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Design and Synthesis of New Mitochondrial Cytotoxin N-Thiadiazolylanilines that Inhibit Tumor Cell Growth

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Abstract—New N-thiadiazolylanilines were designed and synthesized to develop mitochondrial cytotoxins superior to SF 6847. The mitochondrial cytotoxin N-thiadiazolylanilines, TX-108 and TX-109, inhibited EMT6/KU mammary sarcoma cell growth at a low micromolar concentration. Their inhibitory activities were parallel to their mitochondrial cytotoxicity, such as uncoupling oxidative phosphorylation and inhibiting ATP synthesis. This report also supports the notion that the inhibition of tumor cell growth of inhibitor of protein tyrosine kinase AG17, which is identical to SF 6847, may be due to its mitochondrial cytotoxicity.

Introduction

Mitochondrial cytotoxins have been reinvestigated as promising lead compounds for antitumor drug development.[1-6] The mitochondrial cytotoxins involve most of the uncouplers of oxidative phosphorylation in mitochondria used as reagents for bioenergetics.[7] They were originally developed from biocidal drugs, such as pesticides and bacteriocidal drugs for use in agricultural. Most of the uncouplers represent rather hydrophobic and weakly acidic protonophores such as substituted phenols. Thus, the negative charge of their anionic form is highly delocalized, which decreases hydration and therefore makes the phospholipid bilayer permeable to the anionic form of these uncouplers. The mechanisms of proton transfer by uncouplers have been extensively studied using model phospholipid membranes.[8]

A preclinical approach using mitochondrial uncouplers as drugs to treat human diseases has been considered. The mitochondrial uncoupler, carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP), reportedly modulates the activity of Alzheimer protein kinases and of protein phosphatases through ATP depletion.[2] Although FCCP acts through ATP depletion, it raises intracellular calcium.[3] Mitochondrial uncouplers may therefore have many unknown effects on cells. Secondly, SF 6847 (AG17), which is the most potent uncoupler known, inhibits several tyrosine kinases[9,10] and human pancreatic cancer cell growth at micromolar concentrations.[1,9] We tested SF 6847 as a tyrosine kinase inhibitor, not as an uncoupler, to block

the uncontrolled signalling from receptor and intracellular tyrosine kinases that lead to cancer. We then evaluated the antitumor activities of our anilinic and nonphenolic uncouplers developed as mitochondrial cytotoxins. Thus, we considered that the total antitumor activity of SF 6847 is partially due to its mitochondrial cytotoxicity, because its effective concentration for antitumor activity was much higher (10^3-fold) than that for its activity. SF 6847[11,12] was initially discovered as a pesticidal agent.[13] It also belongs to a class of phenols, in which the phenolic hydroxyl group is sterically hindered by two surrounding bulky hydrophobic tert-butyl groups, which occlude the phenolic hydroxyl or phenoxide groups from the environment.[12] Thus the phenolic compound SF 6847 acts as a protonophore.

To develop new antitumor agents, we constituted electron-affinic arylidenecyclopentenedione derivatives, which acted as antitumor agents,[14] hypoxic cell radiosensitizers and cytotoxins,[15] as well as mitochondrial cytotoxins.[16] In this report, we examined the inhibition by new N-thiadiazolylaniline derivatives on the growth of EMT6/KU mammary sarcoma cell line as well as their mitochondrial cytotoxicity, such as the uncoupling of oxidative phosphorylation and the inhibition of ATP synthesis. N-Thiadiazolylaniline derivatives have not been investigated as uncouplers of oxidative phosphorylation except for the development of herbicides.[17] The EMT6/KU mammary sarcoma cell line is used to screen hypoxic cell radiosensitizers and cytotoxins because it grows as single cells, spheroids, or solid tumors.[18-20] We present here evidence that some N-thiadiazolylaniline derivatives inhibit tumor cell growth in parallel with the uncoupling of mitochondrial oxidative phosphorylation.

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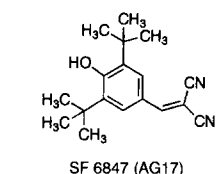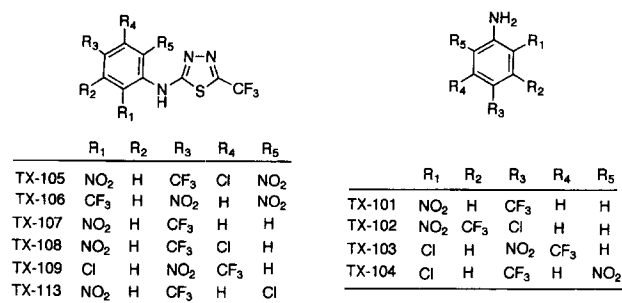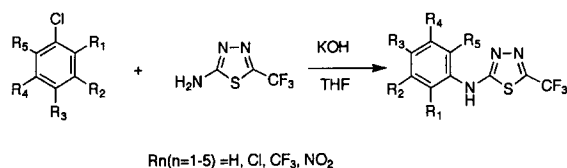
Figure 1. Chemical structures of thiadiazolylanilines and SF 6847 (AG17).

The structures of TX-113 and TX-109 were determined by comparing their spin–spin coupling constants (J value) and chemical shifts of ^{1}H NMR. Thus, the J values of TX-109 (1 Hz) and TX-113 (2 Hz) supported the presence of two protons in the *para* and *meta* position to each other, respectively. We estimated the chemical shifts (δ) of two protons of TX-109 and TX-113 from an approximation based on the parameter of substituent effects.[21] The known parameters of the substituent effects of the nitro and chloro groups were *ortho* = +0.95 and +0.03, *meta* = +0.26 and −0.03 and *para* = +0.38 and −0.02, respectively. The parameter of the substitution effect of trifluoromethyl group was estimated to be *ortho* = +0.12−+0.42 and *meta* = +0.15−+0.23, by calculating from the NMR data[22] of the following compounds: 3,5-bis(trifluoromethyl)aniline, 2-nitro-4-trifluoromethylphenol, *p*-trifluoromethylbenzaldehyde, *p*-trifluoromethylbenzoic acid, 3,5-bis(trifluoromethyl)-benzonitrile, 1-chloro-2,6-dinitro-4-trifluoromethyl-benzene, 1-chloro-2,4-dinitro-6-trifluoromethylbenzene, 1,3-dichloro-2,4-dinitro-6-trifluoromethylbenzene, 1,2-dichloro-4-trifluoromethylbenzene, 1,5-dichloro-4-nitro-2-trifluoromethylbenzene. From these data together with the substituent effects of thiadiazolylamino group deduced from the NMR data of TX-106, TX-107 and TX-108, the structures of TX-113 and TX-109 were determined to be 2-(2-chloro-6-nitro-4-trifluoromethyl-anilino)-5-trifluoromethyl-1,3,4-thiadiazole and 2-(2-chloro-4-nitro-5-trifluoromethylanilino)-5-trifluoro-methyl-1,3,4-thiadiazole, respectively. Finally, this structure of TX-109 was confirmed by X-ray crystallography (data not shown). We also obtained their parent compounds (highly substituted anilines), namely TX-101, 102, 103 and TX-104, under stronger reaction conditions than those used for TX-105–TX-113. All these structures were completely determined by their spectral analyses.

Results

Chemistry

To develop new mitochondrial cytotoxins as chemical modifiers for use in cancer treatment, we designed and synthesized several *N*-thiadiazolylaniline derivatives (Fig. 1) such as TX-105, 106, 107, 108, 109 and 113, from the reaction of 2-amino-5-trifluoromethyl-1,3,4-thiadiazole with the correspnding chloro-trifluoromethylbenzene derivative and potassium hydroxide in THF at room temperature (Scheme 1). The structures of the derivatives were determined by their NMR and IR spectra. The structure of highly substituted aniline derivative TX-105 was confirmed by X-ray crystallographic analysis as shown in Figure 2.
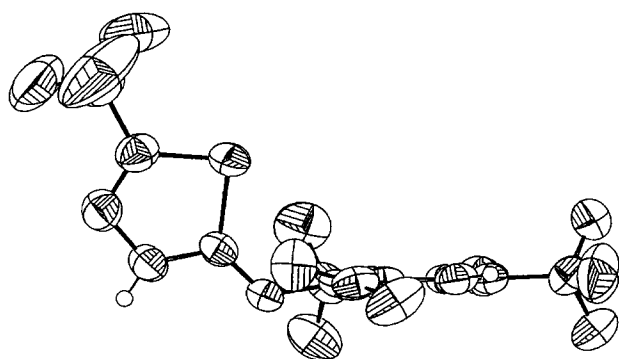
Biological activities

Uncoupling activity. We examined the activities of *N*-thiadiazolylanilines, such as TX-105, 106, 107, 108, 109 and 113, in rat liver mitochondria (RLM). When *N*-thiadiazolylanilines were added to state 4 mitochondria, they all stimulated the respiratory rate, and their activities were not dependent on the concentration of phosphate in the reaction mixture. From their titration curves, we measured their parameters such as the minimal molar concentration of uncouplers required to induce the maximal respiration release (C_{max}), the molar concentration of the uncoupler needed to double the state 4 respiratory rate (C_{200}), and the maximum stimulated respiratory rate (V_{max}). The successive addition of almost all the uncouplers induced an almost linear increase in respiration to reach the maximum stimulated respiratory rate. As shown in Table 1, TX-108 ($C_{max} = 14$ nM, $V_{max} = 210$ natom O mg^{-1} min^{-1}) had higher activity than SF 6847 ($C_{max} = 21$ nM, $V_{max} = 230$ natom O mg^{-1} min^{-1}). TX-109 had lower activity ($C_{max} = 45$ nM) than SF 6847, but highly stimulated the

Scheme 1.

Figure 2. X-Ray crystallographic structure of TX-105.

Table 1. Antitumor activity (EC_{50}), uncoupling activity (C_{max}), and physical data of N-thiadiazoylanilines

Compounds	EC_{50} (μM)	C_{max} (μM)	V_{max} natom O mg^{-1} min^{-1}	pK_a	log P
TX-105	25	7	240	6.1	1.024
TX-106	17	6	205	6.2	0.744
TX-107	2.5	0.11	250	7.6	0.528
TX-108	0.43	0.014	210	7.8	0.262
TX-109	1.3	0.045	340	6.2	0.610
TX-113	2.0	0.095	150	6.6	0.418
SF 6847	1.8	0.021	230	6.8	1.080

EC_{50} (μM): EMT6/KU.
C_{max} (μM); V_{max} natom O mg^{-1} min^{-1}: rat liver mitochondria.

maximal respiratory rate ($V_{max} = 340$ natom O mg^{-1} min^{-1}) compared with SF 6847. The respiratory rate being highly stimulated by TX-109 means that it has less influence upon the respiratory chain than SF 6847. The total evaluation suggested that both TX-108 and TX-109 are potent uncouplers of oxidative phosphorylation comparable to SF 6847, the most potent uncoupler known to date. The further addition of TX-105 and TX-106 caused the titration curve to bend before reaching the V_{max}. We therefore examined whether or not these uncouplers inhibit the respiratory chain in mitochondria. They dose-dependently inhibited the stimulated respiration induced by SF 6847, suggesting that they affect the respiratory chain. To confirm the effects of the thiadiazolyl group on uncoupling, we examined the effects of their parent anilines without a N-thiadiazolyl group, such as TX-101, 102, 103 and TX-104, in RLM. Table 3 shows that all the compounds had less activities at the micromolar concentration of the C_{max} and a low V_{max}, than those of the corresponding N-thiadiazolylanilines, suggesting the important contribution of thiadiazolyl group to the properties of these protonophores.

ATP synthesis. We further examined the inhibitory activities (I_{100}) of these anilines on ATP synthesis in

Table 2. ATP Synthesis inhibition by N-thiadiazoylanilines

Compounds	I_{100} (μM)
TX-105	7
TX-106	5
TX-107	0.12
TX-108	0.07
TX-109	0.05
TX-113	0.25
SF 6847	0.03

RLM. All the aniline derivatives inhibited ATP synthesis at concentrations from nanomolar to micromolar as shown in Tables 2 and 3. TX-108 and TX-109 completely inhibited ATP synthesis at concentration of 0.07 μM and 0.05 μM, respectively. These concentrations were higher than those required for uncoupling activity.

Inhibition of tumor cell growth. We evaluated the inhibitory activities of TX-series mitochondrial cytotoxins on the growth of EMT6/KU mammary sarcoma cells by means of the MTT assay. As shown in Table 1, we found that the inhibitory activities of the TX-series of mitochondrial cytotoxins were proportional to those of their ability to uncouple oxidative phosphorylation and inhibit ATP syntheses in rat liver mitochondria. Thus, their antitumor activities (EC_{50}) were graded as follows: TX-108 > TX-109 > SF 6847 > TX-113 > TX-107 > TX-106 > TX-105. The highly potent mitochondrial cytotoxins such as TX-108 and TX-109 inhibited growth at concentrations of 0.43 and 1.3 μM, respectively. They were more potent inhibitors of tumor cell growth than SF 6847, which has an EC_{50} of 1.8 μM.

Discussion

Many mitochondrial cytotoxins, especially protonophoric uncouplers of oxidative phosphorylation, have been developed only as agricultural chemicals, not as drugs that may be useful in the treatment of human disease. There are a few reports describing the carcinostatic effect of benzal malononitriles on mice[23] and the use of 2,4-dinitrophenol as an anti-obesity drug.[24] Many studies on the bioenergetics of cancer cells have not yielded anti-cancer drugs that will selectively inhibit the energy metabolism of cancer cells,[25-27] despite the progress of biochemical

Table 3. Uncoupling activity ATP synthesis inhibition, and physical data of electron-affinic anilines

Compounds	C_{max} (μM)	C_{200} (μM)	V_{max} (natom O mg^{-1} min^{-1})	I_{100} (μM)	log P
TX-101	52	17	170	104	0.841
TX-102	5.3	1.0	220	8	0.896
TX-103	75	23	190	82	0.620
TX-104	33	10	130	57	0.754

oncology.[28] We designed N-thiadiazolylanilines which are new potent uncouplers of oxidative phosphorylation as mitochondrial cytotoxins which might be as valuable for clinical use as the tyrosine kinase inhibitors, AG17 or SF 6847, which might be applied as antitumor drugs.[1,9] A large number of uncouplers are rather hydrophobic, weakly acidic compounds. TX-108, which is weakly basic, was a potent uncoupler comparable with SF 6847. TX-109 was also a unique uncoupler with a high maximal stimulated respiratory rate of $V_{max} = 340$ natom O mg^{-1} min^{-1} as well as a potent uncoupling activity of $C_{max} = 45$ nM. The aniline-type uncouplers, 2-anilino-1,3,4-thiadiazole derivatives,[17] 1,3,6,8-tetranitrocarbozole,[29] flufenamic acid[29] and fluazinam[30] have been described. However, they were designed as herbicides and their activities of photosynthetic phosphorylation were mainly discussed. The amine-type hypoxic cell cytotoxin[20] SR-4233 (3-amino-1,2,4-benzotriazine 1,4-dioxide, tirapazamine) uncouples oxidative phosphorylation in human MCF-7 breast carcinoma cells.[31] The uncoupling effect of SR-4233 upon oxidative phosphorylation might decrease the rate of ATP synthesis, leading to the partial depletion of the ATP pool, which might be its mechanism of cytotoxicity. Although the cytotoxicity of SR-4233 toward oxygenated cells is not the therapeutic focus of the drug as a hypoxic cell cytotoxin,[20] its cytotoxcity against cancer cells is very useful as a basis for the design of mitochondrial drugs for use as antitumor agents. In summary, we showed that TX-108 and TX-109 are potential antitumor agents as well as mitochondrial cytotoxins, which inhibit ATP syntheses and potently uncouple oxidative phosphorylation.

Experimental
Chemistry

All melting points were determined in a glass capillary tube without correction. Infrared (IR) spectra were recorded in KBr pellets on a Perkin-Elmer 1600 spectrometer and ultraviolet (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 Hitachi R-24B spectrometer (60 MHz) and a JEOL JNM-EX400 (400 MHz) spectrometer with tetramethylsilane as an internal standard, chemical shifts are given in δ values. ^{13}C NMR spectra were recorded on a JEOL JNM-EX400 (100.4 MHz) spectrometer. Electron impact mass spectra (EIMS) were measured on a Shimadzu Model GCMS-QP1000 mass spectrometer. High resolution (HR) EIMS (HREIMS) and fast atom bombardment mass spectra (FABMS) were measured on a JEOL JMS-SX 102A instrument. Elemental analyses were performed with a Yanaco CHN Corder (MT-5). Column chromatography was proceeded using Merck Kieselgel 60 (230–400 mesh). We purchases 1,5-dichloro-4-nitro-2-trifluoromethylbenzene from Lancaster Synthesis Inc. (Windham, New Hampshire, U.S.A.). The partition coefficient (Log P) in octanol/water was determined at pH 7.4 as to the method of Fujita et al.[32] The pK_a values of the compounds were determined by their UV spectra of their compounds in water at pH adjusted with HCl, NaOH and ammonia.[33,34]

General procedure for the synthesis of N-thiadiazolylanilines TX-105–TX-113

To a solution of 2-amino-5-trifluoromethyl-1,3,4-thiadiazole (5.91 mmol) and potassium hydroxide (10.7 mmol) in THF (30 mL) at 0 °C, a substituted trifluoromethylbenzene (5.90 mmol) in THF (15 mL) was added. The mixture was stirred for an appropriate period at room temperature. The reaction mixture was poured into water (300 mL), acidified with 6 N hydrochloric acid, and extracted with EtOAc. Removal of the dried extracts yielded a residue, which was purified by chromatography. Elution with methylene chloride gave TX-105–TX-113.

2-(3-Chloro-2,6-dinitro-4-trifluoromethylanilino)-5-tri-fluoromethyl-1,3,4-thiadiazole (TX-105)

1,3-Dichloro-2,4-dinitro-6-trifluoromethylbenzene. To a mixture of fuming nitric acid (32 mL) and 50% fuming sulfuric acid (32 mL), 1,3-dichloro-4-trifluoromethylbenzene (8 g, 37.2 mmol) was aded gently at 0 °C. The mixture was stirred for 96 h at 80 °C, then poured into ice-water (500 mL) to give crystalline precipitates. These were recrystallized from hexane to yield 1,3-dichloro-2,4-dinitro-6-trifluoromethylbenzene (6.96 g, 60%) as pale yellow crystals, mp 63–65 °C. ^{1}H NMR (CDCl$_3$): δ 8.41 (1H, s). HREIMS m/z: 305.9263 (calcd for C$_7$HCl$_2$F$_3$N$_2$O$_4$: 305.9263).

TX-105. Prepared using 1,3-dichloro-2,4-dinitro-6-trifluoromethylbenzene as a substituted trifluoromethylbenzene. The reaction was continued for 20 h. Yield: 75%. Pale yellowish-white crystals, mp 174–175.5 °C (from EtOAC). EIMS m/z 437 (M$^+$), 391 (M$^+$ − NO$_2$) 345 (M$^+$ − 2NO$_2$). ^{1}H NMR (10% CD$_3$OD–CDCl$_3$) δ 8.49 (1H, s). IR (KBr) 1651, 1613, 1557 (NO$_2$) 1524, 1495, 1341 (NO$_2$), 1310, 1279, 1213, 1177, 1144, 1040, 743, 700 cm^{-1}. UV max (EtOH) 388.2 nm (ε 1390), 233.4 (19984). Anal. calcd for C$_{10}$H$_2$ClF$_6$N$_5$O$_4$S: C, 27.43; H, 0.46; N, 16.00. Found: C, 27.34; H, 0.63; N, 16.17. The structure of TX-105 was established by X-ray analysis.[35]

2-(2,4-Dinitro-6-trifluoromethylanilino)-5-trifluorome-thyl-1,3,4-thiadiazole (TX-106). Prepared using 1-chloro-2,4-dinitro-6-trifluoromethylbenzene as a substituted trifluoromethylbenzene. The reaction was continued for 72 h. Yield: 65%. Pale yellowish-white crystals, mp 187–189 °C (from CH$_2$Cl$_2$). EIMS m/z: 403 (M$^+$), 357 (M$^+$ − NO$_2$), 334 (M$^+$ − CF$_3$), 311 (M$^+$ − 2NO$_2$).^{1}H NMR (10% CD$_3$OD–CDCl$_3$) δ 8.70 (1H, d, $J = 3$ Hz), 8.42 (1H, d, $J = 3$ Hz). IR (KBr) 3040, 1605 (NO$_2$), 1520, 1490, 1465, 1440, 1350, 1330 (NO$_2$), 1315, 1300, 1260, 1215, 1185, 1150, 1140, 1105, 1090, 1055, 1040, 935, 850, 810, 790 cm^{-1}. UV max

(EtOH) 426.0 nm (ε 10682), 296.0 (9180), 221.0 (15989). Anal. calcd for $C_{10}H_3F_6N_5O_4S$: C, 29.78; H, 0.74; N, 17.37. Found: C, 29.69; H, 0.86; N, 17.46.

2-(2-Nitro-4-trifluoromethylanilino)-5-trifluoromethyl-1,3,4-thiadiazole (TX-107).

Prepared using 1-chloro-2-nitro-4-trifluoromethylbenzene as a substituted trifluoromethylbenzene. The reaction was continued for 72 h. Yield: 25%. Pale yellowish-white crystals, mp 121.5–123.0 °C (from CH_2Cl_2). EIMS *m/z*: 358 (M$^+$), 312 (M$^+$ – NO$_2$), 289 (M$^+$ – CF$_3$). ^{1}H NMR (10% CD$_3$OD–CDCl$_3$) δ 8.78 (1H. dd, *J* = 9 and 1 Hz), 8.40 (1H, d, *J* = 3 Hz), 7.88 (1 H, dd, *J* = 9 and 2 Hz). IR (KBr) 3295, 3260, 1638, 1582, 1539 (NO$_2$), 1528, 1493, 1482, 1325 (NO$_2$), 1308, 1282, 1157, 1088, 1041, 917, 907, 852, 835, 765, 747, 695 cm^{-1}. UV max (EtOH) 351.5 nm (ε 6747), 289.5 (12954), 240.5 (10672). HREIMS *m/z*: 357.9853 (calcd for $C_{10}H_4F_6N_4O_2S$: 357.9959).

2-(5-Chloro-2-nitro-4-trifluoromethylanilino)-5-trifluoromethyl-1,3,4-thiadiazole (TX-108).

Prepared using 1,5-dichloro-4-nitro-2-trifluoromethylbenzene as a substituted trifluoromethylbenzene. The reaction was continued for 72 h. Yield: 60%. Pale yellowish-white crystals, mp 161–163 °C (from CH_2Cl_2). EIMS *m/z*: 392 (M$^+$), 346 (M$^+$ – NO$_2$). ^{1}H NMR (10% CD$_3$OD–CDCl$_3$) δ 9.13 (1H, s), 8.55 (1H, s). IR (KBr) 3280, 3110, 1635, 1550 (NO$_2$), 1525, 1490, 1455, 1415, 1350 (NO$_2$), 1315, 1295, 1235, 1205, 1170, 1155, 1090, 1040, 960, 865, 760, 745, 665 cm^{-1}. UV max (EtOH) 351.5 nm (ε 10441), 288.0 (15277), 272.5 (15442), 245.0 (11265). Anal. calcd for $C_{10}H_3ClF_6N_4O_2S$: C, 30.58; H, 0.76; N, 14.27. Found: C, 30.62; H, 0.87; N, 14.49.

2-(2-Chloro-6-nitro-4-trifluoromethylanilino)-5-trifluoromethyl-1,3,4-thiadiazole (TX-113) and 2-(2-chloro-4-nitro-5-trifluoromethylanilino)-5-trifluoromethyl-1,3,4-thiadiazole (TX-109)

1,2-Dichloro-3-nitro-5-trifluoromethylbenzene and 1,2-dichloro-5-nitro-4-trifluoromethylbenzene.

To a mixture of fuming nitric acid (5 ·g, 79.4 mmol) and concd sulfuric acid (10 g, 102.8 mmol), 1,2-dichloro-4-trifluoromethylbenzene (10 g, 46.5 mmol) was added gently at 0 °C. The mixture was stirred for 120 h at room temperature, then poured into ice-water (30 mL) to give oily yellow precipitates. These were washed with water and dried to yield a mixture of 1,2-dichloro-3-nitro-5-trifluoromethylbenzene and 1,2-dichloro-5-nitro-4-trifluoromethylbenzene (1,2-dichloro-3-nitro-5-trifluoromethylbenzene:1,2-dichloro-5-nitro-4-trifluoromethylbenzene = 57: 43, determined by integration of the signal of NMR; 6.3 g, 52%) as a yellow oil. 1,2-Dichloro-3-nitro-5-trifluoromethylbenzene: ^{1}H NMR (CDCl$_3$) δ 8.068 (1H, s), 7.936 (1H, s); 1,2-dichloro-5-nitro-4-trifluoromethylbenzene: ^{1}H NMR (CDCl$_3$) δ: 7.977 (1H, s). These products were used without separation as the starting materials for the synthesis of TX-109 and TX-113.

TX-113 and TX-109.

Prepared using the mixture of 1,2-dichloro-3-nitro-5-trifluoromethylbenzene and 1,2-dichloro-5-nitro-4-trifluoromethylbenzene as a substituted trifluoromethylbenzene. The reaction was continued for 72 h. Yield of TX-113: 26%. Pale yellowish-white crystals, mp 183.5–185.0 °C (from CH_2Cl_2). Yield of TX-109: 4%. Yellowish-white crystals, mp 125–128 °C (from CH_2Cl_2).

TX-113.

EIMS *m/z* 392 (M$^+$), 357 (M$^+$ – Cl), 346 (M$^+$ – NO$_2$), 311 (M$^+$ – Cl – NO$_2$). ^{1}H NMR (10% CD$_3$OD–CDCl$_3$) δ 8.13 (1H d, *J* = 2 Hz), 7.95 (1H, d, *J* = 2 Hz). IR (KBr) 1625, 1585, 1555 (NO$_2$), 1530, 1510, 1495, 1475, 1410, 1355, (NO$_2$), 1330, 1295, 1185, 1145, 1105, 1050, 1035, 905, 750 cm^{-1}. UV max (EtOH) 339.5 nm (ε 8137). Anal. cald for $C_{10}H_3ClF_6N_4O_2S$: C, 30.58; H, 0.76; N, 14.27. Found: C, 30.29; H, 0.89; N, 14.30.

TX-109.

EIMS *m/z*: 392 (M$^+$), 357 (M$^+$ – Cl), 311 (M$^+$ – Cl – NO$_2$). ^{1}H NMR (10% CD$_3$OD–CDCl$_3$) δ: 9.24 (1H, d, *J* = 1 Hz), 8.05 (1H, d, *J* = 1 Hz). IR (KBr) 3295, 3260, 1638, 1582, 1539 (NO$_2$), 1528, 1493, 1482, 1448, 1357, 1325 (NO$_2$), 1308, 1282, 1267, 1232, 1212, 1192, 1157, 1138, 1088, 1041, 917, 907, 852, 835, 765, 747, 695 cm^{-1}. UV max (EtOH): 444.5 nm (ε 8471), 312.0 (9185). HREIMS *m/z*: 391.9578 (calcd for $C_{10}H_3ClF_6N_4O_2S$: 391.9569).

General procedure for the synthesis of *N*-thiadiazoyl-anilines TX-101–TX-104

A solution of 2-amino-5-trifluoromethyl-1,3,4-thiadiazole (1.77 mmol), a substituted trifluoromethylbenzene (2.22 mmol) and 4-dimethylaminopyridine (10 mg) in pyridine (5 mL) was refluxed for 85 h with stirring. The solvent was removed under reduced pressure then the residue was poured into water and extracted with EtOAc. Removal of dried solvents gave a residue that was purified by chromatography. Elution with methylene chloride gave TX-101–TX-104.

4-Trifluoromethyl-2-nitroaniline (TX-101).

Prepared using 1-chloro-2-nitro-4-trifluoromethylbenzene as a substituted trifluoromethylbenzene. Yield: 35%. Yellow crystals, mp 103–105 °C (from CH_2Cl_2). ^{1}H NMR (CDCl$_3$) δ 8.35 (1H, d, *J* = 2 Hz), 7.52 (1H, dd, *J* = 9 and 2 Hz), 6.84 (1H, dd, *J* = 9 and 1 Hz), 6.40 (2H, m). IR (KBr) 3505, 3380, 3220, 1620, 1582 (NO$_2$), 1485, 1429, 1365 (NO$_2$), 1333, 1285, 1270, 1164, 1125, 1082, 918, 840, 775, 605 cm^{-1}. UV max (EtOH) 388.8 nm (ε 9550), 236.4 (15480). Anal calcd for $C_7H_5N_2O_2F_3$: C, 40.77; H, 2.42; N, 13.59. Found: C, 40.81; H, 2.63; N, 13.44.

4-Chloro-3-trifluoromethyl-2-nitroaniline (TX-102).

Prepared using 1,5-dichloro-4-nitro-2-trifluoromethylbenzene as a substituted trifluoromethylbenzene. Yield: 10%. Yellow crystals, mp 108–110 °C (from CH_2Cl_2). ^{1}H NMR (CDCl$_3$) δ 8.46 (1H, s), 6.98 (1H, s), 6.44 (2H, m). IR (KBR) 3540, 3425, 3090, 1648, 1534 (NO$_2$), 1496, 1427, 1348 (NO$_2$), 1303, 1267,

1170, 1152, 1123, 1105, 925, 852, 773, 665 cm^{-1}. UV max (EtOH) 378.4 nm (ε 9197), 241.2 (12508). Anal calcd for $C_7H_4N_2O_2F_3Cl$: C, 34.94; H, 1.66; N, 11.65. Found: C, 34.86; H, 1.78; N, 12.09.

2-Chloro-5-trifluoromethyl-4-nitroaniline (TX-103) and 2-chloro-4-trifluoromethyl-6-nitroaniline (TX-104).

Prepared using a mixture of 1,2-dichloro-5-nitro-4-trifluoromethylbenzene and 1,2-dichloro-3-nitro-5-trifluoromethylbenzene. Yield of TX-103: 9%. Yellow crystals, mp 80–83 °C (from CH_2Cl_2). Yield of TX-104: 20%. Yellow crystals, mp 60–62 °C (from CH_2Cl_2).

TX-103. ^{1}H NMR (CDCl$_3$) δ 8.06 (1H, s), 7.09 (1H, s), 4.92 (2H, m). IR (KBr) 3545, 3505, 3390, 3200, 3080, 1630, 1575, 1525 (NO$_2$), 1500, 1438, 1335 (NO$_2$), 1320, 1282, 1260, 1160, 1143, 1132, 1084, 915, 890, 876, 857, 760, 735, 710, 654 cm^{-1}. UV max (EtOH) 356.6 nm (ε 15074), 237.8 (13192). HREIMS m/z: 239.9918 (calcd for $C_7H_4ClF_3N_2O_2$: 239.9913).

TX-104. ^{1}H NMR (CDCl$_3$) δ 8.30 (1H, d, $J = 2$ Hz), 7.66 (1H, d, $J = 2$ Hz), 6.77 (2H, m). IR (KBr) 3510, 3395, 3110, 1817, 1645, 1589, 1535 (NO$_2$), 1470, 1357 (NO$_2$), 1308, 1295, 1285, 1168, 1133, 1120, 1098, 910, 865, 792, 775, 759, 680 cm^{-1}. UV max (EtOH) 385.8 nm (ε 8499), 240.4 (30015). FABMS m/z: 241 (MH$^+$). HREIMS m/z: 240.9913 (calcd for $C_7H_4ClF_3N_2O_2$: 240.9947).

Biological activity

Uncoupling activity. Mitochondria were isolated from adult male Wistar rats as reported by Myers and Slater.[36] The amount of mitochondrial protein was determined by the Biuret method[37] with bovine serum albumin as the standard. The respiration of mitochondria was monitored polarographically with a Clark-type oxygen electrode (Yellow Spring, YSI 5331). The incubation medium consisted of 200 mM sucrose and 2 mM MgCl$_2$ in 10 mM potassium phosphate buffer (pH 7.4). Mitochondria were added at 0.7 mg protein ml^{-1} in a total volume of 2.53 mL. Succinate (10 mM, plus rotenone at 1 µg mg^{-1} protein) was the respiratory substrate. The activity was determined by measuring changes in the rate of state 4 respiration upon addition of the test compound.

ATP synthesis. ATP synthesis by mitochondria was determined by measuring the increase in pH of the medium associated with ATP synthesis as reported by Nishimura et al.[38] at 25 °C in medium consisting of 200 mM 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 400 µM 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 change was monitored using a pH meter (Horiba, Kyoto, Japan).

Tumor cell growth assay using MTT.[39] The EMT6/KU mammary sarcoma cell line was maintained at exponential growth in spinner culture. MTT [3-(4,5-dimethylthiazol-2-yl)-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 a 48-well culture plate. Drugs (3 µL) were added and the cells were incubated at 37 °C for 48 h. Thereafter, 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 EC$_{50}$ value was estimated as the index of the inhibition of drug to tumor cell growth.

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