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RESEARCH ARTICLE

An iron(II)- and α-ketoglutarate-dependent halogenase acts on nucleotide substrates

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Abstract: While halogenated nucleosides are used as common anti-cancer and anti-viral drugs, naturally occurring halogenated nucleosides are rare. Adechlorin (ade) is a 2'-chloro nucleoside natural product first identified from *Actinomadura* sp. ATCC 39365. However, the installation of chlorine in ade biosynthetic pathway still remains elusive. In this article, we report a Fe^{2+}-α-ketoglutarate halogenase AdeV that can install a chlorine atom onto the C-2' position of 2'-deoxyadenosine monophosphate to afford 2'-chloro-2'-deoxyadenosine monophosphate. Furthermore, we found that 2',3'-dideoxyadenosine-5'-monophosphate and 2'-deoxyinosine-5'-monophosphate can also be converted, less efficiently by 20-fold and 2-fold respectively, relative to the conversion of 2'-deoxyadenosine monophosphate. AdeV represents the first example of a Fe^{2+}-α-ketoglutarate-dependent halogenase that converts nucleotides into chlorinated analogues.

Introduction

Organic compounds containing one or more halogen atoms are extremely common and structurally diverse; many of these are highly important medicinal, agricultural and industrial materials[1]. While most are produced synthetically, nature also produces a rich library of halogenated natural products that are widespread in the biosphere, especially from marine territory. Biological halogenations are known to take place by one of three established mechanisms[2] [3]: 1) S_N2 nucleophilic substitution, exemplified by the S-adenosyl methionine (SAM)-dependent fluorinase[4] [5], chlorinase [6] and halide methyltransferase[7]; 2) via an oxidative strategy exemplified by the flavin adenine dinucleotide (FAD)-dependent halogenase [8] [9] [10] and haloperoxidases [11]; and 3) via iron(II)- and α-ketoglutarate (α-KG)-dependent metalloenzymes utilizing halogen radical (X•) equivalents [12] [13] [14]. Intriguingly, iron(II) (Fe^{2+})-α-KG-dependent halogenases usually accept only substrates that have been tethered to an acyl carrier protein (ACP) or a peptidyl carrier protein (PCP), with WelO5[15] [16] and BesD[17] [18] being the very rare exceptions known to act directly on stand-alone substrates.

Halogenated nucleosides represent an important class of synthetic drugs used in anticancer and antiviral treatments, including Cladribine (**1**, anti-leukaemia), Clofarabine (**2**, anti-leukaemia), Fludaradine (**3**, anti-leukaemia), Fluorouracil (**4**, antitumor), Gemcitabine (**5**, anticancer), and Sofosbuvir (**6**, anti-Hepatitis C) (Figure 1A). In contrast, there are relatively few known halogenated nucleoside natural products, with nucleocidin [19] (**7a**, fluorinated at C-4' of ribose), AT-265 (**7b**, dealanylascamycin, chlorinated at C-2 of adenosine) [20] and adechlorin[21] (**8**, chlorinated at C-2' of ribose) (Figure 1B) being rare examples. Adechlorin is the 2'-chloro derivative of pentostatin (**9**), a competitive inhibitor of adenosine deaminase that is approved as a drug for leukaemia treatment [21]. While the biosynthetic gene clusters for both adechlorin and pentostatin have been characterized[22], it remains unclear how pentostatin is chlorinated to give adechlorin. Here, we report the results of both *in vivo* and *in vitro* studies on the halogenase AdeV involved in the enzymatic chlorination step of adechlorin biosynthesis, and the substrate scope of AdeV is also discussed.

Results and Discussion

Identification of an iron(II)- and α-ketoglutarate-dependent halogenase gene *adeV* from the adechlorin gene cluster

Previous work by Gao *et al*. [22a] revealed that the adechlorin biosynthetic gene cluster (*ade* BGC) was composed of 13 genes (*ade*A-*ade*M), but none of these were identified to encode the required chlorinase (Figure 2A). We then obtained *Actinomadura* sp. ATCC 39365 from the American Type Culture Collection (ATCC) and found that it indeed produces both adechlorin (**8**), pentostatin (**9**) and 2'-amino deoxyadenosine (2'-amino dA, **10**, Figure 1C) in our laboratory conditions (Figure 2B). To pursue the chlorinase involved in adechlorin biosynthesis, we re-sequenced the genome of *Actinomadura* and discovered that eight genes downstream of the *ade* BGC lies a gene annotated as an Fe(II)-α-ketoglutarate-dependent oxygenase (for adechlorin gene cluster organization and annotation, see Figure 2A and Supplementary 1), which exhibits only 15% amino acid similarity to the previously identified Fe(II)-α-ketoglutarate-dependent chlorinase WelO5 (for gene and protein sequence, see Supplementary 2; for BLAST analysis, see Supplementary 3). We therefore took an interest in this gene and its encoded protein as a candidate chlorinase enzyme and designated this gene as *ade*V.

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To verify our hypothesis whether *ade*V is indeed the chlorinase involved in the biosynthesis of adechlorin, gene inactivation was carried out (See *Experimental Section*). HPLC analysis of the metabolites from the Δ*ade*V mutant could not detect any adechlorin (**8**) being produced, while the yield of pentostatin (**9**) increased five-fold when compared to that of the wild-type strain (concentration of 6μg/mL vs 1.2μg/mL in fermentation broth) (Figure 2B), thus strongly suggesting that *ade*V is responsible for the chlorination during the adechlorin biosynthesis (Scheme 1). We also observed that the production of 2'-amino deoxyadenosine (2'-amino dA) (**10**) improved sharply (about ten fold) (concentration of 50μg/mL vs 5μg/mL in fermentation broth) in contrast to ATCC 39365 (Figure 2B). It is then believed that **8** and **10** may share the same precursor possibly from the primary metabolite pathway. The semi-purified fractions containing **8**, **9** and **10** are subjected to the analysis of high resolution mass spectrometry (HRMS) with the mass-to-charge ratio (*m/z*) of 303.0837, 269.1244 and 267.1168, respectively, fully consistent with the previous results. [22a].

Biochemical assay of AdeV

To confirm whether AdeV indeed catalyses the chlorination reaction, we set up *in vitro* enzymatic assays. Overexpression of the entire *ade*V gene, which encodes 310 amino acids, in *E. coli* BL21 (DE3) allowed us to isolate and purify the recombinant protein to near homogeneity with the estimated molecular weight of 34 kDa as observed by SDS-PAGE analysis (Supplementary 4).

To determine the substrates of AdeV, we incubated purified AdeV protein with **9**, Fe^{2+} and α-KG, but found that **9** was not converted into **8** (Supplementary 5), demonstrating that **9** is not the direct substrate of AdeV. This result also suggested that **9** is not the last intermediate of the biosynthesis of **8**. We then considered the possibility that the halogenation step occurs in the early stage of the biosynthesis of **8**. To this end, we incubated 2'-deoxyadenosine (2'-dA, **11**) and 2'-deoxyadenosine monophosphate (2'-dAMP, **12**), both of which are earlier proposed intermediates from the pathway with AdeV. We found that, while AdeV showed activity not to 2'-dA (Supplementary 5), a new peak (labelled compound **13**) emerged in the UV trace of our HPLC analyses at a retention time of 20.8 min when 2'-dAMP (**12**, retention time = 12.7 min) was employed as a substrate (Scheme 2A and Figure 2C). HR-MS analysis of this peak is consistent with 2'-chloro-2'-deoxyadenosine monophosphate (2'-Cl-2'-dAMP, **13**) (calculated [$M+H^+$] 366.03988; observed [$M+H^+$] 366.03586), which also exhibited a characteristic isotopic pattern of mono substitution of chlorine (Figure Supplementary 6B). Tandem mass spectrometry (MS_2) analysis further confirmed the fragmentation pattern is also fully consistent with the predicted one (for detailed raw data and analysis, see Supplementary 6).

To further affirm the chemical identity of **13**, we carried out a scale-up biochemical conversion. The reaction was composed of 40 mg of AdeV protein purified from a 2 L culture of *E. coli*, 0.5 mg Fe^{2+}, 1.5 mg α-KG and 20 mg 2'-dAMP (**12**) in 10 ml of reaction buffer (see *Experimental Section*). Compound **13** was separated from the mixture by semi-preparative HPLC, and the fractions containing **13** were combined and lyophilized, affording 1.9 mg of **13** as a dry powder for proton NMR analysis. we also compared the differences of H chemical shifts between compounds 12 and 13 as shown in Figure S7 as compound 12 is a well-characterized molecule. Two H signals at C2' (2.74 ppm (m, 1H, 2'-H), 2.32 ppm (m, 1H, 2'-H)) of 12 disappeared in 13, instead, a new H signal at 4.21 ppm (m, 1H, 2'-H) emerged. Due to electronegative chlorine installed at C2', other Hs in different carbons (C3' and C4') also move to upfield, i.e. 4.43 ppm vs 5.14 ppm (m, 1H, 4'-H), 4.01 ppm vs 4.44 ppm (m, 1H, 3'-H). Taken together with our HR-MS and NMR analyses, **13** was confirmed to be 2'-chloro-2'deoxyadenosine.

To determine the effect of incubation time on the enzymatic reaction, we incubated 2'-dAMP (**12**), Fe^{2+} and α-KG at room temperature and terminated the reaction after 1 h, 2 h, and 3 h by adding an equal volume of methanol to denature the protein. HPLC profile analysis showed that the amount of conversion increased over time (Figure 2C). When α-KG was absent from the reaction system, no product could be detected, while when Fe^{2+} was not supplemented into the full reaction system, the amount of conversion sharply decreased, which could be accounted for by a trace amount of ferron ions from the purified protein. These results is consistent with our hypothesis that AdeV is an iron(II)-α-ketoglutarate-dependent halogenase (Supplementary 12).

Enzyme kinetic parameters of AdeV toward compound 12

A detailed steady-state kinetic analysis was carried out on AdeV toward **12**. A series of biochemical reactions were performed with a range of concentrations of **12** (0.12 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM, 4 mM, 8 mM) in duplicate. These reactions were then performed at 25°C for 1 hr and terminated by heat inactivation at 65°C. HPLC analysis was carried out as described in *Experimental Section*. The concentrations of the products were determined based on the standard curve of what correlated with HPLC peaks. The Michaelis-Menten equation was least-squares fitted to plots of the initial formation rate of products to extract the K_m and k_{cat} parameters (Supplementary 8). Finally, we calculated that apparent kinetic parameters for AdeV are as followed: K_m=2.5*10^{-3} M, k_{cat}=0.6 min^{-1}, k_{cat}/K_m=240 $min^{-1}{\cdot}M^{-1}$. In contrast, another stand-alone halogenase WelO5 halogenates its preferred substrate 12-epi-fischerindole U at 1.8±0.2 min^{-1} [23].

Biochemical assay of AdeV on different substrates

AdeV can convert 2'-dAMP (**12**) rather than 2'-dA (**11**) or pentostatin (**9**) hints that the 5'-phosphate moiety in 2'-deoxyadenosine scaffold is essential for enzyme activity. We also tested whether 2'-deoxyadenosine-5'-diphosphate (2'-dADP, **14**) or 2'-deoxyadenosine-5'-triphosphate (2'-dATP, **15**) (Figure 3A) could be utilized as substrate of AdeV, and HRMS analysis showed that no chlorinated counterpart could be detected, but

new UV peaks with molecular weights of 366.03 and 368.03 emerged, which could be considered halogenation of 2'-dAMP which comes from minor hydrolysis of 2'-dADP (**14**) or 2'-dATP (**15**) (Supplementary 9).

To check whether chemical alteration of the ribose ring of the nucleotide will affect the activity of AdeV, 2', 3'-dideoxyadenosine monophosphate (**16**) (Figure 3A) was used as substrate, and a new UV peak at a retention time of 29.8 min was observed (Scheme 2B and Figure 3B). HRMS analysis of this peak is consistent with 2'-chloro-2',3'-dideoxyadenosine monophosphate (2'-Cl-2',3'-ddAMP, **22**) (calculated [M+H$^+$] 350.0421; observed [M+H$^+$] 350.0418), which also exhibited a characteristic Cl isotopic pattern (Supplementary 11BC). Tandem mass spectrometry (MS_2) analysis further confirmed a typical fragmentation pattern [MS_2 136.0], fully consistent with the predicted fragmentation pathway (Supplementary 11DE).

To check whether different nucleosides will affect the activity of AdeV, four 2'-deoxy nucleoside monophosphates that are common primary metabolites (2'-deoxyguanine-5'-monophosphate (2'-dGMP, **18**), 2'-deoxyuracil-5'-monophosphate (2'-dUMP, **19**), 2'-deoxythymine-5'-monophosphate (2'-dTMP, **20**) and 2'-deoxycytosine-5'-monophosphate (2'-dCMP, **21**)) (Figure 3A) were used as substrates. no new UV peak was detected, indicating that these four compounds are not the substrates of AdeV (Supplementary 10). With 2'-deoxyinosine-5'-monophosphate (2'-dIMP, **17**), in which adenine had been replaced by inosine, a new UV peak at a retention time of 26.2 min was observed (Scheme 2C and Figure 3B). HRMS analysis of this peak is consistent with 2'-chloro-2'-deoxyinosine monophosphate (2'-Cl-2'-dIMP, **23**) (calculated [M+H$^+$] 367.0210; observed [M+H$^+$] 367.0200), which exhibited a characteristic isotopic pattern of monosubstituted chlorine atom (Supplementary 11FG). Tandem mass spectrometry (MS_2) analysis further confirmed a typical fragmentation pattern [MS2 137.0], fully consistent with the predicted fragmentation pathway (Supplementary 11HI).

Since the above three compounds **12**, **16** and **17** can all be converted by AdeV, we then examined the substrate specificity of AdeV toward them. A competition experiment [24] with equimolar **12** and **16** (0.2 mM each) was carried out with AdeV (20 μM). Subsequent HPLC analysis revealed that the conversion of **12** to **13** under these experimental conditions is approximately 20 times higher than the conversion of **16** to **22** (Figure 3C). Additionally, a similar competition experiment with equimolar **12** and **17** (0.5 mM each) was carried out with AdeV (20 μM). The conversion of **12** to **13** is approximately 2 times higher than that of **17** to **23** (Figure 3C). These results clearly demonstrated that **12** is the preferred substrate for AdeV. These data are reasonable since **12** originates from the primary metabolite pathway and may act as a native substrate of AdeV, which is abundant among cell metabolites.

SCHEMES

Site-directed mutagenesis of active amino acids of AdeV

AdeV showed very weak similarity to the previously identified iron-α-KG-dependent halogenase WelO5 (Supplementary 3). Like most other enzymes of this family, AdeV has a conserved H*A***H motif (two histidine 170 and 251), a common feature of iron-α-KG halogenases compared to the conserved ones for iron-α-KG-dependent hydroxylases (H*D/E***H) (Supplementary 2). H251 was believed to coordinate iron(II) to facilitate a halogenation reaction. To this end, we changed this histidine residue into phenylalanine by site-directed mutagenesis [25]. The new protein variant (H251F) was tested for its activity toward compound **12**, and its halogenation activity was completely abolished (Figure 2D). These results are fully consistent with those of the previously reported enzyme WelO5 [15]. Thus, we proposed the mechanism of AdeV and the key reaction intermediates involved in the chlorination of **12** (Supplementary 12).

Phylogenetic tree analysis and cross comparison of KG-dependent enzymes

A phylogenetic tree was also generated between function-known α-KG-dependent oxygenases and halogenases. Interestingly, the reported substrate ACP-bound halogenases are clustered (highlighted in dark green) (Figure 4). However, AdeV (nucleotide halogenases) together with other ACP-independent halogenases (WelO5/AmbO5 [23] and BesD) are of more evolutionary relevance with oxygenases such as GloF (L-proline hydroxylase) and VldW (aminoglycoside hydroxylase). We also carried out a detailed comparison among three halogenases that act on free-standing substrates along with the classical substrate ACP-tethered halogenase SyrB2 (Scheme 3 and Table 1).

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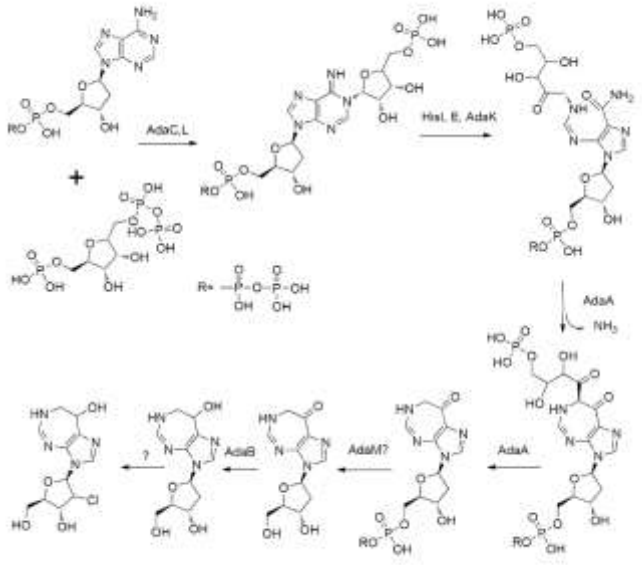

Scheme 1. Schematic representation of the adechlorin biosynthetic pathway by Gao et al. in a previous study.

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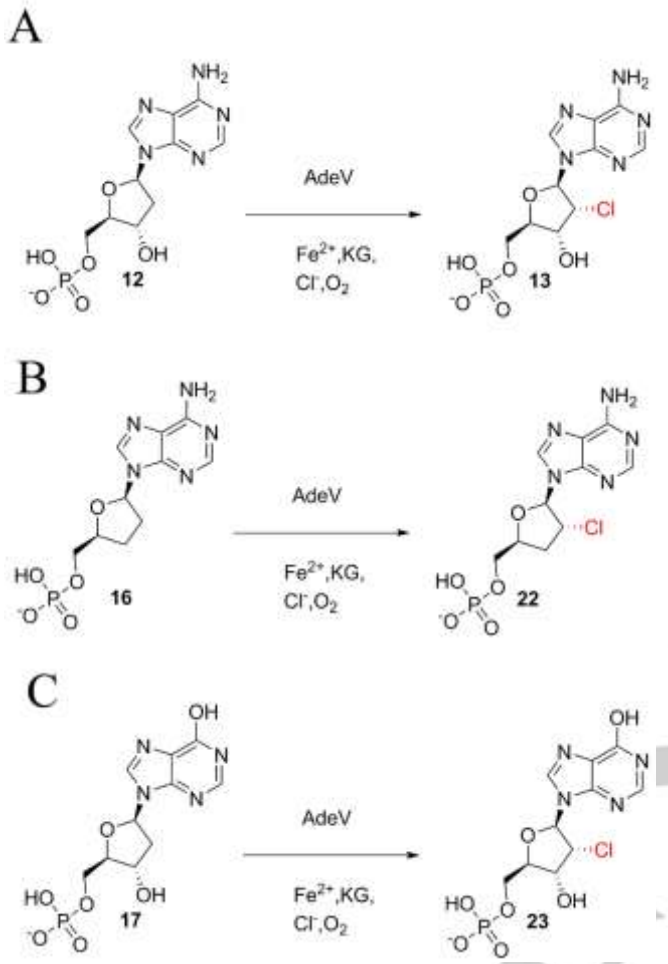
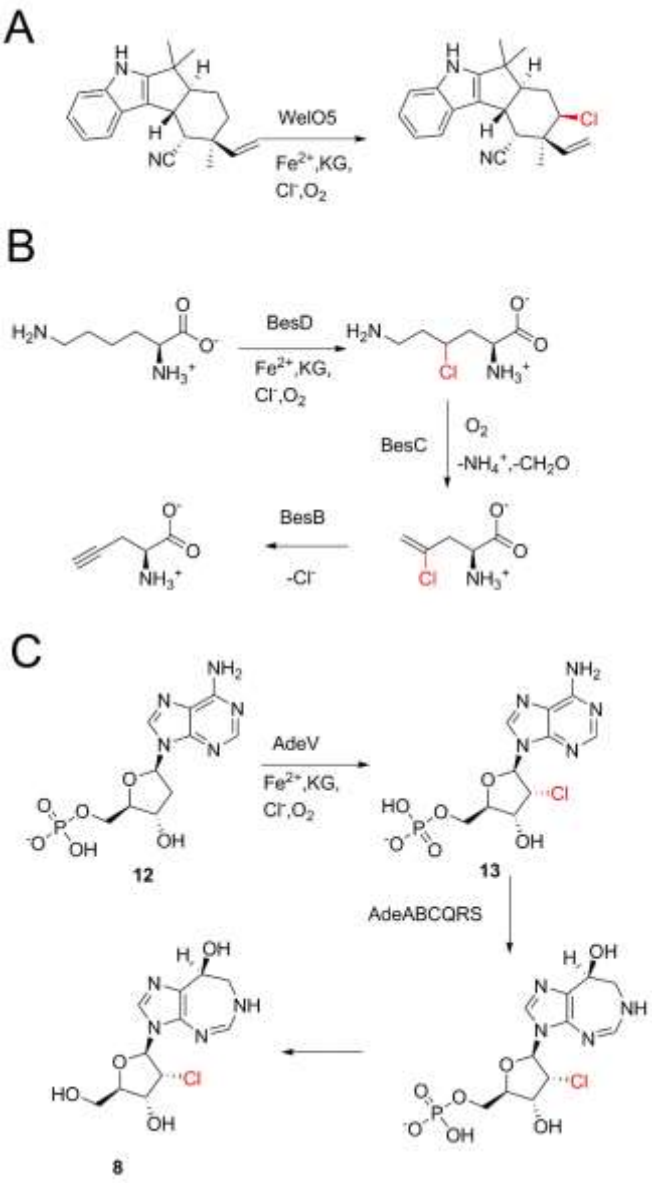


Scheme 2. A) AdeV catalyses the conversion from 2'-dAMP (**12**) to 2'-Cl-2'-dAMP (**13**). B) AdeV catalyses the conversion from 2',3'-ddAMP (**16**) and to 2'-Cl-2',3'-ddAMP (**22**). C) AdeV catalyses the conversion from 2'-dIMP (**17**) to 2'-Cl-2'-dIMP (**23**).



Scheme 3. Schematic representation of three iron-α-ketoglutarate-dependent halogenases that act on ACP (PCP)-free substrates. A) WelO5 acts upon alkaloid as a late-stage modification, B) BesD halogenate lysine to initiate the natural compound ethylyneserine biosynthetic pathway, and C) AdeV halogenate 2'-dAMP (**12**) to initiate adechlorin biosynthesis.

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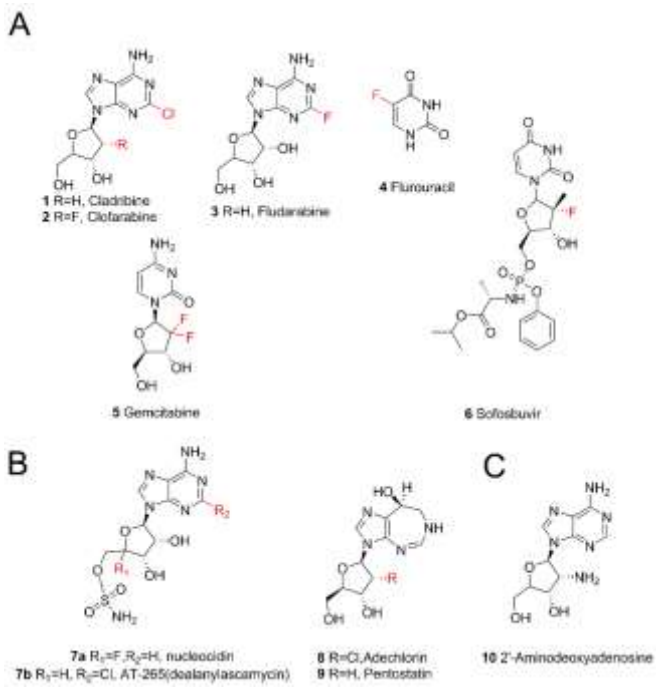

Figure 1. Selected nucleosides: A） Top-selling halogenated nucleosides for clinical use. B) Natural halogenated nucleosides. C) 2'-amino-dA produced by ATCC 39365.

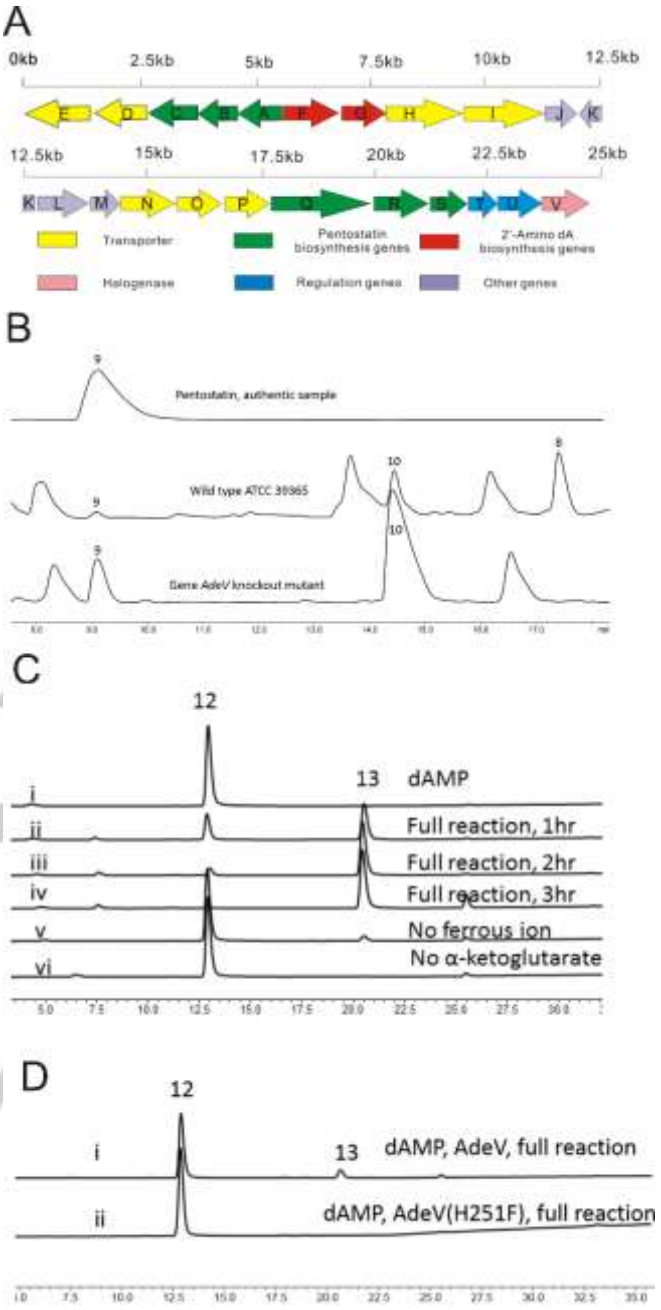

Figure 2. A) Gene organization of the adechlorin biosynthetic pathway. B) HPLC profiles of metabolites. From upper to bottom: pentostatin authentic sample; ATCC 39365; Δ*ade*V. C) HPLC analysis of enzymatic reactions catalysed by AdeV. i) dAMP; ii) full reaction (AdeV, 2'-dAMP, α-KG, Fe^{2+}), 1 hr; iii) full reaction, 2 hr; iv) full reaction, 3 hr; v) reaction in the absence of Fe^{2+}, vi) reaction in the absence of α-KG. D) HPLC analysis of enzymatic reactions catalysed by i) AdeV and ii) AdeV(H251F).

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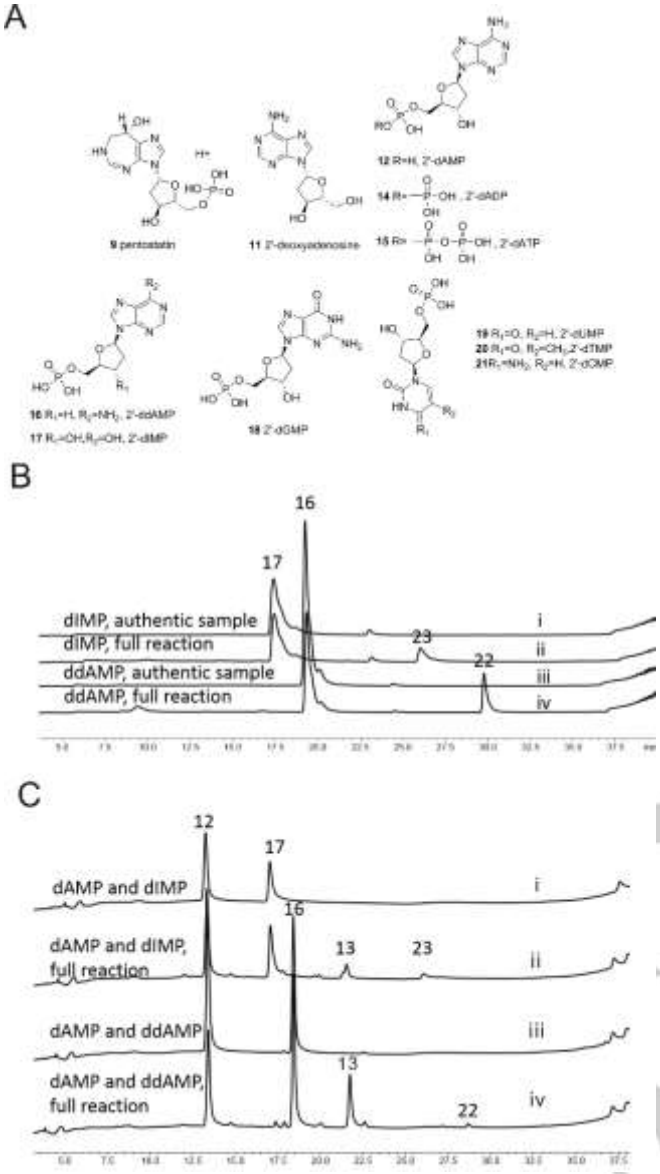

Figure 3. A) 11 substrates used for AdeV enzyme activity test in this study. B) HPLC profiles of enzymatic reactions catalysed by AdeV. i) 2'-dIMP; authentic sample. ii) 2'-dIMP; full reaction (AdeV, 2'-dIMP, α-KG, Fe^{2+}), 1 hr. iii) 2'-ddAMP; authentic sample. iv) 2'-ddAMP; full reaction (AdeV, 2'-ddAMP, α-KG, Fe^{2+}), 1 hr. C) HPLC analysis of enzymatic reaction catalysed by AdeV. i) Equimolar 2'-dIMP and 2'-dAMP. Authentic sample. ii) Equimolar 2'-dIMP and 2'-dAMP; full reaction (AdeV, mixed substrates, α-KG, Fe^{2+}), 1 hr. iii) Equimolar 2'-ddAMP and 2'-dAMP; authentic sample. iv) Equimolar 2'-ddAMP and 2'-dAMP; full reaction (AdeV, mixed substrates, α-KG, Fe^{2+}), 1 hr.

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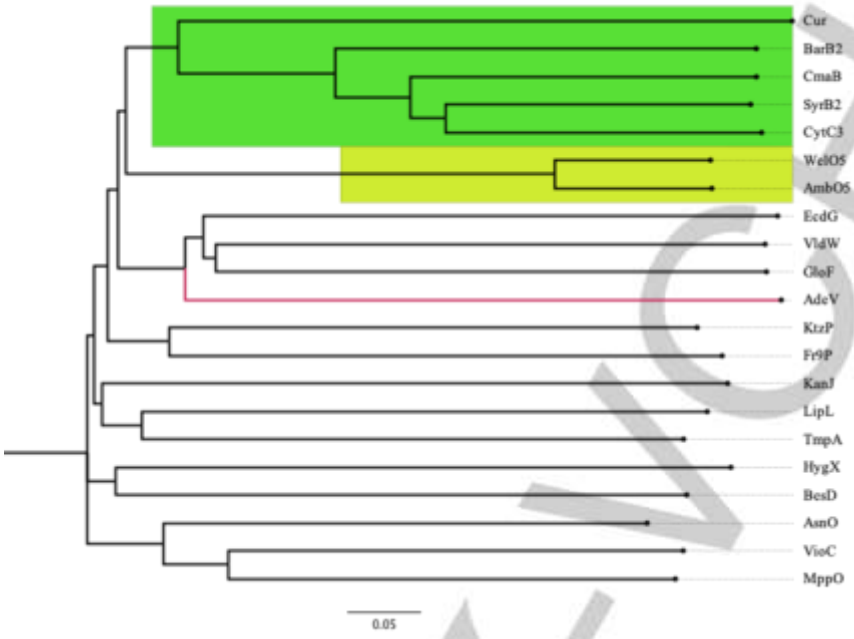

Figure 4. Phylogenetic tree between function-known α-KG-dependent oxygenases and halogenases. Cur, BarB2, CmaB, SyrB2 and CytC3 are CP-tethered halogenases, which are highlighted in dark green. WelO5/AmbO5 (alkaloids highlighted in yellow), BesD and AdeV are CP-independent halogenases, while EcdG , GloF, KtzP, AsnO, VioC, MppO, VldW, Fr9P, LipL, KanJ, HygX and TmpA are oxygenases.

Table 1. Cross-comparison of AdeV with previously characterized iron-α-ketoglutarate-dependent halogenases SyrB2, WelO5 and BesD.

	SyrB2 [12] [13]	WelO5 [15] [16] [23]	BesD [17] [18]	AdeV
Halogenase type	Fe-α-KG	Fe-α-KG	Fe-α-KG	Fe-α-KG
ACP(PCP)-free?	No	Yes	Yes	Yes
Substrates	Amino acid-CP	Alkaloid	Amino acid	Nucleoside
Substrate phosphate-tethered?	No	No	No	Yes
Chlorination timing	After CP-tethered	Late-stage modification	Early stage	Early stage
Cryptic enzyme?	No	No	Yes	No

Conclusion

Iron(II)-α-KG-dependent halogenases act through free radical mechanisms and in all examples, except WelO5 and BesD, act upon substrates tethered with ACP or PCP [15-16] (Scheme 3A). The chlorinase enzyme identified in our report, AdeV, selectively acts upon 2'-dAMP as its native substrate. We also identified that AdeV can accept 2'-ddAMP and 2'-dIMP as substrates but not 2'-dA, showing that the nucleotide form containing a phosphate group at 5' position is crucial for AdeV substrate reactivity. To the best of our knowledge, AdeV represents the first halogenase to selectively act upon nucleotide 5'-phosphates. A phylogenetic tree of all characterized α-KG-dependent oxygenases and halogenases was generated and we could speculate that, using the AdeV sequence as a probe, we could find more halogenated nucleotide natural products through genome mining. The

nucleoside halogenase may also aid us in creating new nucleoside analogues through biological, chemical or biochemical routes [26] [27] [28] [29] for clinical use.

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Keywords: biological halogenation, adechlorin biosynthesis, nucleotide antibiotic, Fe^{2+}-α-ketoglutarate dependent halogenase, C-H functionalization

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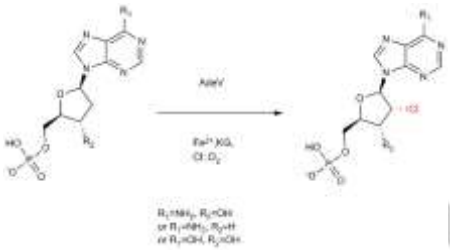

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