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Design, Synthesis, and Biological Activity of Anti-angiogenic Hypoxic Cell Radiosensitizer Haloacetylcarbamoyl-2-nitroimidazoles

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Abstract—We designed, synthesized, and evaluated haloacetylcarbamoyl-2-nitroimidazoles, including chloro (KIN-1800, TX-1835, and TX-1836) and bromo derivatives (TX-1844, TX-1845, and TX-1846), as potential hypoxic cell radiosensitizers with anti-angiogenic activities. To establish biological function owing to the haloacetylcarbamoyl group in the side-chain, we compared their in vitro radiosensitizing activities with those of their parent 2-nitroimidazoles without haloacetylcarbamoyl groups: misonidazole (MISO), TX-1831, and TX-1832, respectively. Both *tert*-butoxy substituted derivatives, TX-1835 and TX-1845, were more potent radiosensitizers than TX-1831. The *p-tert*-butylphenoxy-substituted derivatives, TX-1836 and TX-1846, and the methoxy-substituted derivatives, KIN-1800 and TX-1844, were stronger radiosensitizers than TX-1832 and MISO. We examined the anti-angiogenic activities of these 2-nitroimidazole derivatives containing haloacetylcarbamoyl group by the rat lung endothelial (RLE) cell proliferation assay and chick embryo chorioallantoic membrane (chick CAM) angiogenesis assay and showed that haloacetylcarbamoyl-2-nitroimidazoles were more potent angiogenic inhibitors than the corresponding desacetylcarbamoyl-2-nitroimidazoles. The in vivo chick CAM angiogenesis assay showed that the strong bromoacetylcarbamoyl-2-nitroimidazole radiosensitizers, such as TX-1845 and TX-1846, were the strongest angiogenic inhibitors among them. We concluded that the bromoacetylcarbamoyl-2-nitroimidazole radiosensitizers, such as TX-1845 and TX-1846, are promising as anti-angiogenic hypoxic cell radiosensitizers.

Introduction

Most hypoxic cell radiosensitizers were designed to improve the radiosensitizing activity and neurotoxicity of misonidazole (MISO), primarily by modifing the side-chains.[1–3] We reported that KIH-802: potassium 2-nitroimidazole-1-acetohydroxamate, characterized by a biologically active moiety, hydroxamic acid (–CO–NH–OH) unit, included in its side-chain, shows great promise as an effective radiosensitizer.[4–7] One of the best hydroxamate radiosensitizers was KIN-804,[8] which was highly active in vivo.[9] One of the higher homologues of the hydroxamate radiosensitizers, KIN-841, methyl-2-nitroimidazole-1-butanoyl hydroxamate, inhibited angiogenesis so much that it was evaluated in clinical trials.[10]

The fact that the efficiency of hypoxic cell radiosensitizers seems to depend on the energy status of the tumor cells, indicates that the modification of tumor bioenergetics assists in the development of selective and potent radiosensitizers. For example, 2-arylidene-4-cyclopentene-1,3-diones having antitumor activities[11] were designed as non-nitro hypoxic cell radiosensitizers and hypoxic cell cytotoxins.[12] Among them, the best candidate was KIH-201, a 2-vanillidene-cyclopentene-1,3-dione with significant antitumor activity,[11] that also potently inhibited the P_i-transporter in mitochondria.[13] We have developed new antitumor mitochondrial cytotoxin *N*-thiadiazolylanilines.[14]

Clinical and experimental studies suggest that angiogenesis is a prerequisite for solid tumor growth.[15] Inefficient vascular supply and the resultant hypoxia in tumor tissue often leads to neovascularization to satisfy the needs of surviving tumor tissues. The link between the tumor tissue hypoxia and angiogenesis was not well established. Shweiki et al.[16] reported that hypoxia specially induced the production of vascular endothelial growth factor (VEGF), which mediates hypoxia-initiated angiogenesis. The advantage of the combination of the radiosensitizing activity and anti-angiogenic activity is a synergistic interaction between the hypoxic cell radiosensitizers as hypoxic cytotoxic/radiosensitiz-

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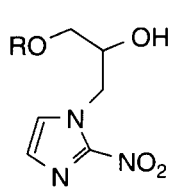
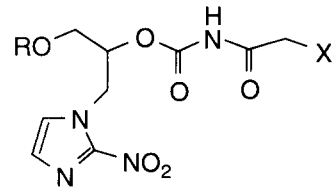


R = Me	MISO
R = *tert*-Bu	TX-1831
R = *p-tert*-Bu-Ph	TX-1832



R = Me, X = Cl	KIN-1800
R = *tert*-Bu, X = Cl	TX-1835
R = *p-tert*-Bu-Ph, X = Cl	TX-1836
R = Me, X = Br	TX-1844
R = *tert*-Bu, X = Br	TX-1845
R = *p-tert*-Bu-Ph, X = Br	TX-1846

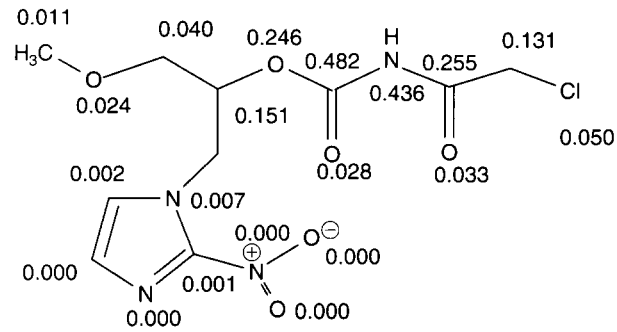
Figure 1. Chemical structure of designed anti-angiogenic hypoxic cell radiosensitizers. Haloacetylcarbamoyl-2-nitroimidazoles and their parent compounds (deshaloacetylcarbamoyl-2-nitroimidazoles).

ing drugs and subsquent treatment with anti-angiogenic agents that destroyed hypoxia-initiated neovasculature.[17] In this paper, we describe that our newly designed and synthesized hypoxic cell radiosensitizers, haloacetylcarbamoyl-2-nitroimidazoles, TX-1835[18,19] and TX-1845[19,20] inhibit angiogenesis, which is related to metastasis induced by proteases in tumor cells.[21,22]

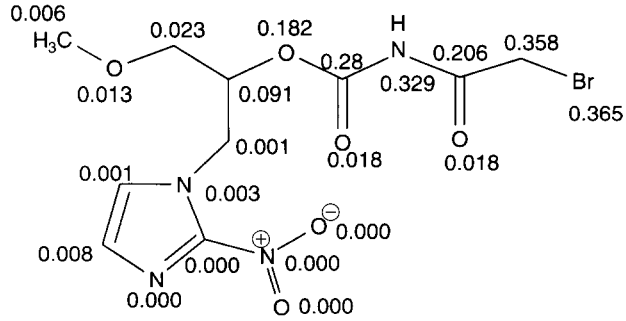
Results

Design and synthesis

To develop new hypoxic cell radiosensitizers as chemical modifiers for use in cancer therapy, we designed and synthesized several haloacetylcarbamoyl-2-nitroimidazoles (Fig. 1). These consisted of chloro (KIN-1800, TX-1835, and TX-1836) and bromo derivatives (TX-1844, TX-1845, and TX-1846). KIN-1800 and TX-1844, TX-1835 and TX-1845, and TX-1836 and TX-1846, are methoxy-substituted, *tert*-butoxy-substituted, *p-tert*-butylphenoxy-substituted haloacetylcarbamoyl-2-nitroimidazoles, respectively. To predict their electron affinities (EAs) as a measure of hypoxic cell radiosensitizing potency,[23] we calculated the orbital energies of the lowest unoccupied molecular orbital (LUMO) [EA = −(LUMO)] of the simple haloacetylcarbamoyl-2-nitroimidazoles, KIN-1800 and TX-1844, with a MOPAC V. 7.00. The EAs of KIN-1800 and TX-1844 were 0.91885 and 1.09502 eV, respectively. Furthermore, we calculated the LUMO coefficients of the haloacetylcarbamoyl-2-nitroimidazoles, KIN-1800 and TX-1844, with the MOPAC V.7.00 to predict which sites are prefered by biological nucleophiles during protease action, as shown in Figure 2. Thus, the difference between the LUMO coefficients of bromo- and chloroacetylcarbamoyl-2-nitroimidazoles predicted that the nucleophiles preferably attack the methylene moiety (the LUMO coefficients = 0.358) of the bromoacetylcarbamoyl group in bromoacetylcarbamoyl-2-nitroimidazoles, TX-1844 and the ester-carbonyl moiety (the LUMO coefficients = 0.482) of the chloroacetylcarbamoyl group in the chloroacetylcarbamoyl-2-nitroimidazole, KIN-1800. The haloacetylcarbamoyl-2-nitroimidazoles were synthesized for a short period (30 min) at high yield (ca. 70–99%) by treating the corresponding 2-nitroimidazole-propanol derivatives such as MISO, TX-1831, and TX-1832, with highly purified, fresh chloroacetyl isocyanate and bromoacetyl

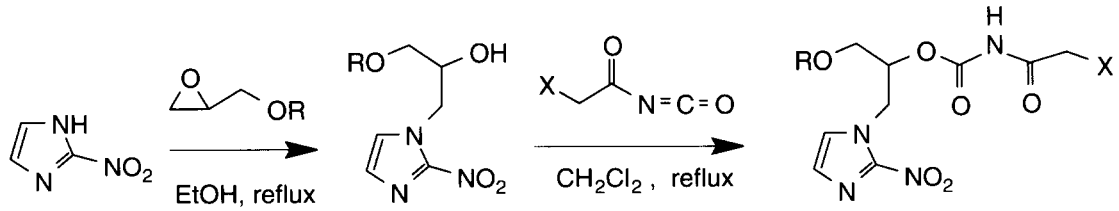


Chloroacetylcarbamoyl-2-nitroimidazole KIN-1800



Bromoacetylcarbamoyl-2-nitroimidazole TX-1844

Figure 2. The LUMO coefficients of haloacetylcarbamoyl-2-nitroimidazoles: chloroacetylcarbamoyl-2-nitroimdiazole KIN-1800 and bromoacetylcarbamoyl-2-nitroimdiazole TX-1844.



R = Me, *tert*-Bu, *p*-*tert*-Bu-Ph
X = Cl, Br

Scheme 1. Synthesis of haloacetylcarbamoyl-2-nitroimidazole radiosensitizers.

isocyanate, as shown in Scheme 1. The 2-nitroimidazole-pronanol derivatives, TX-1831 and TX-1832, were also synthesized from 2-nitroimidazole with the corresponding glycidyl ether in good yield.

Anti-angiogenic activity and its related biological activities such as protease and ATP-synthesis inhibitions

First, we examined in vitro anti-angiogenic activities of haloacetylcarbamoyl-2-nitroimidazole radiosensitizers by the rat lung endothelial (RLE) cell proliferation assay (anti-proliferation acitivity: IC_{50}) as shown in Table 1. The growth of vascular endothelial cells is an important event during angiogenesis. The anti-proliferation activities (IC_{50}) tended to parallel their radiosensitizing effects [$C_{1.6}$: drug concentration required to achieve an ER (enhancement ratio) of 1.6, see Table 5]. The most potent radiosensitizer, TX1846, was also the best proliferation inhibitor with an IC_{50} value of 1.0 among the compounds tested. Table 2 shows the in vivo anti-angiogenic activities (inhibition percent) of the haloacetylcarbamoyl-2-nitroimidazole radiosensitizers by the chick CAM angiogenesis assay. All the haloacetylcarbamoyl-2-nitroimidazoles potently inhibited angiogenesis, whereas none of their parent compounds did so. Bromoacetylcarbamoyl-2-nitroimidazoles were more potent angiogenesis inhibitors than the corresponding chloroacetylcarbamoyl derivatives. Bromoacetylcarbamoyl-2-nitroimidazoles, TX-1845 and TX-1846, were the most potent angiogenesis inhibitors.

Table 1. Inhibition of haloacetylcarbamoyl-2-nitroimidazole radiosensitizers and their deschloroacetylcarbamoyl radiosensitizers on RLE cell proliferation

Radiosensitizer	IC_{50} (μM)
KIN-1800	170
TX-1835	125
TX-1836	6.7
TX-1844	9.0
TX-1845	6.0
TX-1846	1.0
MISO	N.I.
TX-1831	N.I.
TX-1832	30

N.I.: no inhibition.

We evaluated the anti-angiogenesis-related biological activities of the haloacetylcarbamoyl group. First, for the protease-inhibitory activity of chloroacetylcarbamoyl group, we tested the ability of the chloroacetylcarbamoyl-2-nitroimidazoles to porcine pancreatic (PP) elastase as a simple model of serine protease-related angiogenesis. The inhibitory activities of TX-1835 and TX-1836 to PP elastase were determined in terms of K_i values, which were 7.5 and 1.1 mM, respectively, as given in Table 3. The activities were comparable with those of the known PP elastase inhibitor Ac-Ala-Ala-CH_2Cl (K_i, 2.0 mM). KIN-1800, however, was not inhibitory even at 50 mM. The potencies of these chloroacetylcarbamoyl-2-nitroimdiazoles to PP elastase can thus be graded as follows: TX-1836 > TX-1835 > KIN-1800. The order could be correlated with both lipophilicity (log *P*) [log *P*: TX-1836, 2.879; TX-1835, 1.808; KIN-1800, −0.445] and bulkiness of the side-chain of compounds [*p*-*tert*-Bu-Ph (TX-1836) > *tert*-Bu (TX-1835) > Me (KIN-1800)].[24] Their parent compounds, MISO, TX-1831, and TX-1832, did not inhibit PP elastase, even at a concentration of 50 mM. To determine the bioenergetics-modifying activity of the choloroacetylcarbamoyl group, we tested the inhibitory activities of choloroacetylcarbamoyl-2-nitroimidazoles on state 3 respiration of rat liver mitochondria (RLM). All these compounds inhibited state 3 respiration as shown in Table 4. This was caused by the inhibition of ATP synthesis, verified by monitoring the effects on the pH of the reaction mixture. Both TX-1835 and TX-1836 inhibited ATP synthesis more than KIN-1800, MISO, and TX-1831, according to their effects on the IC_{50} values: 0.2 mM for TX-1835 and TX-1836, 2.4 mM for KIN-1800. However, TX-1836 completely inhibited ATP synthesis at 0.4 mM (IC_{100} value), so TX-1836 was the most powerful ATP-synthesis inhibitor among them. TX-1835 and KIN-1800 were rather moderate ATP-synthesis inhibitors, as seen from the IC_{100} values at 1.5 and 5.0 mM.

Radiosensitizing activity

The in vitro radiosensitizing activities ($C_{1.6}$) of the haloacetylcarbamoyl-2-nitroimidazole radiosensitizers, KIN-1800, TX-1835, TX-1836, TX-1844, TX-1845, and TX-1846, are shown in Table 5. All the haloacetylcarbamoyl-2-nitroimidazoles showed higher $C_{1.6}$ values

Table 2. Inhibition of haloacetylcarbamoyl-2-nitroimidazoles and their deschloroacetylcarbamoyl derivativer on angiogenesis

Radiosensitizer	Dose/μg/disk	Embryos evaluated (positive/total)	Inhibition/%
KIN-1800	100	10/11	90
	10	2/11	20
TX-1835	100	8/11	77
	10	6/16	38
TX-1836	100	7/10	70
	10	6/12	50
TX-1844	100	4/5	80
	10	2/4	50
TX-1845	100	5/5	100
	10	4/5	80
TX-1846	100	5/5	100
	10	3/5	60
MISO	100	0/5	0
TX-1831	100	1/5	20
TX-1832	100	1/5	20

Table 3. Inhibitory effects of chloroacetylcarbamoyl-2-nitroimidazole radiosensitizers on the activity of PP elastase

Radiosensitizer	K_i[a] (mM)
KIN-1800	N.I.[b]
TX-1835	7.52
TX-1836	1.13

[a]Enzyme: PP elastase; Substrate: succinyl-Ala-Ala-Ala-pNA.
[b]N.I.: no inhibition.

Table 4. Inhibitory effects of chloroacetylcarbamoyl-2-nitroimidazole radiosensitizers and their deschloroacetylcarbamoyl radiosensitizers on ATP synthesis in RLM

Radiosensitizer	IC_{50} (mM)
KIN-1800	2.4
TX-1835	0.2
TX-1836	0.2
MISO	4.5
TX-1831	2.3

than those of their parent 2-nitroimidazoles. Thus the $C_{1.6}$ values of KIN-1800 (60 μM), TX-1835 (40 μM), TX-1836 (6 μM), TX-1844 (20 μM), TX-1845 (7 μM), and TX-1846 (3 μM) showed that they were more potent than those of their parent compounds: MISO (1 mM), TX-1831 (0.7 mM), and TX-1832 (0.65 mM), respectively. As shown in Table 5, the order of each radiosensitizing activity among all the compounds investigated was not strictly correlated with the lipophilicity estimated by log *P* as a parameter of the radiosensitization,[25] whereas the order of each radiosensitizing activity among each series of compounds, such as methoxy, *tert*-butoxy, *p-tert*-butylphenoxy series, was moderately correlated with the log *P*. The bromoacetylcarbamoyl-2-nitroimidazole, TX-1846, was the strongest radiosensitizer among these tested compounds.

Table 5. In vitro radiosensitizing effects (EMT 6/KU cells) and log *P* of haloacetylcarbamoyl-2-nitroimidazole radiosensitizers and their deshaloacetylcarbamoyl-2-nitroimidazole radiosensitizers

Radiosensitizer	log *P*	$C_{1.6}$/μM[a]
MISO	−0.007	1000
TX-1831	1.185	700
TX-1832	1.620	650
KIN-1800	−0.445	60
TX-1835	1.808	40
TX-1836	2.897	6
TX-1844	−0.701	20
TX-1845	0.885	7
TX-1846	2.265	3

[a]Drug concentration required to achieve an ER of 1.60.

Discussion

Hypoxic cell radiosensitizers are generally compounds with high electron affinity and which consist of electron-deficient heterocyclic rings and polar side-chains that decrease lipophilicity.[26] However, most nitroazole hypoxic cell radiosensitizers are not suitable for clinical use because the nitro group is neurotoxic.[2,27–29] Exceptions include certain mercaptoimidzales[30] and 2-nitroimidazole hydroxamates.[4–6,8,9] The link between tumor tissue hypoxia and angiogenesis was not well established. However, we found Shweiki and co-workers' report[16] on how hypoxia specially induced the production of VEGF, which mediates hypoxia-initiated angiogenesis. We expected that the hypoxic cell radiosensitizer having anti-angiogenic activity would be a synergistic interaction between the hypoxic cytotoxic/hypoxic cell radiosensitizing agents and subsequent treatment with anti-angiogenic agents that destroyed hypoxia-initiated neovasculature.[17] We evaluated their anti-angiogenic activity, adding a haloacetylcarbamoyl

moiety to the side-chain of the 2-nitroimidazole skeleton, which increased the sensitizing efficiency and allowed the development of micromolar radiosensitizers. Both haloacetyl and halomethylketone groups should be a potent biologically alkylating moiety. In fact, molecules containing haloacetyl groups are protease inhibitors,[31-35] ribonuclease inhibitors,[36] and DNA-cleaving agents.[37] There are, however, few reports on the drugs containing the haloacetylcarbamoyl moiety. We found that Ingber and co-workers[38] reported an angiogenesis-inhibitor fumagillin analogue AGM-1470 which had the chloroacetylcarbamoyl group in its structure. However, they did not mention the contribution of chloroacetylcarbamoyl group for the anti-angiogenic activity of AGM-1470 (TNP-470). As described here, the induced biofunction of TX-1845, TX-1835, and KIN-1800 is responsible for the increased sensitizing efficiency compared with their parent 2-nitroimidazoles: TX-1832, TX-1831, and MISO. For the rational design of hypoxic cell radiosensitizers, we use the electron affinities (EAs) calculated from LUMO [EA= −(LUMO)] a measure of hypoxic cell radiosensitization.[23] On the basis of the electron affinity calculated by the MO method, we predicted that bromoacetylcarbamoyl-2-nitroimidazoles such as TX-1844 are more potent hypoxic cell radiosensitizers than chloroacetylcarbamoyl-2-nitroimidazoles such as KIN-1800. Furthermore, on the basis of their LUMO coefficients, we predicted the preferred biological-nucleophile-attacked sites of chloroacetylcarbamoyl-2-nitroimidazoles. Nucleophiles may preferentially attack the methylene moiety of the bromoacetylcarbamoyl group in the bromoacetylcarbamoyl-2-nitroimidazole, TX-1844, and the ester-carbonyl moiety of the chloroacetylcarbamoyl group in the chloroacetylcarbamoyl-2-nitroimidazole, KIN-1800. Unexpectedly, the preferred biological-nucleophile-attacked sites in bromo- and chloro-acetylcarbamoyl-2-nitroimidazoles differed from those predicted from organic electronic theory. Stereochemical factors would also control nucleophile-attacked sites of haloacetylcarbamoyl group to give general preference to haloacetyl moiety as a nucleophile-attacked site. However, further studies such as an approach designed to isolate the adducts of haloacetylcarbamoyl-2-nitroimidazoles with biological nucleophiles are needed to resolve this problem. These results show that the new bromoacetylcarbamoyl-2-nitroimidazoles may be hypoxic cell radiosensitizers that inhibit angiogenesis because of the bromoacetylcarbamoyl group being preferentially attacked by the nucleophiles. In fact, the bromoacetylcarbamoyl-2-nitroimidazole, TX-1844, was a more potent anti-angiogenic compound than the chloroacetylcarbamoyl-2-nitroimidazole, KIN-1800. Thus the difference in the in vitro anti-angiogenic activities between TX-1844 and KIN-1800 was greater than that of the radiosensitizing activities, of which the former was a ratio of about 20 and the latter was a ratio of three. Adding an anti-angiogenic haloacetylcarbamoyl moiety to standard hypoxic cell radiosensitizers may provide a new approach to designing drugs for cancer therapy, because the growth of solid tumors is thought to be dependent on angiogenesis.[39]

In addition, extracellular proteolysis is an essential component of the angiogenesis.[15,22,40] In the studies of the biological activity of the haloacetylcarbamoyl group, we chose the chloroacetylcarbamoyl-2-nitroimidazoles as an example and tested their ability to inhibit PP elastase. Both the *p-tert*-butylphenoxy derivative, TX-1836 and the *tert*-butoxy derivative, TX-1835 inhibited protease, but the methoxy analogue KIN-1800 did not. These results agree with the finding that inhibitors containing the hydrophobic benzyloxycarbonyl group are bound to elastase more tightly than the corresponding acetyl derivatives, probably due to the hydrophobic environment on the enzyme surface.[41] The viability of cells irradiated with X-rays depends on their physiological condition, such as their ATP level and rate of synthesis in mitochondria.[36,42,43] Since ATP is required in many metabolic processes including repair processes, a large change in the cellular ATP level may affect the viability of cells to survive stresses such as radiation or drug toxicity.[43] The 2-nitroimidazoles have many effects on cellular metabolism, such as depleting nonprotein and protein thiols, stimulating oxygen consumption, inhibiting glycolysis and perturbing purine metabolism.[13,43,44] Thus radiosensitizers that inhibit energy transduction should be more effective than those that do not. One of the protease-inhibiting radiosensitizers, TX-1835, completely inhibited the ATP synthesis in RLM at 1.5 mM. Thus these compounds must play the roles of ATP synthesis inhibitors as well as those of hypoxic cell radiosensitizers. Since depleting ATP from cells sensitizes them to the cytotoxicity of X-ray irradiation,[43] the inhibitory activity of TX-1835 on ATP synthesis may enhance its radiosensitizing effects. From these results, we expect that the potent radiosensitizing activity of haloacetylcarbamoyl-2-nitroimidazole such as TX-1845 and TX-1846 as the potent angiogenesis inhibitors would be partially due to the contribution of the haloacetylcarbamoyl moiety for the biological activities such as protease and ATP synthesis inhibitions.

Conclusion

We designed a new hypoxic cell radiosensitizer with anti-angiogenic activities, by introducing haloacetylcarbamoyl groups into the side-chain moieties of 2-nitroimidazoles. The synthetic haloacetylcarbamoyl-2-nitroimidazole radiosensitizers also had anti-angiogenic activities. The in vivo chick CAM angiogenesis assay showed that the strong bromoacetylcarbamoyl-2-nitroimidazole radiosensitizers, such as TX-1845 and TX-1846, were the strongest angiogenic inhibitors among them. We concluded that the bromoacetylcarbamoyl-2-nitroimidazole radiosensitizers, such as TX-1845 and TX-1846, were promising as unique dual-function, hypoxic cell radiosensitizers for cancer therapy.

Experimental

Porcine pancreatic (PP) elastase, HEPES, and succinyl-Ala-Ala-Ala-*p*NA were obtained from Sigma Chemical

Co. (St Louis, USA), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and the Peptide Institute, Inc. (Osaka, Japan), respectively. MISO was synthesized in our laboratory as reported.[45] All other chemicals and drugs were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Sigma-Aldrich Japan (Tokyo, Japan). Oxygen consumption and pH were monitored with a YTS multi-ion monitor (Yamashita-Giken Ltd., Tokushima, Japan), that included a Clark oxygen electrode and a pH electrode. The octanol–water partition coefficient (log *P*) was measured according to the method of Fujita et al.[46] All melting points were determined in a glass capillary tube without correction. IR spectra were recorded in KBr pellets on a Perkin-Elmer 1600 spectrometer. UV absorption spectra were determined in ethanol on a Hitachi U-2000 spectrophotometer. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a JEOL JNM-EX400 (400 MHz) spectrometer with tetramethylsilane as the internal standard, and chemical shifts are given in δ values. Fast atom bombardment mass spectra (FABMS) and high-resolution mass spectra (FABHRMS) were measured on a JEOL JMS-SX 102A instrument. Elemental analyses were performed with a Yanaco CHN Corder (MT-5). Chromatographic separations were performed on silica gel columns (Kieselgel 60, 230-400 mesh, Merck). The semi-empirical molecular orbital (MO) calculation was applied by the PM3 method of J. J. P. Stewart with his program MOPAC V. 7.00 revised for the DOS-extender environment on a microprocessor.

Syntheses of drugs

Synthesis of 3-*tert*-butoxy-1-(2-nitroimidazolyl)-2-propanol (TX-1831). To a suspension of 2-nitroimidazole (904 mg, 8 mmol) and sodium carbonate (828 mg, 6 mmol) in anhydrous EtOH (30 mL), *tert*-buthylglycidyl ether (2.09 g, 16.4 mmol) was added. After being refluxed for 7 h, the reaction mixture was filtered and washed with CH_2Cl_2. The filtrate was evaporated in vacuo to give a semi-solid residue, which was chromatographed on silica gel with CH_2Cl_2 and MeOH to afford TX-1831 (1.08 g, 55.5%) as a pale yellow solid: mp 75–77 °C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.19 (s, 9H, *tert*-butyl), 1.63 (s, 1H, OH), 3.36 and 3.51 (each m, each 1H, OCH_2), 4.07 (s, 1H, CH), 4.46 and 4.63 (each m, each 1H, NCH_2), 7.13 and 7.21 (each s, each 1H, imidazole); IR (KBr) ν_{max} 3341, 2972, 1532, 1489, 1360, 1280, 1190, 1162, 1100, 918, 836, 798 cm^{-1}. Anal. calcd for $C_{10}H_{17}N_3O_4$: C, 49.38; H, 6.99; N, 17.28. Found: C, 49.79; H, 6.97; N, 17.05.

Synthesis of 3-(4-*tert*-butylphenoxy)-1-(2-nitroimidazolyl)-2-propanol (TX-1832). To a suspension of 2-nitroimidazole (1130 mg, 10 mmol) and sodium carbonate (500 mg, 4.7 mmol) in anhydrous EtOH (40 mL), 4-*tert*-butylphenyl-2,3-propylether (4.12 g, 20 mmol) was added. After being refluxed for 6 h, the reaction mixture was filtered and washed with distilled water and CH_2Cl_2. The filtrate was extracted with CH_2Cl_2, and then the organic phase was dried by anhydrous sodium sulfate and evaporated in vacuo to give a pale yellow powder (TX-1832, 2.25 g, 70.7%): mp 157–158 °C ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.29 (s, 9H, *tert*-butyl), 2.59 (s, 1H, OH), 3.91 and 4.01 (each dd, each 1H, J = 5.5 and 4.4 Hz, OCH_2), 4.26 (m, 1H, CH), 4.48 and 4.84 (each dd, each 1H, J = 13.5 and 10.8 Hz, NCH_2), 6.84 and 7.29 (each d, each 2H, J = 8.8 and 8.0 Hz, Ar), 7.10 and 7.36 (each s, each 1H, imidazole); IR (KBr) ν_{max} 3358, 2961, 1529, 1531, 1486, 1360, 1239, 1185, 1044, 832, 796, 554 cm^{-1}. Anal. calcd for $C_{16}H_{21}N_3O_4$: C, 60.19; H, 6.58; N, 13.17. Found: C, 60.42; H, 6.58; N, 13.25.

General procedure for the synthesis of haloacetylcarbamoyl-2-nitroimidazoles (KIN-1800, TX-1835, 1836, 1844, 1845, and 1846). To a solution of a 3-substituted-1-(2-nitroimidazolyl)-2-propanol (such as MISO, TX-1831, and TX-1832, 1.5 mmol) in anhydrous CH_2Cl_2 (15 mL), was added haloacetyl isocyanate (either chloroacetyl isocyanate or bromoacetyl isocyanate) (3 mmol), which was prepared imidiately before use by a modification of the method of Speziale and Smith.[47] After being refluxed for 30 min, the reaction mixture was filtered and the filtrate was washed with CH_2Cl_2. The residue remaining after evaporation in vacuo was purified by silica gel column chromatography (CH_2Cl_2–AcOEt) to give haloacetylcarbamoyl-2-nitroimidazoles such as KIN-1800, TX-1835, 1836, 1844, 1845, and 1846.

Synthesis of 2-chloroacetylcarbamoyl-1-(2-nitroimidazolyl)-3-methoxypropane (KIN-1800). Prepared with misonidazole and chloroacetyl isocyanate as a 3-substituted-1-(2-nitroimidazolyl)-2-propanol and haloacetyl isocyanate, respectively. Yield: 99.0% as a pale yellow solid, mp 101–102 °C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.36 (s, 3H, CH_3O), 3.62 (m, 2H, OCH_2), 4.33 and 4.38 (AB type, each 1H, J = 15.5 Hz, $ClCH_2$), 4.59 and 4.93 (each dd, each 1H, J = 13.9 and 14.4 Hz, NCH_2), 5.32 (m, 1H, CH), 7.14 and 7.28 (each s, each 1H, imidazole); IR (KBr) ν_{max} 3177, 1783, 1719 1537, 1484, 1360, 1231, 1164, 1049, 837, 808, 767, 614 cm^{-1}; FABHRMS *m/z* 321.0573 (MH^+) [calcd for $C_{10}H_{13}ClN_4O_6$: 321.0602 (MH^+)].

Synthesis of 2-chloroacetylcarbamoyl-1-(2-nitroimidazolyl)-3-*tert*-butoxypropane (TX-1835). Prepared with 3-*tert*-butoxy-1-(2-nitroimidazolyl)-2-propanol (TX-1831) and chloroacetyl isocyanate as a 3-substituted-1-(2-nitroimidazolyl)-2-propanol and haloacetyl isocyanate, respectively. Yield: 72.3% as a pale yellow solid, mp 49–50 °C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.20 (*s*, 9H, *tert*-butyl), 3.49 and 3.60 (each dd, each 1H, J = 9.9 and 10.6 Hz, OCH_2), 4.37 and 4.41 (AB type, each 1H, J = 16.0 Hz, $ClCH_2$), 4.59 and 4.93 (each dd, each 1H, J = 14.6 and 14.8 Hz, NCH_2), 5.25 (m, 1H, CH), 5.30 (s, 1H, NH), 7.16 and 7.27 (each s, each 1H, imidazole); IR (KBr) ν_{max} 3140, 2976, 1787, 1726, 1541, 1491, 1364, 1208, 1164, 1095, 838, 771, 651 cm^{-1}; FABHRMS *m/z* 363.1052 (MH^+) [calcd for $C_{13}H_{19}ClN_4O_6$: 363.1071 (MH^+)].

Synthesis of 2-chloroacetylcarbamoyl-1-(2-nitroimidazolyl)-3-(4-*tert*-butyl)-phenoxypropane (TX-1836). Prepared with 3-(4-*tert*-butylphenoxy)-1-(2-nitroimidazolyl)-2-propanol and chloroacetyl isocyanate as a 3-substituted-1-(2-nitroimidazolyl)-2-propanol and haloacetyl isocyanate, respectively. Yield: 68.8% as a pale yellow solid, mp 104–106 °C; ^{1}H NMR ($CDCl_3$–CD_3OD, 400 MHz): δ 1.29 (s, 9H, *tert*-butyl), 4.19 (s, 2H, OCH_2), 4.38 (s, 2H, $ClCH_2$), 4.76 and 5.04 (each m, each 1H, NCH_2), 5.34 (s, 1H, NH), 5.46 (m, 1H, CH), 6.83 and 7.32 (each d, each 2H, each J = 8.8 Hz, Ar), 7.14 and 7.40 (each s, each 1H, imidazole); IR (KBr) ν_{max} 3242, 2961, 1787, 1764, 1707, 1540, 1513, 1489, 1365, 1208, 1164, 1109, 1090, 832, 625, 552 cm^{-1}; FABHRMS *m/z* 439.1398 (MH^+) [calcd for $C_{19}H_{23}ClN_4O_6$: 439.1384 (MH^+)].

Synthesis of 2-bromoacetylcarbamoyl-1-(2-nitroimidazolyl)-3-methoxypropane (TX-1844). Prepared with 3-(4-*tert*-butylphenoxy)-1-(2-nitroimidazolyl)-2-propanol and bromoacetyl isocyanate as a 3-substitued-1-(2-nitroimidazolyl)-2-propanol and haloacetyl isocyanate, respectively. Yield: 84.4% as a pale yellow solid, mp 108–110 °C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.32 (s, 3H, CH_3O), 3.62 (m, 2H, OCH_2), 4.12 and 4.16 (AB type, each 1H, J = 12.8 Hz, $BrCH_2$), 4.59 and 4.93 (each dd, each 1H, J = 14.4 and 14.8 Hz, NCH_2), 5.32 (m, 1H, CH), 7.14 and 7.24 (each s, each 1H, imidazole); IR (KBr) ν_{max} 3177, 2989, 1783, 1719, 1542, 1484, 1360, 1231, 1008, 837, 761, 596 cm^{-1}; FABHRMS *m/z* 365.0079 (MH^+) [calcd for $C_{10}H_{13}BrN_4O_6$: 365.0097 (MH^+)].

Synthesis of 2-bromoacetylcarbamoyl-1-(2-nitroimidazolyl)-3-*tert*-butoxypropane (TX-1845). Prepared with 3-*tert*-butoxy-1-(2-nitroimidazolyl)-2-propanol and bromoacetyl isocyanate as a 3-substitued-1-(2-nitroimidazolyl)-2-propanol and haloacetyl isocyanate, respectively. Yield: 70.0% as a pale yellow solid, mp 98–99 °C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.20 (s, 9H, *tert*-butyl), 3.51 and 3.60 (each dd, each 1H, J = 10.6 and 10.2 Hz, OCH_2), 4.20 and 4.24 (AB type, each 1H, J = 13.2 Hz, $BrCH_2$), 4.59 and 4.93 (each dd, each 1H, J = 14.8 and 14.4 Hz, NCH_2), 5.25 (m, 1H, CH), 5.30 (s, 1H, NH), 7.16 and 7.28 (each s, each 1H, imidazole); IR (KBr) ν_{max} 3427, 1977, 1786, 1720, 1539, 1488, 1362, 1287, 1219, 1190, 1092, 1026, 839, 770 cm^{-1}. FABHRMS *m/z* 407.0583 (MH^+) [calcd for $C_{13}H_{19}BrN_4O_6$: 407.0566 (MH^+)].

Synthesis of 2-bromoacetylcarbamoyl-1-(2-nitroimidazolyl)-3-(4-*tert*-butyl)-phenoxypropane (TX-1846). Prepared with 3-(4-*tert*-butylphenoxy)-1-(2-nitroimidazolyl)-2-propanol and bromoacetyl isocyanate as a 3-substitued-1-(2-nitroimidazolyl)-2-propanol and haloacetyl isocyanate, respectively. Yield: 92.7% as a pale yellow solid, mp 94–95 °C. ^{1}H NMR ($CDCl_3$-CD_3OD, 400 MHz): δ 1.31 (s, 9H, *tert*-butyl), 4.15 (s, 2H, OCH_3),4.15 and 4.20 (each 1H, s, $BrCH_2$), 4.77 and 5.05 (each d, each 1H, each J = 14.4 Hz, NCH_2), 5.34 (s, 1H, NH), 5.48 (m, 1H, CH), 6.83 and 7.32 (each d, each 2H, each J = 8.8 Hz, Ar), 7.14 and 7.40 (each s, each 1H, imidazole); IR (KBr) ν_{max} 3435, 2962, 1784, 1702, 1539, 1513, 1488, 1364, 1211, 834, 766, 553 cm^{-1}; FABHRMS *m/z* 483.0892 (MH^+) [calcd for $C_{19}H_{23}BrN_4O_6$: 483.0879 (MH^+)].

Endothelial cell proliferation assay

Rat lung endothelial (RLE) cells were provided by Dr G. L. Nicolson, Texas M.D. Anderson Hospital, Houston, Texas, and maintained at exponential growth in spinner culture. MTT[48] [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] was purchased from Dojindo Laboratories (Kumamoto, Japan). Optical density (OD) was measured on a microplate reader (BioRad model 450, Japan Bio-Rad Laboratories, Tokyo, Japan) using a 570 nm filter with blanking at 700 nm. The MTT assay was performed by suspending the cells in Eagle's MEM containing 7% $NaHCO_3$ and 4.7% FCS (pH 7.4), then pouring 300 μL into the wells of 48-well culture plates. Drugs (3 μL) were added and the cells were incubated at 37 °C for 48 h. Drugs were washed out with culture medium. The culture medium (270 μL) and 30 μL of the MTT reagent (MTT, 5 mg mL^{-1} in phosphate buffered saline without potassium and magnesium ions) were added and the cells were incubated for 3 h at 37 °C. Formazan was extracted with 300 μL of 0.04 N HCl in isopropanol and the OD was measured at a wavelength of 570 nm using a microplate reader. From the surviving fraction (OD % control) as a function of drug concentration, the IC_{50} value was estimated as an index of the ability of the drug to inhibit endothelial cell proliferation.

Chick CAM angiogenesis assay

The anti-angiogenic activity was evaluated using chick embryo chorioallantoic membranes (chick CAM), as described.[49] Onto four-day-old fertilized chick embryos (Ohmiya Kakin Laboratory Ohmiya, Japan) in the shells, 10 μL aliquots of samples mixed in 1% methylcellulose/0.9% NaCl were applied into rings placed on the surface of the growing CAM. After an incubation at 37 °C for 48 h, a fat emulsion was injected into the CAM to visualize the blood vessels. The angiogenic inhibition was indicated by an avascular zone around a ring of 3 mm diameter. The results are expressed as the percentages of embryos showing inhibition. Actinonin was the positive control in the CAM assay.[50]

Protease inhibition assay[31–33]

All enzyme assays were performed spectrophotometrically using a Hitachi U-2000 spectrophotometer. The concentration of PP elastase was determined with *p*-nitroanilide (*p*NA), succinyl-Ala-Ala-Ala-*p*NA.[51] This chromogenic compound was also applied as a substrate for PP elastase. The rate of hydrolysis of succinyl-Ala-Ala-Ala-*p*NA was measured in 50 mM HEPES buffer containing 9% DMSO and 0.25 M NaCl at pH 7.5 and 25 °C. All K_i values were obtained from the values of K_m

and $K_{m'}$ the observed K_m value in the presence of an appropriate inhibitor (I) by means of the equation: $K_i = [I] \cdot K_m/(K_{m'} - K_m)$ where [I] is the inhibitor concentration. K_m and $K_{m'}$ were determined by their Lineweaver–Burk plots. The inhibitor concentration was always 10-fold higher than enzyme concentration and was usually 100–1000-fold higher.

Measurement of ATP synthesis in mitochondria

Mitochondria were isolated from the livers of Wistar rats by a modification of the reported procedure.[52] The amount of mitochondrial protein was determined by the Biuret method[53] with BSA as a standard. The respiration of mitochondria (0.7 mg protein mL^{-1}) was monitored polarographically with a Clark oxygen electrode at 25 °C in a medium consisting of 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, and 3 mM potassium phosphate buffer, pH 7.4, in a total volume of 1.68 mL. The reaction was started by adding 0.4 mM ADP to the mitochondrial suspension (0.7 mg protein mL^{-1}) energized with 5 mM succinate (plus rotenone at 1 μg mg^{-1} protein). The pH was monitored with an electrode and calibrated using 20 μM oxalic acid. The ATP synthesis in the presence of drugs is shown as a percentage of that in the absence of drugs. The drug concentrations required to inhibit 50% (IC_{50}) (for all drugs) and 100% of ATP synthesis (IC_{100}) (for some drugs) were determined from their dose-response plots.

In vitro radiosensitization assay

In vitro radiosensitization was measured in EMT6/KU single cells under hypoxic conditions, as described.[4–7] Enhancement ratios (ERs) were determined at an appropriate dose from the ratio of radiation doses required to reduce the surviving fraction of EMT6/KU cells to 1%. Radiosensitization efficiency ($C_{1.6}$) is the concentration of drug required to produce an ER of 1.6 in vitro.

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