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Synthesis and biological activity of 1-methyl-tryptophan-tirapazamine hybrids as hypoxia-targeting indoleamine 2,3-dioxygenase inhibitors

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ABSTRACT

We have designed and synthesized new hypoxic-neoplastic cells-targeted indoleamine 2,3-dioxygenase (IDO) inhibitors. 1-Methyl-tryptophan (1MT)-tirapazamine (TPZ, 3-amino-1,2,4-benzotriazine 1,4-dioxide) hybrid inhibitors including **1** (TX-2236), **2** (TX-2235), **3** (TX-2228), and **4** (TX-2234) were prepared. All of these compounds were uncompetitive IDO inhibitors. TPZ-monoxide hybrids **1** and **3** showed higher IDO inhibitory activities than TPZ hybrids **2** and **4**. Among these hybrids, hybrid **1** was the most potent IDO inhibitor. TPZ hybrids **2** and **4** showed stronger hypoxia-selective cytotoxicity than TPZ to EMT6/KU cells. These data suggest that TPZ hybrids **2** and **4** may act through their dual biological functions: first, they function as hypoxic cytotoxins in hypoxic cells, and then are metabolized to their TPZ-monoxide (3-amino-1,2,4-benzotriazine 1-oxide) hybrids, which function as IDO inhibitors.

1. Introduction

Indoleamine 2,3-dioxygenase (IDO) is a monomeric 45 kDa heme-containing dioxygenase that catalyzes the initial and rate-limiting step of L-tryptophan (L-Trp) catabolism in the kynurenine pathway.[1,2] This step involves the oxidative cleavage of the 2,3 double bond in the indole moiety, resulting in the production of *N*-formylkynurenine. Heme iron that exists in the active site of IDO is active as its ferrous (Fe^{2+}) form, whereas the ferric (Fe^{3+}) form is inactive.[3,4] IDO-mediated tryptophan catabolism is shown in Scheme 1.[5] These kynurenine pathway metabolites, such as 3-hydroxykynurenine, 3-hydroxyanthranilic acid, anthranilic acid, and quinolinic acid, suppress T cell proliferation and differentiation, and accelerate T cell apoptosis.[6] Allogenic fetal rejection by pregnant maternal mice is prevented by IDO-mediated tryptophan catabolism.[7] For this reason, IDO is associated with immunosuppression and immunotolerance. Expression of IDO has been reported in various neoplastic cells, such as lung, prostatic, pancreatic, and colorectal carcinomas.[8] Especially in lung cancer, IDO mRNA can be constitutively expressed, and the higher IDO expression was observed in some patients' samples.[9] IDO is associated with tumor immunosuppression.

1-Methyl-tryptophan (1MT; **5** in Fig. 1) is a competitive inhibitor of IDO with a K_i = 19 μM.[10] Hypoxic-neoplastic cells in a neoplasm indicate resistance to chemo-radiotherapy, probable tumor metastasis, and an overall poor prognosis.[11–13] TPZ (**6** in Fig. 1) is a hypoxic cytotoxin, which is currently under phase II/III clinical trials in combination with radiotherapy and cisplatin-based chemotherapy.[14] TPZ is reduced by one-electron reductases such as cytochrome P450 reductase to form a radical, which mediates the induction of lethal double strand breaks in cellular DNA.[12] Further metabolism produces the TPZ-monoxide (**7** in Fig. 1). From the above considerations, we reasoned that a hybrid molecule possessing both IDO inhibitory activity and hypoxia-selective cytotoxicity could be an effective and novel antineoplastic agent.[15] Recently naphthoquinone analogues[16] and exiguamine A[17] were shown to have higher IDO inhibitory activities than 1MT. They, however, may affect other enzymes such as cytochrome P450 reductase.[18] To develop IDO-specific inhibitors, we chose the substrate (L-Trp) analogue 1MT, indoleamine as the IDO inhibitory unit for our designed hybrids. We present here the synthesis and biological activity of 1MT-TPZ hybrids, which was defined as hybridization of 1MT with TPZ or TPZ-monoxide **1** (TX-2236), **2** (TX-2235), **3** (TX-2228), and **4** (TX-2234) as hypoxia-targeting IDO inhibitors.

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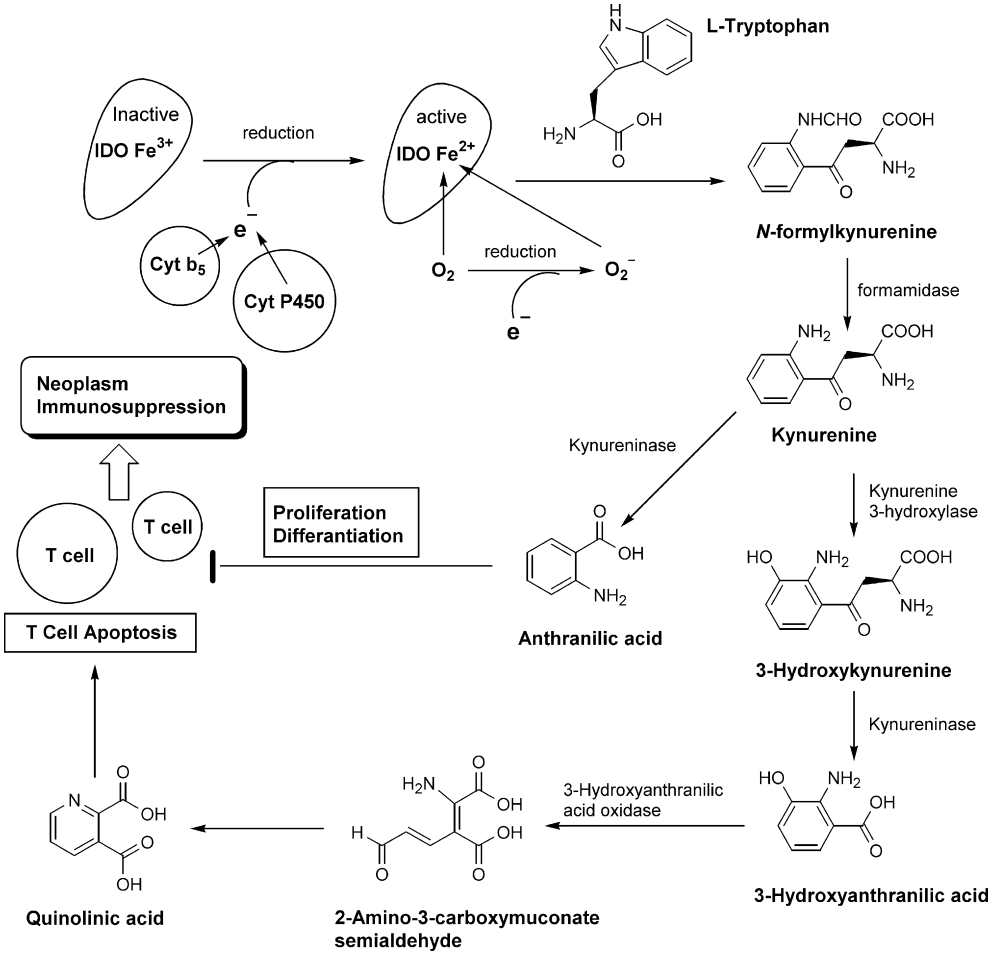


Scheme 1. IDO-mediated tryptophan catabolism.

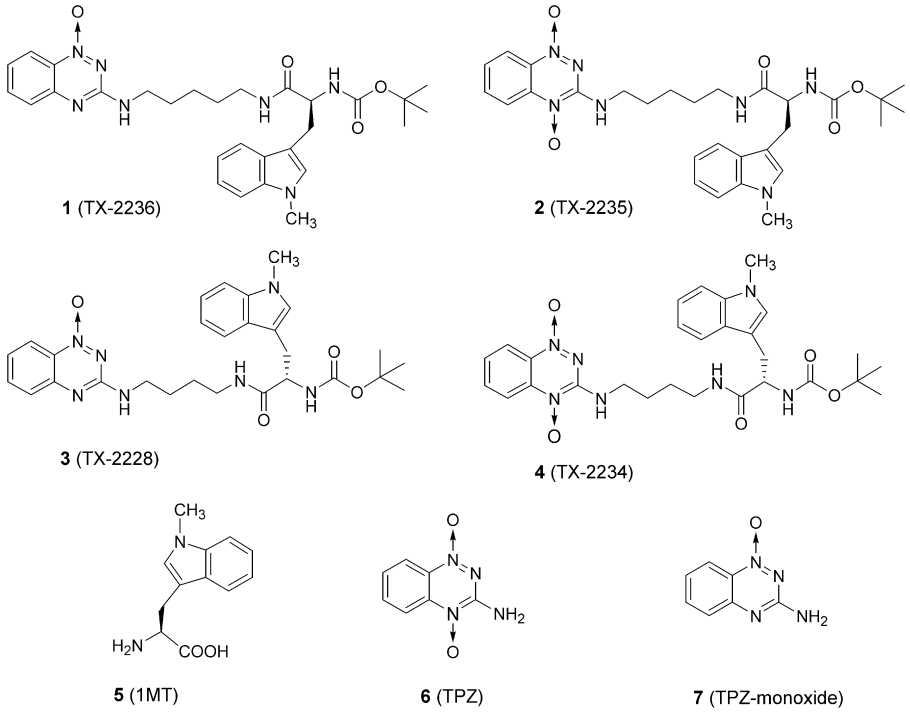


Figure 1. Structure of 1MT-TPZ hybrids **1**–**4**, 1MT **5**, TPZ **6** and TPZ-monoxide **7**.

2. Results

2.1. Molecular design

The 1MT-TPZ hybrids were prepared by conjugation of the L-isomer of 1MT with TPZ or TPZ-monoxide using flexible alkyl chains as linking moieties. The amino group of 1MT was protected with the *tert*-butyloxycarbonyl (Boc) group. The molecular orbitals (MO) of all these newly constructed 1MT-TPZ hybrids were calculated with WinMOPAC 3.0 (Fujitsu) by the PM3 method. The results including highest occupied molecular orbital (HOMO) and Eigenvalue (E_{HOMO}) and lowest unoccupied molecular orbital (LUMO) and Eigenvalue (E_{LUMO}) are shown in Figure 2. The HOMO coefficient of hybrid **1** is localized at the 1MT moiety of the hybrid, while the that of hybrid **2** localized at the TPZ moiety of the hybrid. On the other hand, the LUMO coefficient of hybrids **1** and **2** are localized at the TPZ moiety of the hybrid. The MO coefficients of hybrids **3** and **4** (data not shown) are also localized in a similar region as found in hybrids **1** and **2**.

2.2. Synthesis

To conjugate 1MT and TPZ with amide bonds, we synthesized the chloride **9** as previously described.[19] The chloride **9** underwent nucleophilic displacement with appropriate alkyl diamines to give the corresponding 3-(aminoalkylamino) monoxides **10** and **11** (alkyl linker chain: $n = 5$ and 4, respectively) as shown in Scheme 2.[20] The amino group of 1MT was protected with a Boc group to give Boc-*N*-1MT **8** quantitatively. The coupling reactions of Boc-*N*-1MT **8** with amines **10** and **11** were carried out using DIPCDI (*N*,*N*′-diisopropylcarbodiimide) in the presence of HOBt (1-hydroxybenzotriazole) to give the corresponding TPZ-monoxide hybrids **1** (TX-2236) and **3** (TX-2228) in good yields (99% and 89%, respectively). To synthesize TPZ hybrids, the amino groups of **10** and **11** were protected with the trifluoroacetyl group to give the corresponding trifluoroacetamides **12** (TX-2239) and **13** (TX-2237) as shown in Scheme 3.[20] Compounds **12** and **13** were then oxidized with CF_3CO_3H to give the corresponding dioxide trifluoroacetamides **14** (TX-2240) and **15** (TX-2238) in moderate yields (50–52%). Compounds **14** and **15** were deprotected under basic conditions, and then treated with HCl to give the corresponding dioxide amine hydrochlorides **16** and **17**. The coupling reactions of Boc-*N*-1MT **8** with amines **16** and **17** were carried out using DIPCDI in the presence of HOBt and Et_3N to give the corresponding TPZ hybrids **2** (TX-2235) and **4** (TX-2234) in good yields (98% and 93%, respectively).

2.3. Biological activity

2.3.1. IDO inhibitory activity

The inhibition assays were performed in a 96-well microtiter plate as described by Matin et al.[21] and Takikawa et al.[22] with a minor modification. To quantify the concentration of kynurenine, a range of final concentrations of 0–500 μM kynurenine were measured in standard medium. The absorption data plotted against the corresponding concentrations of kynurenine showed good linearity of $Y = 0.0054X$ ($r^2 = 0.9999$, data not shown). The production of kynurenine was detected spectrophotometrically, and the K_m and K_i values were determined using a Lineweaver–Burk plot. The K_m value of L-Trp was 207 μM, the IDO inhibition constants K_i of 1MT-TPZ hybrids are shown in Table 1. 1MT as a unit of the hybrid had an IDO inhibitory activity of $K_i = 53.2$ μM (lit.[9] $K_i = 19$ μM), while the other units including TPZ **6**, TPZ-monoxide **7**, and Boc-*N*-1MT **8** showed no inhibitory activities. As expected, the 1MT-TPZ hybrids, including **1**–**4**, displayed IDO inhibitory activities. The K_i of these 1MT-TPZ hybrids **1**, **2**, **3**, and **4**, were 76.3, 197, 87.1, and 367 μM, respectively. The TPZ-monoxide hybrids **1** and **3** showed stronger IDO inhibitory activities than the corresponding TPZ hybrids **2** and **4**. The length of alkyl linker chains of hybrids did not influence their IDO inhibitory activities. The trifluoroacetamido TPZ analogues without 1MT units, **12**–**15**, also displayed no IDO inhibitory activities. The combination of 1MT **5** with TPZ **6** gave more effective inhibition than 1MT alone (1MT **5** + TPZ **6**: $K_i = 30.0$ μM; 1MT **5**: 53.2 μM). On the other hand, the combination of 1MT **5** with TPZ-monoxide **7** did not enhance the inhibition of 1MT **5** (1MT **5** + TPZ-monoxide **7**: $K_i = 45.0$; 1MT **5**: $K_i = 53.2$ μM). The inhibition mode of these 1MT-TPZ hybrids were shown to be uncompetitive, while 1MT was competitive as shown in Figure 3.

2.3.2. Hypoxia-selective cytotoxicities[23]

Hypoxia-selective cytotoxicities of 1MT-TPZ hybrids **1**–**4** were determined as shown in Table 2. The efficacies of the compounds in killing aerobic or hypoxic EMT6/KU cells were determined by clonogenic cell survival. The cytotoxicity was evaluated as the

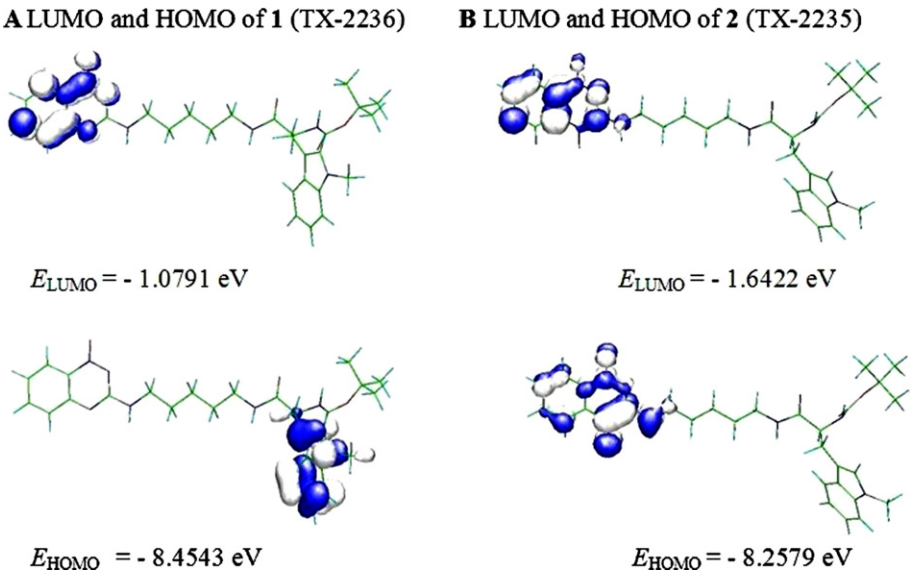
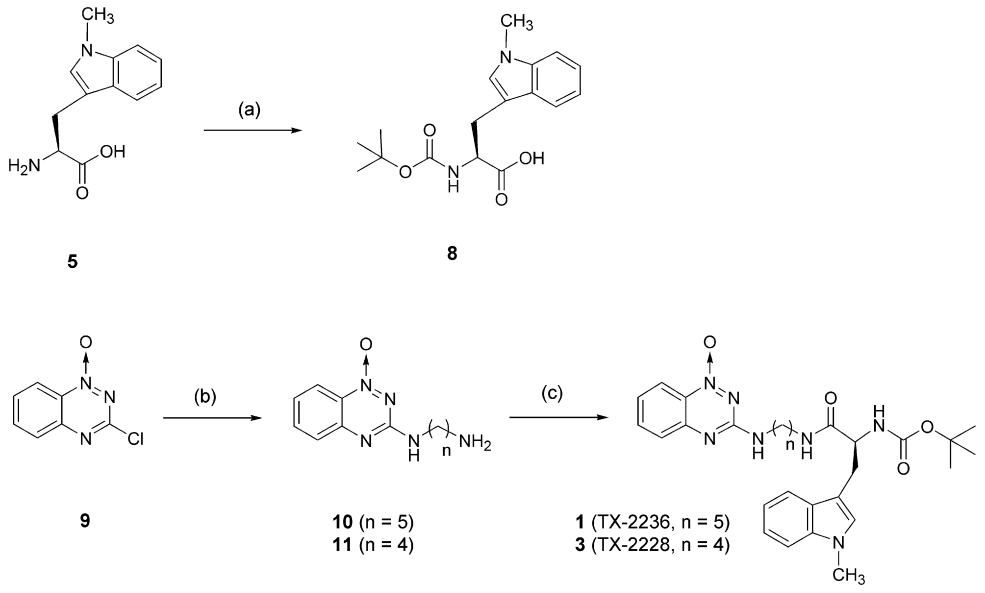
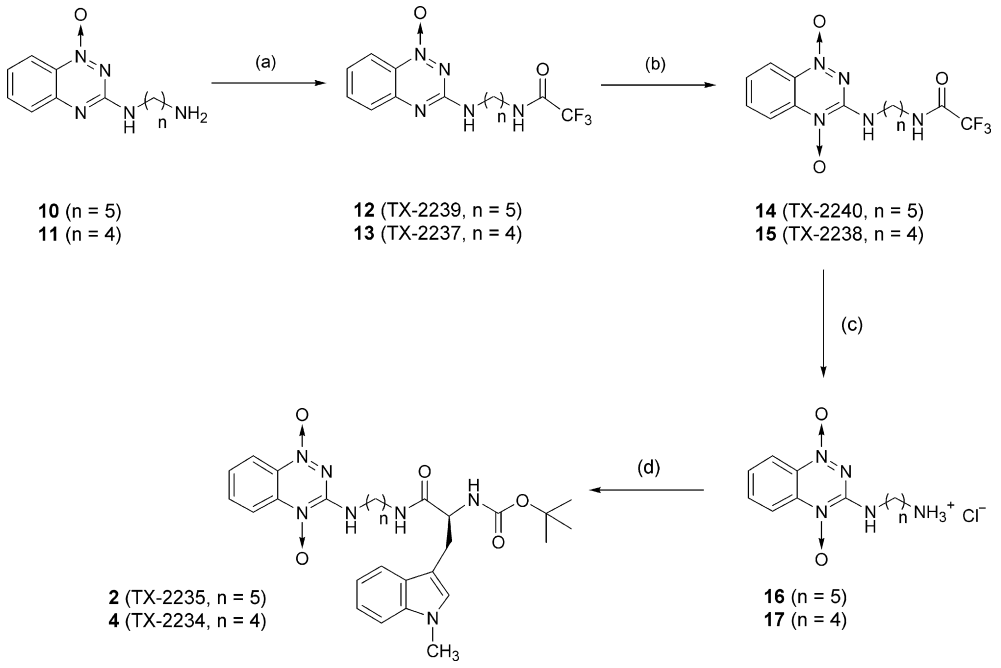


Figure 2. Molecular orbital of 1MT-TPZ hybrids **1** (A) and **2** (B) calculated by WinMOPAC 3.0 (PM3).



Scheme 2. Synthesis of TPZ-monoxide hybrids **1** and **3**. Reagents and conditions: (a) $(Boc)_2O$, 1 M NaOH aq, H_2O/1,4-dioxane = 1:2, rt; (b) alkyldiamine, Et_3N, CH_2Cl_2, rt; (c) **8**, DIPCDI, HOBt, CH_2Cl_2, rt.



Scheme 3. Synthesis of TPZ hybrids **2** and **4**. Reagents and conditions: (a) $(CF_3CO)_2O$, CH_2Cl_2, rt; (b) 35% H_2O_2, $(CF_3CO)_2O$, CH_2Cl_2, rt; (c) 28% NH_3 aq, MeOH, rt; (d) **8**, DIPCDI, HOBt, Et_3N, CH_2Cl_2, rt.

IC_{50} value, and the hypoxic cytotoxicity ratio (HCR) was calculated as the HCR = IC_{50}(Aero)/IC_{50}(Hypo). TPZ **6** displayed hypoxic cytotoxicity (IC_{50}(Hypo) = 12 μM) and hypoxic selectivity (HCR = 3.2), whereas TPZ-monoxide **7** displayed no cytotoxicity in either aerobic and hypoxic conditions (IC_{50}(Aero) = >80 μM; IC_{50}(hypo) = >40 μM). TPZ hybrids **2** and **4** have higher hypoxic cytotoxicity (**2**: IC_{50}(Hypo) = 7.1 μM; **4**: IC_{50}(Hypo) = 8.0 μM) and hypoxic selectivity (**2**: HCR = 4.9; **4**: HCR = >5.0) than TPZ, while TPZ-monoxide hybrids **1** and **3** had lower hypoxic cytotoxicity (**1**: IC_{50}(Hypo) = 33 μM; **3**: IC_{50}(Hypo) = 33 μM) and hypoxic selectivity (**1**: HCR = 1.3; **3**: HCR = 1.1) than TPZ. TPZ-monoxide hybrids **1** and **3**, however, demonstrated cytotoxicity only at concentrations of more than 40 μM, in both aerobic and hypoxic conditions.

3. Discussion

IDO is activated only after its heme iron is reduced from the ferric form to the ferrous form. This reduction takes place more easily in reductive or hypoxic conditions. Ferrous form of heme iron is easily oxidized to the inactive ferric form under aerobic conditions while the reduction of IDO may not take place in aerobic conditions. Maghzal et al. have reported that cytochrome b_5 or cytochrome P450 is involved in the reduction of IDO.[24] Thus,

Table 1
IDO inhibitory activity

Compound	K_i value (μM)
1 (TX-2236)	76.3
2 (TX-2235)	197
3 (TX-2228)	87.1
4 (TX-2234)	367
5 (1MT)	53.2
6 (TPZ)	NI[c]
7 (TPZ-monoxide)	NI[c]
8 (Boc-*N*-1MT)	NI[c]
12 (TX-2239)	NI[c]
13 (TX-2237)	NI[c]
14 (TX-2240)	NI[c]
15 (TX-2238)	NI[c]
5 + **6**[a]	30.0
5 + **7**[b]	45.0

Substrate (L-Trp) concn 0, 10, 20, 40, 80, 160 μM, inhibitor concn 400 μM, IDO concn 0.05 μM.
[a] **5** (1MT) and **6** (TPZ) concn were each 200 μM.
[b] **5** (1MT) and **7** (TPZ-monoxide) concn were each 200 μM.
[c] Not inhibited.

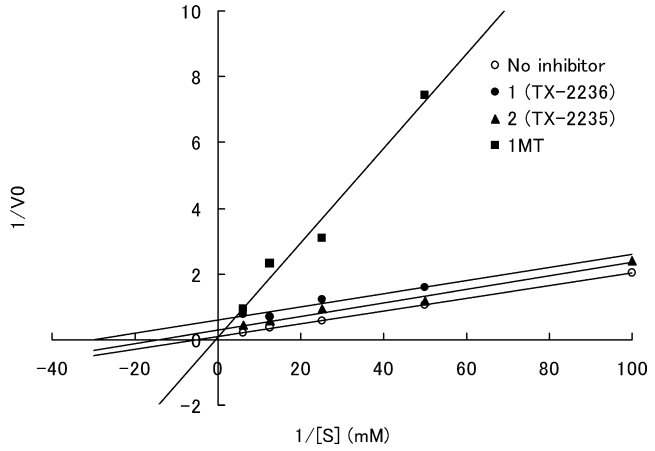


Figure 3. Lineweaver–Burk plot of 1MT-TPZ hybrids **1**, **2**, and 1MT. ○, no inhibitor; ●, **1** (TX-2236); ▲, **2** (TX-2235); ■, 1MT.

Table 2
Hypoxia-selective cytotoxicities

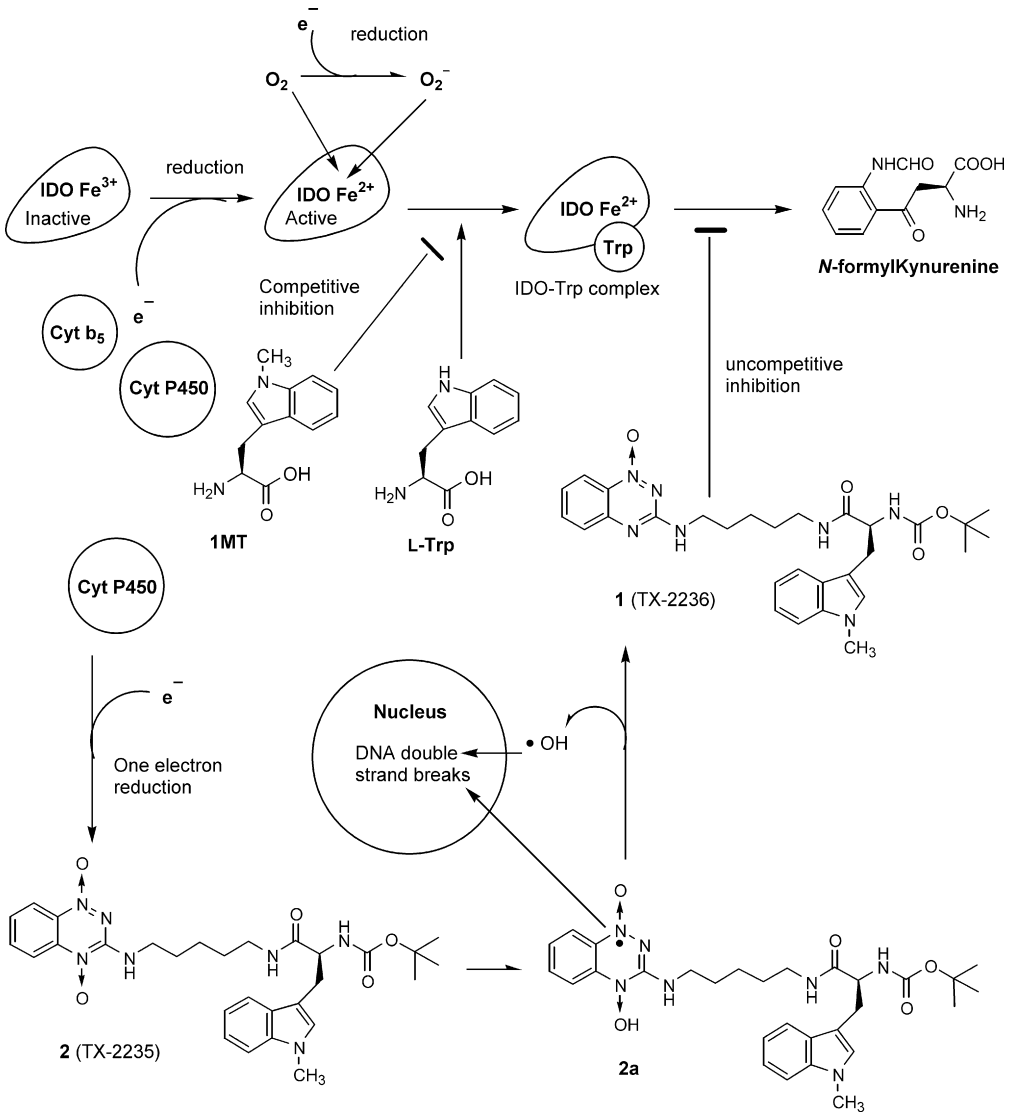
Compound	IC_{50}(Aero)[a] (μM)	IC_{50}(Hypo)[b] (μM)	HCR
6 (TPZ)	38	12	3.2
7 (TPZ-monoxide)	>80	>40	
1 (TX-2236)	42	33	1.3
2 (TX-2235)	35	7.1	4.9
3 (TX-2228)	36	33	1.1
4 (TX-2234)	>40	8.0	>5.0

[a] IC_{50} in aerobic conditions.
[b] IC_{50} in hypoxic conditions (0% O_2, 5% CO_2, 95% N_2).

activation of IDO is favored in hypoxic conditions, but IDO needs molecular oxygen or superoxide for the catalytic reaction, concentrations of which are normally low in hypoxic conditions (hypoxia pO_2 less than 5 mmHg).[25] We reasoned that the hypoxic environment in neoplasm may stabilize the active ferrous form of IDO, and catalyzes tryptophan degradation by using molecular oxygen or superoxide existing in the hypoxic-neoplastic cells. Because of the oxygen affinity of rhIDO with $K_d^{O2} = 54$ μM (Sugimoto, unpublished data), IDO still can function in hypoxic conditions. In anoxic environment, this catalytic reaction does not take place because of the lack of molecular oxygen. Also hypoxic cytotoxins undergo one-electron reduction to produce cytotoxic radicals, but do not undergo one-electron reduction in aerobic conditions.[12] We focused on this point to develop hypoxia-targeting IDO inhibitors.

To our knowledge, hypoxia-targeting IDO inhibitors have not been previously reported. Our strategy involved the hybridization of the hypoxic cytotoxin TPZ to the known IDO inhibitor 1MT to develop hypoxia-targeting IDO inhibitors. The TPZ moiety involved in the hybrids is also expected to work as a hypoxic cytotoxin. First, we examined whether 1MT-TPZ hybrids have IDO inhibitory activity or not. Even though the 1MT-TPZ hybrids have the 1MT unit in the molecules, they nonetheless showed uncompetitive inhibition, different from that of 1MT (competitive inhibition, as shown in Fig. 3). An uncompetitive inhibitor is able to bind to only an enzyme–substrate complex, not to an enzyme active site.[26] Brastianos et al. have reported that exiguamine A derived from a marine sponge possesses strong IDO inhibitory activity.[17] Furthermore, Carr et al. have reported that analogues of exiguamine A, compounds that are not indoleamine analogue, showed uncompetitive inhibition.[27] 1MT-TPZ hybrids have IDO inhibitory activities, and the TPZ-monoxide hybrids are stronger IDO inhibitors. We found that the HOMO coefficients of TPZ-monoxide hybrids **1** and **3** are localized at their 1MT moieties, in contrast to the HOMO coefficients of TPZ hybrids **2** and **4** that are localized at the TPZ moieties. The latter have lower IDO inhibitory activities (Fig. 2A and B). Based on the evidence that the combination of 1MT with other chemotherapeutic agents, such as paclitaxel and cyclophosphamide, increased their therapeutic effects,[10,28] we examined the combinations of 1MT **5** with TPZ **6** or that of 1MT **5** with TPZ-monoxide **7**. The combination of 1MT **5** with TPZ **6** was more effective than a single treatment of 1MT **5**, whereas the combination of 1MT with TPZ-monoxide is the same as a single treatment of 1MT **5**. Further in vivo studies should be carried out to evaluate effects of the combination 1MT **5** with TPZ **6**.

We also examined hypoxia-selective cytotoxicity of the 1MT-TPZ hybrids by clonogenic cell survival. Hypoxic cytotoxicities of TPZ hybrids **2** and **4** were up to about 1.5-fold higher compared to their lead compound TPZ, and we observed preferential hypoxic cytotoxicities. Nagasawa et al. have reported that hypoxic selectivity of TPZ to EMT6/KU cells at 10 μM concentration was 2.9, which was estimated by the ratio of the colony number formed under the aerobic and hypoxic conditions.[23] Consistent with this, TPZ showed similar hypoxic selectivity (HCR = 3.1). TPZ hybrids have higher hypoxia-selective cytotoxicity than their metabolite TPZ-monoxide hybrids as shown in Table 2. The TPZ metabolite TPZ-monoxide **7** and TPZ-monoxide hybrids **1** and **3** did not display cytotoxicity. However, hybrids **1** and **3** showed cytotoxicities at the higher drug concentration of more than 40 μM. These cytotoxicities may be due to the action of the *N*-methyl indole and benzotriazine moieties as DNA intercalators.[29] These data were consistent with their molecular orbital calculations in that their LUMO coefficients were localized at the TPZ moieties, as shown in Figure 2. In addition, E_{LUMO} values of the TPZ hybrids were lower than those of the TPZ-monoxide hybrids (**2**: $E_{LUMO} = -1.6422$ eV; **1**: $E_{LUMO} = -1.0791$ eV). For this reason, TPZ hybrids were more easily reduced to produce their cytotoxic radicals, and these data suggest that 1MT-TPZ hybrids can work effectively not only as IDO inhibitors but also as hypoxic cytotoxins. TPZ hybrids have stronger hypoxia-selective cytotoxicities than TPZ itself. Based on the above observations and considerations, we propose the following explanation for our results, shown in Scheme 4: first, TPZ hybrids **2** are taken into hypoxic cells and suffer one-electron reduction by reductases to generate hydroxyl radicals or TPZ radicals **2a** that cause DNA double strand breaks. At the same time, TPZ hybrids **2** are metabolized to TPZ-monoxide hybrids **1**, which are stronger IDO inhibitors compared to the parent TPZ hybrids **2**. TPZ hybrids **4** also function in the same manner as **2**. TPZ hybrids **2** and **4** can attack hypoxic cells as dual antineoplastic agents functioning as hypoxic cytotox-



Scheme 4. Proposed mechanism for IDO inhibition of 1MT-TPZ hybrid **2**.

ins and IDO inhibitors. These 1MT-TPZ hybrids will be expected to increase therapeutic benefit for cancer patients because of the decreased patient's burden and increased effective and selective therapeutic effects. In this study, to investigate our hypothesis we evaluated the biological activities of 1MT-TPZ hybrids. Further studies on their mode of action and their in vivo effects remain to be studied. Many previous medicinal chemistry studies on hypoxic cytotoxins have been focused only on the development of more clinically effective TPZ analogues, especially Hay et al. have reported pharmacokinetics and pharmacodynamics studies of TPZ analogues.[12,13] We suggest in our present studies that not only TPZ and TPZ analogues, but also TPZ-monoxide analogues have potential as promising antineoplastic agents. An additional important consideration for developing hybrid drugs is the fact that our designed 1MT-TPZ hybrids retained both IDO inhibitory activities and hypoxic cytotoxicities. We expect that additional design of hybrid drugs such as these will increase in importance as a strategy for development of antineoplastic agents.

In conclusion, 1MT-TPZ hybrids **1**–**4** were potent uncompetitive IDO inhibitors. TPZ-monoxide hybrids **1** and **3** were stronger inhibitors than TPZ hybrids **2** and **4**. Hybrid **1** was the most potent among them. TPZ hybrids **2** and **4** have higher hypoxia-selective cytotoxicity than TPZ. We have developed hypoxia-targeting IDO inhibitors with unique modes of action in that TPZ hybrids work first as hypoxic cytotoxins and then their metabolites TPZ-monoxide hybrids function as IDO inhibitors.

4. Experimental

4.1. Chemistry

All chemicals and drugs were purchased from Wako Pure Chemical industries, Ltd (Osaka, Japan) and Sigma–Aldrich Japan (Tokyo, Japan). Melting points were determined on a Yanaco melting point apparatus (MP-S3 model) or Round Science RFS-10. NMR spectra were obtained on a JEOL JNM-EX400 spectrometer at 400 MHz for ^{1}H spectra. Spectra were obtained in $CDCl_3$ unless otherwise specified, and are referenced to Me_4Si. Chemical shifts and coupling constants were recorded in units of ppm and Hz, respectively. IR spectra were measured in KBr pellets on a Perkin-Elmer spectrum RX1-T1 spectrometer. Mass spectra were determined on a JEOL JMS-AX-500 mass spectrometer using an ionizing potential of 20 eV. Fast atom bombardment mass spectra (FABMS) and high-resolution mass spectra (FABHRMS) were

measured on a JEOL JMS-DX-303 instrument. Elemental analyses were performed with a Yanaco CHN Corder (MT-5). Solvents were evaporated under reduced pressure on a rotary evaporator. Thin-layer chromatography was carried out on aluminum-backed silica gel (Merck 60 F_{254}) with visualization of components by UV light (254 or 365 nm). Column chromatography was carried out on KANTO Chemical silica gel 60 N (spherical, neutral) 40–50 μm. EtOAc refers to ethyl acetate; MeOH refers to methanol; DIPCDI refers to *N*,*N*′-diisopropylcarbodiimide; HOBt refers to 1-hydroxybenzotriazole. All solvents were freshly distilled. Tirapazamine **6**,[30] TPZ-monoxide **7**,[11,31,32] 3-Cl **9**[19] were synthesized as previously described.

4.1.1. Boc-*N*-1-Methyl-L-tryptophan (8)

1-Methyl-L-trp **5** (54 mg, 0.25 mmol) was suspended in H_2O/1,4-dioxane = 1:2 (0.75 mL), and 1 M NaOH (250 μL) and $(Boc)_2O$ (45 μL, 0.20 mmol) were added. The reaction mixture was stirred at room temperature for 4 h. The solvent was evaporated and the residue was cooled in an ice-bath and acidified with 1 M HCl aq (pH 2). The reaction mixture was extracted with EtOAc (3× 15 mL) and the organic layer was washed with brine, dried with Na_2SO_4, and the solvent was evaporated. The residue was purified by column chromatography, eluting with a gradient (5–10%) of MeOH/CH_2Cl_2 to give **8** (76.5 mg, 96%) as a pale purple powder: mp 140–142 °C; ^{1}H NMR δ 7.59 (d, J = 8.0 Hz, 1H), 7.33 (d, J = 8.3 Hz, 1H), 7.22 (ddd, J = 7.6, 7.0, 1.0 Hz, 1H), 7.11 (ddd, J = 7.4, 7.1, 1.0 Hz, 1H), 6.91 (s, 1H), 3.74 (s, 3H, Me), 3.33 (m, 2H, CH_2), 1.42 (s, 9H, *t*-Bu); IR (KBr) 3326, 2985, 1726, 1660, 1480, 1406, 1367, 1250, 1160, 743 cm^{-1}; EIMS *m*/*z*: M^+ 318 (4.8), 245 (2.0), 201 (1.6).

4.1.2. [5-(1-Oxido-1,2,4-benzotriazine-3-yl)pentyl]-1,5-pentanediamine (10)

Chloride **9** (20 mg, 0.11 mmol) was dissolved in CH_2Cl_2 (2 mL). Et_3N (46 μL, 0.33 mmol) and 1,5-pentanediamine (50 μL, 0.40 mmol) were added. The reaction mixture was stirred at room temperature for 2 days. The reaction mixture was partitioned between CH_2Cl_2 and 0.1 M HCl aq, and the aqueous layer was washed with CH_2Cl_2 (3× 30 mL). The aqueous layer was made basic with 2 M NaOH and extracted with CH_2Cl_2 (3× 20 mL). The organic layer was washed with brine, dried with $MgSO_4$, and the solvent was evaporated. The residue was dried in vacuo to give 1-oxide **10** (27.7 mg, quant.y) as a yellow solid: ^{1}H NMR [CD_3OD] δ 8.22 (dd, J = 8.5, 0.7 Hz, 1H), 7.76 (ddd, J = 8.5, 6.9, 1.2 Hz, 1H), 7.59 (d, J = 8.3 Hz, 1H), 7.34 (ddd, J = 8.4, 7.2, 1.2 Hz, 1H), 3.48 (t, J = 7.1 Hz, 2H, CH_2NH_2), 2.93 (t, J = 7.6 Hz, 2H, CH_2NH), 1.72 (m, 4H, CH_2× 2), 1.51 (m, 2H, CH_2); IR (KBr) 3308, 2934, 1571, 1420, 1326, 761 cm^{-1}.

4.1.3. [4-(1-Oxido-1,2,4-benzotriazine-3-yl)butyl]-1,4-butanediamine (11)

Chloride **9** (20 mg, 0.11 mmol) was dissolved in CH_2Cl_2 (2 mL). Et_3N (46 μL, 0.33 mmol) and 1,4-butanediamine (34.6 mg, 0.33 mmol) were added. The reaction mixture was stirred at room temperature for 14.5 h. The reaction mixture was partitioned between CH_2Cl_2 and 0.1 M HCl aq, and the aqueous layer was washed with CH_2Cl_2 (3× 20 mL). The aqueous layer was made basic with 2 M NaOH and extracted with CH_2Cl_2 (3× 25 mL). The organic layer was washed with brine, dried with $MgSO_4$, and the solvent was evaporated. The residue was dried in vacuo to give 1-oxide **11** (25.9 mg, 100%) as a yellow solid: ^{1}H NMR [CD_3OD] δ 8.20 (ddd, J = 8.5, 1.5, 0.5 Hz, 1H), 7.75 (ddd, J = 8.5, 6.9, 1.5 Hz, 1H), 7.58 (d, J = 8.1 Hz, 1H), 7.32 (ddd, J = 8.5, 7.1, 1.2 Hz, 1H), 3.48 (t, J = 7.1 Hz, 2H, CH_2NH_2), 2.72 (t, J = 7.1 Hz, 2H, CH_2NH), 1.66–1.75 (m, 2H, CH_2), 1.56–1.63 (m, 2H, CH_2); IR (KBr) 3294, 2936, 1575, 1497, 1417, 1322, 761 cm^{-1}.

4.1.4. 5-{(1-Oxido-1,2,4-benzotriazin-3-yl)aminopentyl}-2,2,2-trifluoroacetamide (12)

Amine **10** (121.8 mg, 0.50 mmol) was suspended in CH_2Cl_2 (3 mL), and $(CF_3CO)_2O$ (690 μL, 5.00 mmol) was added. The reaction mixture was stirred at room temperature for 24 h, after which time the solvent was evaporated. The residue was purified by reprecipitation to give **12** (165 mg, 98%) as a yellow solid: mp 183–184 °C; ^{1}H NMR δ 8.30 (dq, J = 8.6, 0.5 Hz, 1H), 7.74 (ddd, J = 8.5, 7.2, 1.2 Hz, 1H), 7.62 (dd, J = 8.5, 1.0 Hz, 1H), 7.33 (ddd, J = 8.5, 7.2, 1.5 Hz, 1H), 6.44 (br s, 1H, $NHCH_2$), 3.55 (q, J = 6.8 Hz, 2H, CH_2NHCO), 3.40 (q, J = 6.6 Hz, 2H, CH_2NH), 1.64–1.77 (m, 2H, CH_2), 1.44–1.52 (m, 2H, CH_2), 0.84–1.01 (m, 2H, CH_2); IR (KBr) 3315, 2934, 1704, 1572, 1498, 1421, 1207, 1181, 760 cm^{-1}; Anal. Calcd for $C_{14}H_{16}F_3N_5O_2$: C, 48.98; H, 4.70; N, 20.40. Found: C, 48.81; H, 4.92; N, 20.21.

4.1.5. 4-{(1-Oxido-1,2,4-benzotriazin-3-yl)aminobutyl}-2,2,2-trifluoroacetamide (13)

Amine **11** (228.5 mg, 0.97 mmol) was suspended in CH_2Cl_2 (5 mL) and $(CF_3CO)_2O$ (1.35 mL, 9.70 mmol) was added. The reaction mixture was stirred at room temperature for 46 h, after which time the solvent was evaporated. The residue was purified by reprecipitation to give **13** (348.1 mg, quant.y) as a yellow solid: mp 199–203 °C; ^{1}H NMR [CD_3OD] δ 8.21 (dq, J = 8.6, 0.5 Hz, 1H), 7.75 (ddd, J = 8.5, 6.9, 1.5 Hz, 1H), 7.59 (d, J = 8.8 Hz, 1H), 7.32 (ddd, J = 8.5, 7.3, 1.2 Hz, 1H), 3.47–3.52 (m, 2H, CH_2NHCO) 3.30–3.36 (m, 2H, CH_2NH), 1.66–1.73 (m, 4H, 2× CH_2); IR (KBr) 3293, 2939, 1700, 1587, 1497, 1420, 1182, 762 cm^{-1}. Anal. Calcd for $C_{13}H_{14}F_3N_5O_2$: C, 47.42; H, 4.29; N, 21.27. Found: C, 47.21; H, 4.46; N, 21.06.

4.1.6. 5-{(1,4-Dioxido-1,2,4-benzotriazin-3-yl)aminopentyl}-2,2,2,-trifluoroacetamide (14)

Trifluoroacetamide **12** (52.6 mg, 0.15 mmol) was suspended in CH_2Cl_2 (3 mL), and $(CF_3CO)_2O$ (209 μL, 1.50 mmol) was added. The reaction mixture was stirred at 0 °C for 10 min, then 35% H_2O_2 (143 μL, 1.50 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 24 h. The reaction mixture was poured into brine and made basic with 28% aq NH_3, and extracted with CH_2Cl_2 (3× 10 mL). The organic layer was dried with Na_2SO_4 and the solvent was evaporated. The residue was purified by column chromatography eluting with a gradient (0–10%) of MeOH/EtOAc to give dioxide **14** (28.4 mg, 52%) as a red solid and starting material **12** (19.3 mg, 37%) as a yellow solid: mp 197–199 °C; ^{1}H NMR δ 8.34 (dd, J = 8.8, 1.2 Hz, 1H), 8.27 (d, J = 8.8 Hz, 1H), 7.91 (ddd, J = 8.5, 7.2, 1.2 Hz, 1H), 7.54 (t, J = 7.8 Hz, 1H), 6.56 (br s, 1H, $NHCH_2$), 3.65 (q, J = 6.1 Hz, 2H, CH_2NHCO), 3.39 (q, J = 6.6 Hz, 2H, CH_2NH), 1.75–1.87 (m, 2H, CH_2), 1.69 (quin, J = 7.3 Hz, 2H, CH_2), 1.45–1.53 (m, 2H, CH_2); IR (KBr) 3277, 2936, 1702, 1636, 1497, 1412, 1184, 767 cm^{-1}. EIMS *m*/*z* 359 (M^+); Anal. Calcd for $C_{14}H_{16}F_3N_5O_3$: C, 46.80; H, 4.49; N, 19.49. Found: C, 46.64; H, 4.33; N, 19.49.

4.1.7. 4-{(1,4-Dioxido-1,2,4-benzotriazin-3-yl)aminobutyl}-2,2,2,-trifluoroacetamide (15)

Compound **13** (132.1 mg, 0.40 mmol) was suspended in CH_2Cl_2 (4 mL), and $(CF_3CO)_2O$ (556 μL, 4.00 mmol) was added. The reaction mixture was stirred at 0 °C for 5 min, then 35% H_2O_2 (329 μL, 4.00 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 21.5 h. The reaction mixture was poured into brine and made basic with 28% aq NH_3, and extracted with CH_2Cl_2 (3× 30 mL). The organic layer was dried with Na_2SO_4 and the solvent was evaporated. The residue was purified by column chromatography eluting with a gradient (0–10%) of MeOH/EtOAc to give dioxide **15** (69 mg, 50%) as a red solid and starting material **13** (95 mg, crude) as a yellow solid: mp

193–196 °C; ^{1}H NMR δ 8.34 (dd, J = 8.8, 1.2 Hz, 1H), 8.28 (d, J = 8.3 Hz, 1H), 7.91 (t, J = 7.3 Hz, 1H), 7.54 (t, J = 7.9 Hz, 1H), 7.33 (br s, 1H, NHCO), 6.63 (br s, 1H, $NHCH_2$), 3.68 (d, J = 5.4 Hz, 2H, CH_2NHCO), 3.45 (q, J = 6.6 Hz, 2H, CH_2NH), 1.75–1.84 (m, 4H, 2× CH_2); IR (KBr) 3269, 2940, 1703, 1627, 1496, 1415, 1363, 1183, 1090, 722 cm^{-1}; Anal. Calcd for $C_{13}H_{14}F_3N_5O_3$: C, 45.22; H, 4.09; N, 20.28. Found: C, 45.23; H, 4.07; N, 20.44.

4.1.8. [5-(1,4-Dioxido-1,2,4-benzotriazine-3-yl)pentyl]-1,5-pentanediamine hydrochloride (16)

Compound **14** (68.2 mg, 0.19 mmoL) was suspended in MeOH (6.0 mL), and 28% aq NH_3 (6.0 mL) was added. The reaction mixture was stirred at room temperature for 6 days, and the solvent was then evaporated. The residue was acidified with 2 M HCl aq, and the aqueous layer was washed with CH_2Cl_2 (3× 10 mL) and EtOAc (3× 10 mL). The water was distilled off azeotropically at 40 °C to give amine HCl salt **16** (57.5 mg, quant.y) as a yellow solid that was used without further purification: ^{1}H NMR [CD_3OD] δ 8.36 (dd, J = 8.66, 1.22 Hz, 1H), 8.13 (t, J = 8.3 Hz, 1H), 8.02 (d, J = 8.5 Hz, 1H), 7.69 (t, J = 7.6 Hz, 1H), 3.65 (t, J = 7.7 Hz, 2H, ($CH_2NH_3^+$), 2.95 (t, J = 7.3 Hz, 2H, CH_2NH), 1.70–1.84 (m, 4H, 2× CH_2), 1.49–1.57 (m, 2H, CH_2NH).

4.1.9. [4-(1,4-Dioxido-1,2,4-benzotriazine-3-yl)butyl]-1,4-butanediamine hydrochloride (17)

Compound **15** (94.9 mg, 0.27 mmol) was suspended in MeOH (7.0 mL). 28% aq NH_3 (7.0 mL) was added. The reaction mixture was stirred at room temperature for 4 days and the solvent was then evaporated. The residue was acidified with 2 M HCl aq, and the aqueous layer was washed with CH_2Cl_2 (3× 10 mL) and EtOAc (3× 10 mL). The water was distilled off azeotropically at 40 °C to give amine HCl salt **17** (79.5 mg, quant.y) as an orange powder that was used without further purification: ^{1}H NMR [CD_3OD] δ 8.34 (d, J = 7.8 Hz, 1H), 8.20 (d, J = 8.5 Hz, 1H), 8.02 (ddd, J = 8.5, 7.0, 1.2 Hz, 1H), 7.62 (t, J = 7.0 Hz, 1H), 3.62 (t, J = 6.3 Hz, 2H, ($CH_2NH_3^+$), 3.00 (t, J = 6.1 Hz, 2H, CH_2NH), 1.71–1.84 (m, 4H, 2× CH_2).

4.1.10. [5-(1-Oxido-1,2,4-benzotriazin-3-yl)aminopentyl]-(2*S*)-*N*-*tert*-butoxycarbonyl-2-amino-(1-methyl-indole-3-yl)propanamide (1; TX-2236)

Boc-*N*-1MT **8** (40.9 mg, 0.13 mmol), HOBt (20 mg, 0.13 mmol), and **10** (29.6 mg, 0.12 mmol) were dried in vacuo for 30 min. After CH_2Cl_2 (3 mL) was added to the mixture, DIPCDI (21 μL, 0.13 mmol) was added and the reaction mixture was stirred at room temperature for 2.5 h. The solvent was evaporated and the residue was purified by column chromatography eluting with a gradient (75–100%) of EtOAc/hexane to give **1** (64.9 mg, 99%) as a yellow powder: mp 180–183 °C; ^{1}H NMR [CD_3OD] δ 8.28 (d, J = 8.78 Hz, 1H), 8.14 (t, J = 9.02 Hz, 1H), 7.92–8.00 (m, 1H), 7.49–7.60 (m, 2H), 7.29 (d, J = 8.53 Hz, 1H), 7.11 (t, J = 7.31 Hz, 1H), 7.00 (t, J = 7.31 Hz, 1H), 6.96 (s, 1H), 3.47–3.51 (m, 2H, CH_2NHCO), 3.33–3.40 (m, 2H, CH_2NH), 2.96–3.21 (m, 4H, 2× CH_2), 1.62–1.82 (m, 2H, CH_2), 1.22–2.00 (m, 9H, *t*-Bu); IR (KBr) 3338, 2936, 1685, 1653, 1363, 1173, 760 cm^{-1}; Anal. Calcd for $C_{29}H_{37}N_7O_4$: C, 63.60; H, 6.81; N, 17.90. Found: C, 63.36; H, 6.75; N, 17.88.

4.1.11. [5-(1,4-Dioxido-1,2,4-benzotriazin-3-yl)aminopentyl]-(2*S*)-*N*-*tert*-butoxycarbonyl-2-amino-(1-methyl-indole-3-yl)propanamide (2; TX-2235)

Boc-*N*-1MT **8** (61 mg, 0.19 mmol), HOBt (32 mg, 0.21 mmol), and amine **16** (57.5 mg, 0.19 mmol) were dried in vacuo for 30 min. After CH_2Cl_2 (7 mL) was added to the mixture, DIPCDI (33 μL, 0.21 mmol) and Et_3N (79 μL, 0.57 mmol) were added, and the reaction mixture was stirred at room temperature for 7 h. The solvent was evaporated and the residue was purified by column chromatography eluting with a gradient (5–10%) of MeOH/CH_2Cl_2 to give **2** (105.4 mg, 98%) as a red powder: mp 105–107 °C; ^{1}H NMR δ 8.33 (dd, J = 8.7, 1.2 Hz, 1H), 8.29 (d, J = 8.8 Hz, 1H), 7.87 (ddd, J = 8.5, 7.2, 1.2 Hz, 1H), 7.64 (d, J = 8.1 Hz, 1H), 7.50 (ddd, J = 8.7, 7.6, 1.5 Hz, 1H), 7.22 (ddd, J = 7.6, 6.9, 1.2 Hz, 1H), 7.11 (ddd, J = 8.0, 6.9, 1.0 Hz, 2H), 6.92 (s, 1H), 5.79 (br s, 1H, $NHCH_2$), 5.54 (br s, 1H, NHCO), 4.37 (br s, 1H, NHBoc), 3.84 (quin, J = 6.3, 6.6 Hz, 2H, CH_2NHCO), 3.74 (s, 3H, CH_3), 3.50–3.56 (m, 2H, CH_2NH), 3.27 (d, J = 5.1 Hz, 1H), 3.07–3.21 (m, 2H, CH_2), 1.59–1.70 (m, 6H, 3× CH_2), 1.39 (s, 9H, *t*-Bu); IR (KBr) 3322, 2932, 1685, 1652, 1595, 1496, 1414, 1365, 1178, 766 cm^{-1}; HRMS (FAB$^+$) calcd for $C_{29}H_{38}N_7O_5$ (M+H)$^+$ m/z 564.2937. Found 564.2949; Anal. Calcd for $C_{29}H_{37}N_7O_5$: C, 61.80; H, 6.62; N, 17.40. Found: C, 61.57; H, 6.63; N, 17.26.

4.1.12. [4-(1-Oxido-1,2,4-benzotriazin-3-yl)aminobutyl]-(2*S*)-*N*-*tert*-butoxycarbonyl-2-amino-(1-methyl-indole-3-yl)propanamide (3; TX-2228)

Boc-*N*-1MT **8** (41 mg, 0.13 mmol), HOBt (21.7 mg, 0.14 mmol), and **11** (30 mg, 0.13 mmol) were dried in vacuo for 30 min. After CH_2Cl_2 (2 mL) was added to the reaction mixture, DIPCDI (22 μL, 0.14 mmol) was added and the reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated and the residue was purified by column chromatography eluting with a gradient (75–100%) of EtOAc/hexane to give **3** (61.5 mg, 89%) as a yellow powder: mp 181–182 °C; ^{1}H NMR δ 8.25 (dd, J = 1.0, 8.5 Hz, 1H), 7.69 (ddd, J = 1.5, 7.1, 8.5 Hz, 1H), 7.57–7.64 (m, 2H), 7.27–7.36 (m, 2H), 7.20 (ddd, J = 1.0, 6.8, 7.6 Hz, 1H), 7.07 (ddd, J = 1.2, 7.0, 7.9 Hz, 1H), 6.91 (s, 1H), 5.85 (br s, 1H, $NHCH_2$), 5.30 (br s, 1H, NHCO), 4.39 (br s, 1H, NHBoc), 3.72 (s, 3H, CH_3), 3.09–3.48 (m, 6H, 3× CH_2), 1.76 (br, 2H, CH_2), 1.41 (m, 11H, *t*-Bu, CH_2); IR (KBr) 3331, 2937, 1688, 1655, 1360, 1172, 760 cm^{-1}; Anal. Calcd for $C_{28}H_{35}N_7O_4$: C, 63.02; H, 6.61; N, 18.37. Found: C, 62.95; H, 6.52; N, 18.35.

4.1.13. [4-(1,4-Dioxido-1,2,4-benzotriazin-3-yl)aminobutyl]-(2*S*)-*N*-*tert*-butoxycarbonyl-2-amino-(1-methyl-indole-3-yl)propanamide (4; TX-2234)

Boc-*N*-1MT **8** (84 mg, 0.26 mmol), HOBt (44 mg, 0.29 mmol) and **17** (75 mg, 0.26 mmol) were dried in vacuo for 30 min. After CH_2Cl_2 (5 mL) was added to the reaction mixture, DIPCDI (45 μL, 0.29 mmol) and Et_3N (92 μL, 0.79 mmol) were added and the reaction mixture was stirred at room temperature for 7 h. An additional 5 mL of CH_2Cl_2 was added and stirring was continued for 2 h. The solvent was evaporated and the residue was purified by column chromatography eluting with a gradient (5–10%) of MeOH/CH_2Cl_2. The product was reprecipitated from CH_2Cl_2/hexane to give **4** (135.5 mg, 93%) as a red powder: mp 108–110 °C; ^{1}H NMR δ 8.33 (d, J = 8.1 Hz, 1H), 8.29 (d, J = 8.8 Hz, 1H), 7.88 (ddd, J = 8.5, 7.2, 1.2 Hz, 1H), 7.63 (d, J = 7.8 Hz, 1H), 7.52 (t, J = 8.5 Hz, 1H), 7.16 (t, J = 8.1 Hz, 1H), 7.05–7.09 (m, 2H), 6.93 (s, 1H), 5.87 (br s, 1H, $NHCH_2$), 5.26 (br s, 1H, NHCO), 4.41 (br s, 1H, NHBoc), 3.84 (quin, J = 6.3, 6.6 Hz, 2H, CH_2NHCO), 3.74 (s, 3H, CH_3), 3.45 (q, J = 6.3 Hz, 2H, CH_2NH), 3.28–3.33 (m, 1H), 3.09–3.18 (m, 2H, CH_2), 1.66 (br, 4H, 2× CH_2), 1.42 (s, 9H, *t*-Bu); IR 3343, 2970, 1618, 1593, 1496, 1416, 1364, 1171, 768 cm^{-1}; HRMS (FAB$^+$) calcd for $C_{28}H_{36}N_7O_5$ (M+H)$^+$ m/z 550.2781. Found 550.2802; Anal. Calcd for $C_{28}H_{35}N_7O_5$: C, 61.19; H, 6.42; N, 17.84. Found: C, 60.92; H, 6.53; N, 17.98.

4.2. Assay of IDO

4.2.1. Materials

1-Methyl-L-tryptophan and L-kynurenine were purchased from Sigma–Aldrich. L-Tryptophan, ascorbic acid, methylene blue, bovine liver catalase, and *p*-dimethylaminobenzaldehyde were purchased from Wako chemicals (Osaka, JP). RhIDO was supplied by Dr. Sugimoto.

4.2.2. Kynurenine standard curve

The standard medium (200 μL) contained 1 M potassium phosphate buffer (20 μL, pH 6.5) at a final concentration of 100 mM, 0.4 M ascorbic acid (20 μL, neutralized with 1 M NaOH aq) at a final concentration of 40 mM, 1 mM methylene blue (4 μL) at a final concentration of 20 μM, 10 mg/mL catalase (4 μL) at a final concentration of 200 μg/mL, MilliQ water (112 μL), and a series of kynurenine solutions at final concentrations of 0, 10, 50, 100, 500 μM. To the assay mixture was then added 40 μL of 30% (w/v) of TCA. It was then centrifuged (13,000 rpm, 4 °C, 15 min). Aliquots (125 μL) of the supernatant were treated with 2% (w/v) *p*-dimethylaminobenzaldehyde in acetic acid (125 μL) in a 96-well microtiter plate. The yellow pigment derived from kynurenine was measured at 490 nm using a Model 550 microplate reader (Bio-Rad, Tokyo, Japan).

4.2.3. IDO inhibition assay

The enzyme activity assay was carried out according to the method of Matin[21] and Takikawa[22] with a minor modification. All activity assays were carried out in aerobic condition. The standard medium (200 μL) contained 1 M potassium phosphate buffer (20 μL, pH 6.5) at a final concentration of 100 mM, 0.4 M ascorbic acid (20 μL, neutralized with 1 M NaOH aq) at a final concentration of 40 mM, 1 mM methylene blue (4 μL) at a final concentration of 20 μM, 10 mg/mL catalase (4 μL) at a final concentration of 200 μg/mL, L-tryptophan (20 μL) at a final concentration of 0, 10, 20, 40, 80, 160 μM, with or without inhibitor solution in DMSO (10 μL) at a final concentration of 400 μM, MilliQ water (112 μL), and 0.5 μM rhIDO (20 μL) at a final concentration of 0.05 μM. The reaction mixture was incubated at 20 °C for 20 min. To the assay mixture was then added 40 μL of 30% (w/v) TCA. The mixture was incubated at 65 °C for 20 min to hydrolyze *N*-formylkynurenine to kynurenine. It was then centrifuged (13,000 rpm, 4 °C, 15 min). Aliquots (125 μL) of the supernatant were treated with 2% (w/v) *p*-dimethylaminobenzaldehyde in acetic acid (125 μL) in a 96-well microtiter plate. The yellow pigment derived from kynurenine was measured at 490 nm using a Model 550 microplate reader (Bio-Rad, Tokyo, Japan).

4.3. Hypoxia-selective cytotoxicity

EMT6/KU cells, a gift from Dr. Masunaga (Kyoto University, Kyoto), were cultured in Eagle minimum essential medium containing 12.5% (v/v) fetal bovine serum, and kanamycin (60 mg/L) at 37 °C in 5% CO_2. The surviving cell fractions after drug treatment under hypoxic or aerobic condition were determined in a clonogenic assay. Cells in late log phase were seeded in a 60-mm tissue culture dish at a density of 10^3 cells per dish in 1 mL of medium, 5×10^2 cells per dish in 0.5 mL of medium, 2×10^2 cells per dish in 1 mL of medium. All compounds were dissolved in DMSO. Compound solutions were diluted with culture medium at a concentration of 0.1, 1, 5, 10, 20, 40, and 80 μM for aerobic condition, and 0.1, 1, 2.5, 5, 10, 20, 40 μM for hypoxic condition. After growth in a CO_2 incubator for 24 h, culture medium was exchanged to drug containing medium. The cells were then placed in a hypoxic chamber flushed with 5% CO_2 and 95% N_2 at 37 °C for 5 h at flow rates of 1.5–2.0 nL/min for hypoxic condition, for the aerobic condition were placed in a CO_2 incubator at 37 °C for 5 h. After the treatment, drugs were removed by exchanging the medium for a fresh one and the cells were cultured to form colonies for 7–10 days. The colonies obtained were fixed with methanol, stained with 5% Giemsa solutions, and counted. Hypoxic cytotoxicity ratio (HCR) was calculated as HCR = IC_{50} (Aero)/IC_{50} (Hypo).

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