error bars are estimated standard deviations from triplicate measurements.

The results revealed that both anomers of **20** do not inhibit engineered A3A-mimic even at elevated concentrations in comparison with a control ODN containing **FdZ** at pH 6.0. It is very likely that a negative charge on nucleobase **20** prevents binding to the enzyme.

Recently, it was reported that A3A prefers deaminating cytosines present in the short loops of DNA hairpins rather than linear DNA at pH 7.[74-76] We assumed that placing nucleoside  **$\beta$ -20** in much more preferred substrate would allow us to detect inhibitory potential of the resulting DNA hairpin. Nucleoside  **$\beta$ -20** was introduced instead of the target dC in the DNA hairpin with TTC loop and tested in the  $^1\text{H}$  NMR assay monitoring A3A-catalysed deamination of dC-hairpin (T(GC)<sub>2</sub>TTC(GC)<sub>2</sub>T, bold C is deaminated) at 150 mM salt concentration, pH 7.4. Recently, **FdZ**, 5-methyl-2'-deoxyzebularine and diazepinone deoxyriboside inserted in loops of DNA hairpins have shown selective inhibition of A3A with IC<sub>50</sub> and K<sub>i</sub> in low nM range.[56, 61, 77, 78] Unfortunately, no inhibition of A3A by the DNA hairpin carrying  **$\beta$ -20** was detected at concentrations used (20 and 100  $\mu\text{M}$  of inhibitor DNA, 1 mM dC-hairpin as a substrate, 600 nM wtA3A-His<sub>6</sub> in 50 mM Na<sup>+</sup>/K<sup>+</sup> phosphate buffer, supplemented with 100 mM NaCl, 1 mM TCEP, 100  $\mu\text{M}$  sodium trimethylsilylpropanesulfonate (DSS) and 10% D<sub>2</sub>O; at pH 4.7).

### 3. Discussion and Conclusion

Nucleoside and polynucleotide (A3) cytidine deaminases share a universal mechanism of target nucleobase engagement, deamination, and inhibition.[52, 60-65] We have recently demonstrated first inhibition of A3A-induced mutagenesis in cells using a DNA hairpin carrying **FdZ** instead of the target C in the TTC loop.[61] To further improve potency of DNA-based inhibitors of A3 one can use more potent inhibitors of cytosine deamination than previously characterised **FdZ**, **dZ** and diazepinone. There are two obvious choices based on the literature on CDA inhibitors, THU and phosphapyrimidine nucleoside **P** (**Fig. 1**). However, hemiaminal functionality in the nucleobase and fast transformation into pyranose in THU along with instability of **P** in water prevent their incorporation into DNA fragments using conventional DNA synthesis chemistry. Here, we hypothesised that aqueous stability of **P** can be significantly improved by changing of the N3 atom in the nucleobase to the methylene group providing nucleosides **dPC** with and without a double bond between C5-C6 atoms, respectively (**Fig 1**). Towards this end we developed a synthetic strategy for these nucleosides and identified that assembly of the nucleobase on the sugar was more viable than coupling of the final nucleobase to the Hoffer's sugar. It is interesting that only the charge-neutral phosphinamide **22** inhibited hCDA similarly to **dZ** at pH 7.4 whereas negatively charged phosphinic acid **20** showed some inhibition of hCDA only at pH 4.7. Unfortunately, due to low stability of charge-neutral phosphinamide **22** towards nucleophiles we could not incorporate it into DNA.

Synthesis of DMT-protected phosphoramidite of nucleoside **20** and its incorporation into DNA was more straightforward but no inhibition of A3A was observed for these oligodeoxynucleotides. These results suggest that negatively charged nucleobases cannot be accommodated in the active site of hCDA and A3A and other options need to be considered for the development of new nucleobases mimicking transitions states and an intermediate of cytosine deamination to improve potency of DNA-based A3 inhibitors.

**Author Contributions:** M.V.K., G.B.J., E.H., and V.V.F. designed the research, M.V.K. performed the synthesis of nucleosides and linear DNA, H.M.K. performed the synthesis of hairpin DNA, S.H. and H.M.K. performed enzymatic assays. All authors analysed the data, wrote the article, and have read and agreed to the published version of the manuscript.

### **Acknowledgements**

NMR and mass spectrometry facilities at Massey University and the assistance of Dr Patrick J. B. Edwards and Mr David Lun are gratefully acknowledged. We thank Prof. Reuben S. Harris (HHMI and University of Texas Health, San Antonio, TX, USA) and members of his cancer research program for many helpful discussions. We are grateful for the financial support provided by the Worldwide Cancer Research (grant 16–1197), breast cancer partnership of Health Research Council of New Zealand with Breast Cancer Cure, and Breast Cancer Foundation NZ (grant 20/1355), Palmerston North Medical Research Foundation, Maurice Wilkins Centre for Molecular Biodiscovery, Massey University Research Fund (MURF 2015, 7003 and RM20734), Kiwi Innovation Network (KiwiNet) and Massey Ventures Ltd (MU002391) and the School of Natural Sciences, Massey University.

### **Supplementary information:**

Supplementary experimental details about the synthesis of nucleosides and modified ODNs and enzymatic assays;  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{31}\text{P}$  NMR, IR and HRMS (ESI) spectra of new compounds synthesised, RP-HPLC profiles and HRMS (ESI) spectra of ODNs.

### **Data Availability Statement:**

Original NMR spectra, HPLC profiles and details of enzymatic assays are available from corresponding authors upon request.

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