Radiolytic activation of a cytarabine prodrug possessing a 2-oxoalkyl group: one-electron reduction and cytotoxicity characteristics†

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An anti-tumour agent of cytarabine (**ara-C**) was conjugated with a 2-oxopropyl group at the N(4) position to obtain a radiation-activated prodrug (**oxo-ara-C**) that targeted hypoxic tumour tissues with selective cytotoxicity. The parent anti-tumour agent, **ara-C**, was confirmed to be released from **oxo-ara-C** *via* one-electron reduction upon hypoxic X-ray treatment. The prodrug **oxo-ara-C** had dramatically reduced cytotoxicity against human lung adenocarcinoma A549 cells relative to **ara-C** because of the effect of 2-oxopropyl substituent. In contrast, X-ray treatment of hypoxic A549 cells containing **oxo-ara-C** enhanced the cytotoxic effect, indicating that toxic **ara-C** was preferentially released in hypoxic cells *via* radiolytic one-electron reduction by hydrated electrons (e_{aq}^-).

Introduction

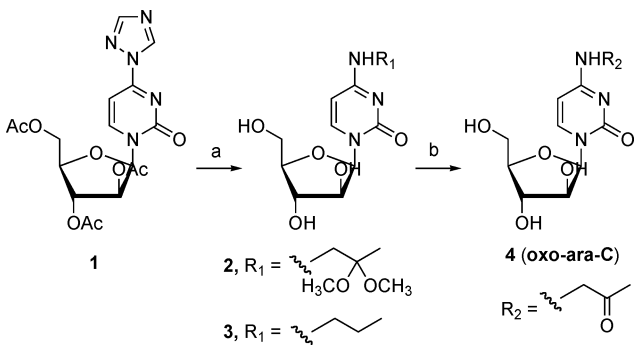
Cytarabine (1-β-D-arabinofuranosyl-cytosine; **ara-C**) synthesized by Walwick and coworkers is one of a family of antimetabolites.[1] This deoxycytidine analog undergoes phosphorylation by intrinsic kinases, and is subsequently incorporated into DNA during chain elongation mediated by polymerase, thereby resulting in the inhibition of DNA replication and elongation.[2] Because of its highly cytotoxic effect, **ara-C** has been widely used as an antineoplastic and antiviral agent. However, **ara-C** has drawbacks in clinical applications: typically, a high dose of **ara-C** causes serious side effects, including encephalopathy and cerebellar dysfunction.[3] Therefore, there is a need for giving a target-specific feature to **ara-C** that would allow it to discriminate between tumour and normal cells. In view of the hypoxic microenvironment specific to tumours (generated from an imbalance between supply and consumption of oxygen[4]), we have attempted to exploit a new class of anti-tumour prodrugs that target the hypoxic microenvironment, resulting in a selective anti-tumour effect.[5]

We have thus far identified a series of 2-oxoalkyl groups that act as effective substituents for conjugation to nucleic acid derivatives, which are removable by X-ray treatment under hypoxic conditions in aqueous solution.[6] An activation mechanism has been proposed by which the 2-oxoalkyl group undergoes one-electron reduction by hydrated electrons (e_{aq}^-)[7] generated *via* radiolysis of water to form the corresponding π* anion radical, followed by thermal activation into the σ* anion radical, which is readily hydrolysed to release the 2-oxoalkyl group.[8] We have applied these characteristics of the 2-oxoalkyl group to develop two prodrugs of anti-tumour agents, 1-(2′-oxopropyl)-5-fluorouracil and 2′-deoxy-5-fluoro-3-(2′-oxoalkyl)uridine, which are activated to release 5-fluorouracil (5-FU) and 5-fluoro-2′-deoxyuridine (5-FdUrd), respectively, upon hypoxic X-ray treatment.[6]

In this study, we designed an **ara-C** derivative possessing a 2-oxopropyl group at the N(4) position (**oxo-ara-C**) to obtain a novel radiation-activated prodrug. We performed radiolytic one-electron reduction of **oxo-ara-C** and evaluated its cytotoxic effect under hypoxic conditions. The 2-oxopropyl group was readily removed from **oxo-ara-C** upon hypoxic X-ray treatment. Analysis of cell viability using an SF assay revealed that **oxo-ara-C** released the toxic parent **ara-C** *via* one-electron reduction when irradiated with X-rays under hypoxic conditions, thereby resulting in cytotoxic effects.

Results and discussion

The synthesis of **oxo-ara-C** is outlined in Scheme 1. The triazole group of **1**[9] was substituted with 1-amino-2,2-dimethoxypropane[10] to give the acetal derivative **2**. Hydrolysis of **2** furnished the desired



Scheme 1 *Reagents and conditions*: (a) 1-amino-2,2-dimethoxypropane, dioxane, 80 °C, 9.5 h (for **2**, 52%); propylamine, ethanol, room temperature, 4.5 h (for **3**, 71%); (b) **2**, oxalic acid, THF, room temperature, 5 h (44%).

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oxo-ara-C. A control compound **3** lacking a carbonyl group at the N(4) position was prepared by the coupling of **1** with propylamine.

We examined the efficiency of radiolytic one-electron reduction of **oxo-ara-C** for controlled release of **ara-C** in an argon-purged aqueous solution containing 2-methyl-2-propanol (10 mM) as the scavenger of oxidizing hydroxyl radicals (•OH).[7,11] Under these radiolysis conditions, reducing hydrated electrons (e_{aq}^-) were generated as the major active species. Fig. 1 shows a representative reaction profile analyzed by HPLC of the radiolytic one-electron reduction of **oxo-ara-C** by the e_{aq}^- under hypoxic conditions. The appearance of a single new peak in Fig. 1 was attributed to the formation of the parent **ara-C**, as confirmed by the overlapped injection of authentic samples in the HPLC analysis. The *G* values[12] were 151 nmol J^{-1} for the decomposition of **oxo-ara-C** and 69 nmol J^{-1} for the formation of the corresponding **ara-C**; thus, 46% of the decomposed **oxo-ara-C** was converted to **ara-C** upon hypoxic X-ray treatment. In contrast, the radiolytic decomposition of **oxo-ara-C** was dramatically suppressed under aerobic conditions; the *G* values were 27 nmol J^{-1} for the decomposition of **oxo-ara-C** and 4 nmol J^{-1} for the formation of the corresponding **ara-C**. These results indicate that **oxo-ara-C** was activated to release **ara-C** in a hypoxia-selective manner, as was observed in the case of the 5-FU and 5-FdUrd prodrugs possessing 2-oxoalkyl groups.[6,11]

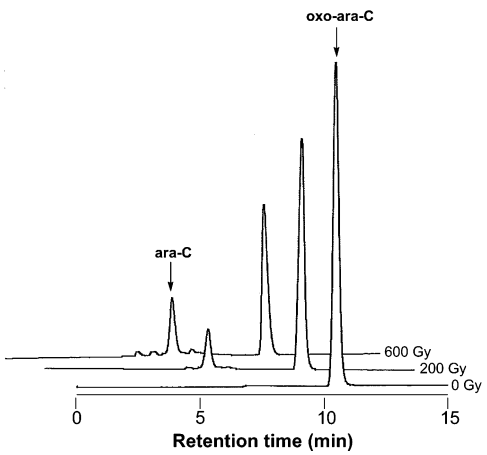

Fig. 1 HPLC profiles for the one-electron reduction of **oxo-ara-C** (100 μM) under hypoxic X-ray treatment (0, 200, and 600 Gy) of an aqueous solution containing 2-methyl-2-propanol (10 mM).

To confirm the mechanistic validity of one-electron reduction of **oxo-ara-C** by e_{aq}^-, we conducted a control experiment in which **oxo-ara-C** was irradiated in an aqueous solution purged with nitrous oxide (N_2O)[13] gas, which efficiently captures the reducing species e_{aq}^- to produce the oxidizing species •OH. As shown in Table 1, the formation of **ara-C** was suppressed to a greater extent in the presence of N_2O relative to the argon-purged aqueous solution, while efficient decomposition of **oxo-ara-C** occurred due to the •OH reaction (see also Fig. S1 and S2†). Thus, it is reasonable to conclude that the reducing species e_{aq}^- is essential for the activation of **oxo-ara-C** and the concomitant release of **ara-C**.

In a separate experiment, we carried out radiolytic reduction of N(4)-substituted **ara-C** lacking a carbonyl group **3** in aqueous solution. As shown in Table 1, hypoxic irradiation of **3** failed to release **ara-C**, indicating that the carbonyl moiety is a key

Table 1 *G* values for the decomposition of **oxo-ara-C** and for the formation of **ara-C** upon X-ray treatment in several gas-saturated solutions

		G value/nmol J^{-1}	
Compound	Saturating gas	Decomposition	Formation
oxo-ara-C	Argon	151	69
oxo-ara-C	Air	27	4
oxo-ara-C	Nitrous oxide	141	5
3	Argon	130	ND[a]

[a] Not detected.

structural unit necessary for the release of **ara-C** *via* one-electron reduction of **oxo-ara-C** by e_{aq}^-.

An understanding of the function of **oxo-ara-C** in living cells is important for its biological application. We therefore assessed the cytotoxic properties of **ara-C** and **oxo-ara-C** toward A549 cells (human lung adenocarcinoma). A549 cells were cultured for 72 h in the presence of various concentrations of **ara-C** or **oxo-ara-C** under aerobic conditions, and were subsequently subjected to a cell viability assay (Fig. 2). The IC_{50} values were 20.7 and 0.38 μM for **oxo-ara-C** and **ara-C**, respectively, indicating that a small modification of **ara-C** by addition of a substituent such as the 2-oxopropyl group can effectively reduce the cytotoxicity of the parent anti-tumour agent. This result may be promising for further investigations to identify whether the dose of **oxo-ara-C** could be more increased without considerable side effects, compared to **ara-C**.

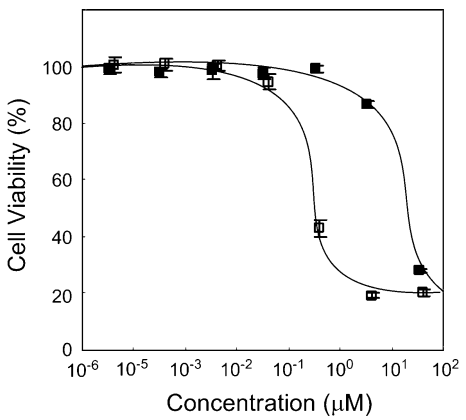

Fig. 2 Cytotoxicity of **ara-C** and **oxo-ara-C** against A549 tumor cells. A549 cells were incubated with indicated concentrations of **ara-C** or **oxo-ara-C** under aerobic conditions for 72 hours. To calculate the cell viability in each conditions, SF counts (OD_{450}) for each drug concentration were compared to those in minimal drug concentrations. □, **ara-C**; ■, **oxo-ara-C**. Results are shown with the mean ±S.D (n = 3).

We subsequently exposed A549 cells to varying doses of X-rays in the presence or absence of **oxo-ara-C** under aerobic or hypoxic conditions, and characterized the hypoxia- and radiation-dependent cytotoxic effects of **oxo-ara-C** (Fig. 3). In accordance with the suppression of cytotoxic effects described above, the cells were viable even in the presence of 3.3 μM **oxo-ara-C** without X-ray treatment (compare entries 1 and 2).[14] Although the A549 cells were practically resistant to radiation under hypoxic conditions (entry 7), **oxo-ara-C** showed the striking property to significantly enhance the radiation sensitivity of A549 cells (entry 8).

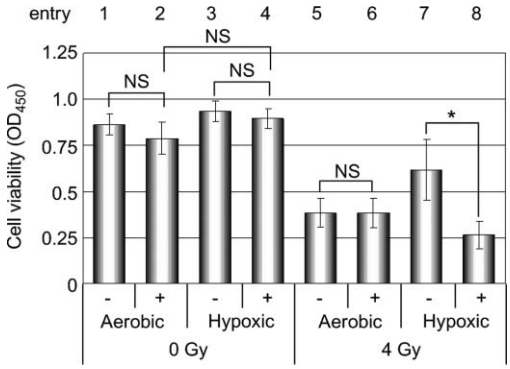

Fig. 3 Radiation-induced cytotoxicity of **oxo-ara-C** against A549 cells under hypoxic conditions. A549 cells were cultured in the presence (+) or absence (–) of 3.3 μM **oxo-ara-C**, and treated with X-rays (4 Gy) under aerobic or hypoxic conditions. Results are shown with the mean ±S.D (n = 3) (* $P < 0.05$; NS: not significant).

In contrast, **oxo-ara-C** had little effect on the radiation sensitivity under aerobic conditions (compare entries 5 and 6). These results strongly suggest that **oxo-ara-C** preferentially releases toxic **ara-C** *via* radiolytic one-electron reduction in hypoxic cells and thereby results in enhanced cytotoxicity, consistent with the chemical reactivity upon irradiation.

We have demonstrated that **oxo-ara-C** is a useful radiation-activated prodrug that shows a cytotoxic effect upon hypoxic tumour cells. Incorporation of a 2-oxoalkyl group into **ara-C** dramatically reduced its cytotoxicity, while the toxic activity was recovered upon hypoxic irradiation. Although the suppression mechanism of the intrinsic cytotoxicity of **oxo-ara-C** in the presence of the 2-oxoalkyl group remains unclear, it can be presumed that the steric hindrance and/or disturbance of hydrogen bonding at the N(4) position may inhibit the recognition of intracellular DNA polymerases, which are key enzymes involved in the cytotoxicity of **ara-C**. Further mechanistic studies using DNA polymerase and triphosphates of **oxo-ara-C** are currently in progress to confirm this hypothesis.

Conclusion

In summary, we designed and synthesized an **ara-C** derivative possessing a 2-oxopropyl group at the N(4) position (**oxo-ara-C**) as a radiation-activated prodrug. **Oxo-ara-C** releases **ara-C** upon reaction with e_{aq}^-, generated during the radiolysis of water under hypoxic conditions. Assessment of the viability of A549 cells revealed that incorporation of the 2-oxoalkyl group into **ara-C** resulted in a dramatic suppression of the cytotoxic effect, while hypoxic X-ray treatment recovered the cytotoxicity *via* removal of the 2-oxoalkyl group to re-generate active **ara-C**. Thus, **ara-C** derivatives possessing 2-oxoalkyl groups are promising candidates as a new class of radiation-activated prodrugs for the treatment of hypoxic tumour tissues.

Experimental

General

All reactions were carried out under a dry nitrogen atmosphere using freshly distilled solvents unless otherwise noted. Reagents were purchased from Aldrich, Wako pure chemical industries and Nacalai tesque, and used as received. Ultrapure water was obtained from YAMATO WR-600A. Precoated TLC (Merck silica gel 60 F_{254}) plates were used for monitoring the reactions. Column chromatography was carried out on Wakogel C-300 (Wako pure chemical industries). ^{1}H NMR spectra were measured with JEOL JMN-AL-300 (300 MHz) or JEOL JMN-AL-400 (400 MHz) spectrophotometers at ambient temperature. ^{13}C NMR spectra were measured with JEOL JMN-AL-300 (75.5 MHz) or JEOL JMN-AL-400 (100 MHz) spectrophotometers at ambient temperature. Coupling constants (J values) are reported in Hertz. The chemical shifts are expressed in ppm downfield from tetramethylsilane, using residual methanol ($\delta = 3.30$ in ^{1}H NMR, $\delta = 49.0$ in ^{13}C NMR) as an internal standard. Multiplicity is designed as singlet (s), doublet (d), triplet (t), doublet-doublet (dd), or multiplet (m). FAB Mass spectra were recorded on JEOL JMS-SX102A spectrometer, using glycerol matrix. A Rigaku RADIOFLEX-350 was used for X-ray treatment. High-performance liquid chromatography (HPLC) was performed with Shimadzu LC-6A system. Sample solutions were injected on a reversed phase column (Inertsil ODS-3, GL Science Inc., Ø 4.6 × 150 nm). The 0.1 M triethylamine (Et_3N) 5 vol% acetonitrile/water solution containing acetic acid, pH 7.0, was delivered as the mobile phase at a flow rate of 0.6 ml/min at 40 °C. The elution peaks were monitored at 260 nm wavelength.

4-(2,2-Dimethyoxypropylamino)-1-(β-D-arabinofuranosyl)pyrimidine-2-(1*H*)-one (2). 1-Amino-2,2-dimethoxypropane[10] (796 mg, 6.68 mmol) was added to a solution of **1**[9] (4-(1,2,4-Triazol-yl)-1-(β-D-2,3,5-tri-*O*-acetylarabinofuranosyl)pyrimidine-2(1*H*)-one, 167 mg, 0.40 mmol) in dioxane (1.2 ml) and stirred for 9.5 h at 80 °C. The solvent was removed under reduced pressure. The crude product was purified by column chromatography (SiO_2 6% methanol-chloroform) to give **2** (71 mg, 52%) as a white solid; mp 171–173 °C; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.75 (d, 1H, $J = 7.6$ Hz), 6.16 (d, 1H, $J = 4.0$ Hz), 5.93 (d, 1H, $J = 7.6$ Hz), 4.16 (dd, 1H, $J = 2.4$, 3.6 Hz), 4.04 (dd, 1H, $J = 2.4$, 2.4 Hz), 3.92 (m, 1H), 3.79 (m, 2H), 3.58 (s, 2H), 3.23 (s, 6H), 1.28 (s, 3H); ^{13}C NMR (CD_3OD, 75.5 MHz) δ 165.9, 158.7, 143.0, 101.8, 95.7, 88.3, 86.4, 78.2, 76.8, 62.8, 48.8, 48.8, 45.3, 20.5; FABMS (glycerol) m/z 346 [(M + H)$^+$]; HRMS calcd. for $C_{14}H_{24}N_3O_7$ [(M + H)$^+$] 346.1614, found 346.1611.

4-Propylamino-1-(β-D-arabinofuranosyl)pyrimidine-2-(1*H*)-one (3). Propylamine (0.23 ml, 2.80 mmol) was added to a solution of **1**[9] (4-(1,2,4-Triazol-yl)-1-(β-D-2,3,5-tri-*O*-acetylarabinofuranosyl)pyrimidine-2(1*H*)-one, 43.5 mg, 0.103 mmol) in ethanol (0.3 ml) at room temperature. After 4.5 h the product was concentrated under reduced pressure. The crude product was purified by column chromatography (SiO_2 1% Et_3N, 9% methanol-chloroform) to give **3** (21 mg, 71%) as a white solid; mp 194–195 °C; ^{1}H NMR (CD_3OD, 300 MHz) δ 7.72 (d, 1H, $J = 7.5$ Hz), 6.17 (d, 1H, $J = 3.9$ Hz), 5.79 (d, 1H, $J = 7.8$ Hz), 4.16 (m, 1H), 4.07 (m, 1H), 3.93 (m, 1H), 3.80 (m, 2H), 3.32 (m, 2H), 1.60 (m, 2H, $J = 7.2$ Hz), 0.96 (t, 3H, $J = 7.2$ Hz); ^{13}C NMR (CD_3OD, 100 MHz) δ 165.4, 158.6, 142.5, 95.6, 88.2, 86.3, 78.2, 76.8, 62.8, 43.4, 23.3, 11.7; FABMS (glycerol) m/z 286 [(M + H)$^+$]; HRMS calcd. for $C_{12}H_{20}N_3O_5$ [(M + H)$^+$] 286.1403, found 286.1394.

4-(2-Oxopropylamino)-1-(β-D-arabinofuranosyl)pyrimidine-2-(1*H*)-one (4) (oxo-ara-C). **2** (20 mg 0.058 mmol) was hydrolyzed by treatment with aqueous 1 M oxalic acid in THF (0.3 ml) at room temperature for 5 h. The solvent was evaporated under reduced pressure and the crude product was purified by column chromatography (SiO_2 1% Et_3N, 12% methanol-chloroform) to give **oxo-ara-C** (7.7 mg, 44%) as a white solid; mp 180–182 °C; ^{1}H NMR (CD_3OD, 300 MHz) δ 7.79 (d, 1H, $J = 7.5$ Hz), 6.15 (d, 1H, $J = 3.8$ Hz), 5.93 (d, 1H, $J = 7.5$ Hz), 4.26 (s, 2H), 4.16 (dd, 1H, $J = 2.4$, 3.6 Hz), 4.04 (dd, 1H, $J = 2.8$, 2.6 Hz), 3.92 (m, 1H), 3.79 (m, 2H), 2.18 (s, 3H); ^{13}C NMR (CD_3OD, 75.5 MHz) δ 206.0, 165.6, 158.5, 143.4, 95.4, 88.4, 86.6, 78.2, 76.8, 62.8, 51.3, 27.1; FABMS (glycerol) m/z 300 [(M + H)$^+$]; HRMS calcd. for $C_{12}H_{18}N_3O_6$ [(M + H)$^+$] 300.1196, found 300.1189.

Radiolytic reduction

Aqueous solutions of **oxo-ara-C** and **3** (100 μM), containing 2-methyl-2-propanol (10 mM), were purged with argon or nitrous oxide for 15 min and then irradiated in a sealed glass ampoule at ambient temperature with an X-ray source (4.0 Gy min^{-1}). After X-ray treatment, the solution was immediately subjected to HPLC analysis.

Assessment of cytotoxicity toward A549 cells

A549 cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were seeded into 96-well plates (2000 cells/well) and cultured at 37 °C in a well-humidified incubator with 5% CO_2 and 95% air (aerobic condition) for 24 hours. The cells were then incubated with the various concentrations of **ara-C** or **oxo-ara-C** under aerobic conditions for 72 hours, and added with 11 μL of Cell Count Reagent SF[15] (Nacalai, Japan). The plates were further incubated at 37 °C for 2 hours and the cell viability assay was performed using Microplate Reader (BIO-RAD).

Radiation-induced cytotoxicity of oxo-ara-C

A549 cells were seeded into 96-well plates (2000 cells/well) and incubated at 37 °C for 24 hours under aerobic or hypoxic conditions. For the hypoxic treatment (<0.02% of oxygen), the cells were treated in a hypoxic chamber, BACTRON-II(Sheldon Manufacturing Inc., Cornelius, OR, USA). The plates kept under aerobic or hypoxic conditions using Anaeron Pack System (Mitsubishi Gas Chemical Company Inc., Japan) were treated with X-rays at a dose of 4 Gy and incubated for 72 hours under aerobic conditions. After adding 12 μL of Cell Count Reagent SF solution (Nacalai, Japan) to each well, and the cell viability assay was performed as described above.

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7 Radiolysis of diluted aqueous solution at around pH 7.0 produces primary water radicals such as oxidizing hydroxyl radicals (•OH), reducing hydrated electrons (e_{aq}^-) and reducing hydrogen atoms (•H) with G values of G(•OH) = 280 nmol J^{-1}, $G(e_{aq}^-)$ = 280 nmol J^{-1} and G(•H) = 60 nmol J^{-1}.

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12 The number of molecules produced or changed per joule of radiation energy absorbed by the reaction system.

13 Dissolved N_2O exclusively scavenges reducing hydrated electrons to produce hydroxyl radicals, as follows: $e_{aq}^- + N_2O \rightarrow$ •OH + OH^- + N_2 ($k = 9.1 \times 10^9$ dm^3 mol^{-1} s^{-1}).

14 Without X-ray treatment, the effect of oxygen on the cell viability is negligible, irrespective of the presence or the absence of **oxo-ara-C** (Fig. 3, entry 1–4).

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