Development of new hypoxic cell sensitizers: Amides of nitrobenzoic acid with spermidine and spermine

Y. Nagao, S. Takao, E. Fujita[1], C. Murayama, T. Mori, T. Asao and T. Suzue

Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611 (Japan), Department of Radiology, School of Medicine, Tokai University, Bohseidai, Isehara, Kanagawa-ken 259-11 (Japan), and Research Laboratory, Taiho Pharmaceutical Co., Ltd, Kawauchi-cho, Tokushima 771-01 (Japan), August 3, 1982

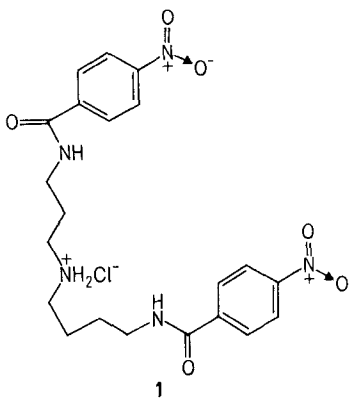
Summary. New type hypoxic cell sensitizers, N^1,N^{10}-bis(4-nitrobenzoyl)spermidine (**1**) and N^1,N^{14}-bis(4-nitrobenzoyl)-spermine (**2**), have been developed. These compounds were shown to be more effective radiosensitizers to hypoxic cells than misonidazole (**3**).

We have been interested in the total synthesis of naturally occurring amides of polyamines (especially spermidine) and of polyamine-containing compounds having potential for biological or physiological activity[2-5]. We wish to report herewith a new type of hypoxic cell sensitizer: FNT-1 [N^1,N^{10}-bis(4-nitrobenzoyl)spermidine] (**1**) and FNT-2 [N^1,N^{14}-bis(4-nitrobenzoyl)spermine] (**2**)[6].

These compounds have been developed based upon the following information. a) The natural polyamines [spermidine (**4**) and spermine (**6**)] show charge-charge affinity to nucleic acid[7-9]. b) Some aromatic compounds bind to nucleic acids by intercalation, i.e., the insertion of a flat molecule between the base pairs of a double helix[9-12]. c) Most excellent hypoxic cell sensitizers possess 1 or 2 nitro group(s) as the electron-accepting group[13-15].

Thus, a speculative intercalator, the 4-nitrobenzoyl group, was linked to the polyamines, spermidine (**4**) and spermine (**6**). Synthesis of compounds **1** and **2** was efficiently carried out utilizing a highly chemoselective acylating reagent, 3-acyl-1,3-thiazolidine-2-thione ('ATT')[16].

A solution of spermidine (**4**) (0.22 g) in CH_2Cl_2 (25 ml) was added to a solution of 3-(4-nitrobenzoyl)-1,3-thiazolidine-2-thione (**5**) (0.8 g, m.p. 166–167 °C) in CH_2Cl_2 (25 ml). After being stirred at room temperature for 1 h under N_2, the reaction mixture was washed with 2% NaOH (100 ml) in order to remove the 1,3-thiazolidine-2-thione (TT) released. The TT-free solution was washed with brine, dried over Na_2SO_4, and evaporated in vacuo to give crude crystals, which were recrystallized from ethanol to afford pure yellow needles (0.65 g, m.p. 126–127 °C, 98% yield).



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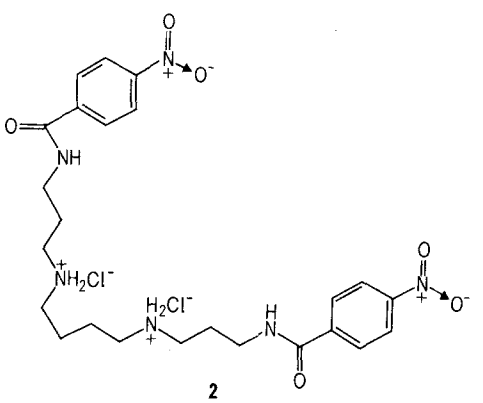

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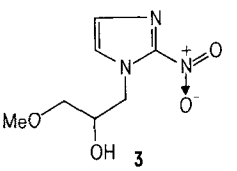

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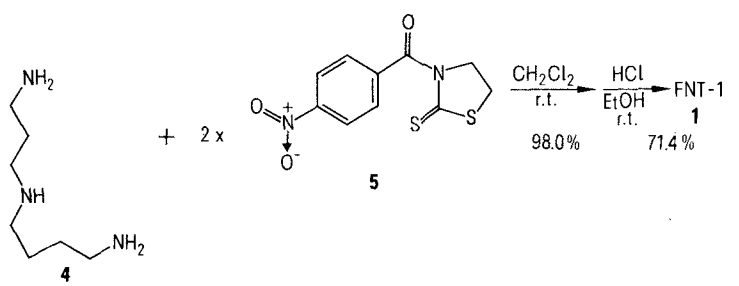
Formulae **1**~**3**



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Scheme

Hydrochloride **1** was obtained as yellow needles, m.p. 167–168 °C (from ethanol).

FNT-2 (**2**) [m.p. 255–258 °C (decomp)] was similarly prepared in good yield.

A radiosensitizing effect of FNT-1 and -2 on hypoxic cells (HeLa S3 Cells) in vitro was investigated according to the method which was previously reported by Tokai authors[15].

Test sample (0.1 ~ 1.0 mM) was added to 0.5 ml of the HeLa cell suspensions $4 \sim 5 \times 10^5$ cells/ml). After hypoxic treatment of the medium by flushing with 95% nitrogen and 5% carbon dioxide gas[17], it was irradiated with ^{60}Co γ-rays (dose rate: 2.34 Gy/min) at 3 doses; 4, 8, and 12 Gy, respectively. The cells were washed with Hanks' balanced salt solution to remove the test samples and diluted in Eagle's minimum essential medium (Nissui, Tokyo) to obtain suitable cell suspension. This diluted cell suspension was plated and incubated 37 °C for 10 ~ 12 days. The surviving fraction was determined by the colony formation method[18].

Figures 1 and 2 show the survival curves for drug-treated or-untreated HeLa S3 cells irradiated at the absence or presence of oxygen. In our system, the D_0-value[19] for hypoxic cells was 5.87 Gy and it was 1.72 Gy for aerobic cells; the calculated oxygen enhancement ratio was 3.41. In the case of FNT-1 (**1**), an increase in the concentration brought about a progressive increase in the sensitization of hypoxic cells. No cell killing was observed when cells were exposed to 0.1 mM of FNT-2 (**2**), but when the concentration was increased up to 1 mM, it showed the similar sensitizing effect to that noted with 0.5 mM of FNT-1 (**1**).

The table shows the enhancement ratios of both compounds, FNT-1 and -2, at the tested drug concentrations. The enhancement ratios were obtained from the D_0-ratios in the absence and presence of the radiosensitizers. The previous data[15] for misonidazole are also presented for comparison. Based on these results, both compounds were shown to have more effective radiosensitizing abilities to hypoxic cells than misonidazole (**3**).

Enhancement ratio (ER) of FNT-1 (**1**), FNT-2 (**2**), and misonidazole (**3**)

Compound	Concentration (mM)	ER
FNT-1	0.1	1.18
	0.5	1.65
	1.0	2.10
FNT-2	1.0	1.45
Misonidazole	0.1	1.05
	0.5	1.12
	1.0	1.32

1 Reprint requests to E.F., Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611 (Japan).
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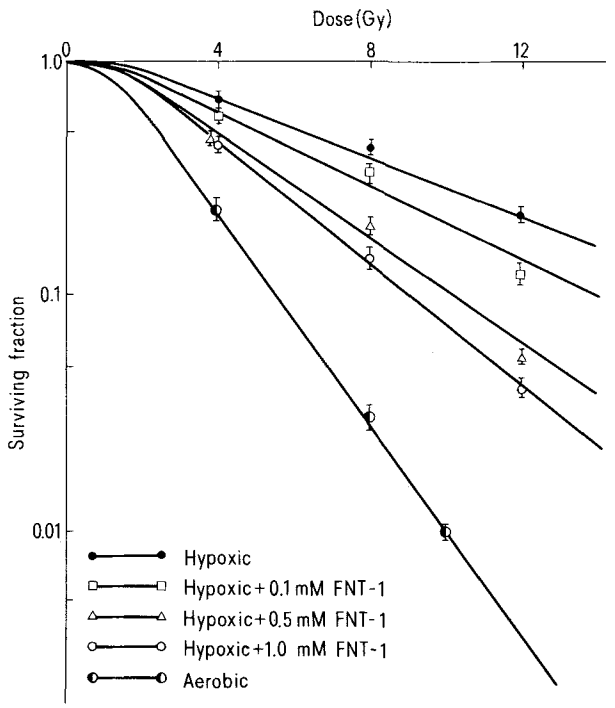

Figure 1. The effect of FNT-1 (**1**) on the radiation survival of HeLa S3 cells.

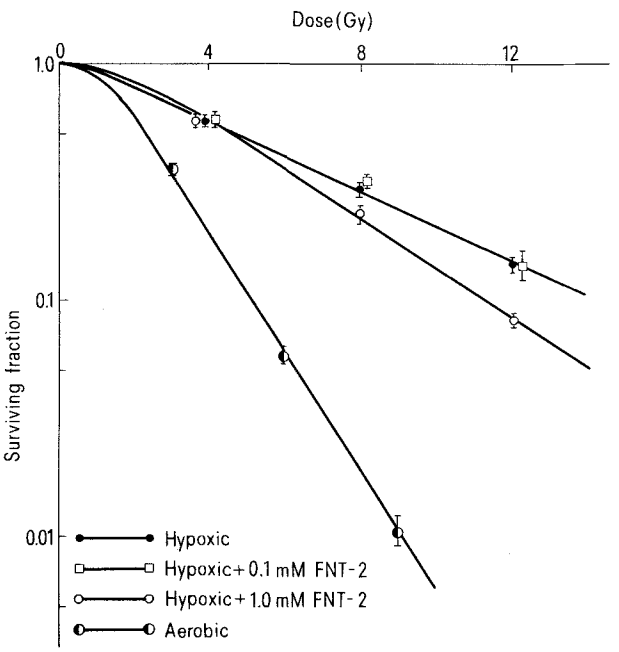

Figure 2. The effect of FNT-2 (**2**) on the radiation survival of HeLa S3 cells.

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17 The hypoxic treatment itself did not show any cytotoxic effect.
18 A colony consists of more than 1000 cells.
19 D_0 means the slope of the exponential portion of the survival curve after initial shoulder; it is the dose required to reduce the surviving fraction to 37%.

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Stimulation of nitrate reductase activity by delta amino levulinic acid in excised maize leaves[1]

S.N. Mishra and H.S. Srivastava[2]

Department of Life Sciences, Vigyan Bhawan, Khandwa Road, University of Indore, Indore-452001 (India), November 2, 1982

Summary. Supply of 0.2 mM delta amino levulinic acid to maize leaf segments in light increased nitrate reductase activity, total organic nitrogen and to some extent chlorophyll also, but it had no effect on glutamate dehydrogenase or peroxidase activities, in the absence or presence of nitrate or ammonium as nitrogen source.

Amino levulinic acid (ALA) is believed to be the rate-limiting precursor for the synthesis of tetrapyrroles such as chlorophylls, heme and bile pigments[3,4]. It stimulates the synthesis of chlorophyll in dark-cultured *Euglena gracilis*[5] and of protochlorophyllides in etiolated wheat[6] and barley[7] leaves. Radioactive ALA is incorporated into peroxidase tetrapyrroles in light, in cultured peanut cells[8]. However, prolonged treatment of plant material with ALA results in a decreased phytochrome content[9]. As a precursor for chlorophyll synthesis, ALA can affect biogenesis and metabolism of chloroplasts, which in turn may affect other plant processes also. However, the effects of ALA on processes other than chloroplast metabolism are also known. For example, it causes swelling of mitochondria from barley leaves[10] and stimulates oxygen uptake in etiolated wheat leaves in the dark[10]. To examine whether the effect of ALA extended to other plant processes as well, the assimilation of nitrate and ammonium and relevant enzyme activities in excised maize leaves, were studied.

Experimental procedure and results. *Zea mays* L. (cv. Ganga safed-2) seeds were purchased from National Seed Corporation, New Delhi. Seeds were surface sterilized, washed thoroughly and grown in continuous light (supplied by 100 W incandescent bulbs and 40 W fluorescent tubes) of about 65 W m^{-2} density for 10 days at 25 ± 2 °C, with modified $^1/_2$ strength Hoagland's solution without nitrogen. Primary leaf segments from uniformly grown seedlings were incubated in $^1/_4$ strength Hoagland's solution, containing either no nitrogen or 10 mM KNO_3 or NH_4Cl as nitrogen source. Delta amino levulinic acid (purchased from Sigma Chemical company, St. Louis, Mo.) was added as indicated at a concentration of 0.2 mM and the incubation was carried on for 24 h at 25 °C, either in light or in dark. The pH of the nutrient solution and of incubation medium was 6.0. Total chlorophyll, peroxidase, in vivo nitrate reductase activity and NADH specific aminating glutamate dehydrogenase activity were measured by the methods of Strain and Svec[11], Maehly[12], Srivastava[13], and Singh and Srivastava[14] respectively. Nitrogen was measured by a modified micro-kjeldahl method[15], of 80% ethanol soluble and insoluble fractions separately. Protein was calculated by multiplying the insoluble nitrogen value by a factor of 6.25[16]. Values are expressed as their mean ± SE for 3 experiments. The paired t-test (between the replicates of −ALA and +ALA) was applied to evaluate the significance of effect of ALA on different parameters.

In the light, the exogenous supply of ALA to excised maize leaves increased protein content, total nitrogen and total chlorophyll slightly (table 1). The increase in protein in the presence of nitrogenous salts was however, insignificant. In the dark also, some increase in protein and organic nitrogen contents was observed in control or in KNO_3. A slight increase (14–29%) was observed in total chlorophyll also, during ALA supply. In light, supply of ALA increased total and specific activities of nitrate reductase significantly and this stimulation was more pronounced in control or KNO_3 than in NH_4Cl leaves (table 2). However, it had either no effect, or inhibited the activity of NADH-glutamate dehydrogenase slightly. The activity of peroxidase increased slightly in the control but was unaffected in the presence of KNO_3 or NH_4Cl. In the dark, supply of ALA did not increase either total or specific activity of nitrate reductase significantly. Total activity of nitrate reductase increased significantly with the supply of glycine, glutamate, glutamine and alpha ketoglutarate as well (table 3). The effect of glutamate was most pronounced and the effects varied slightly with the variation in concentration of glutamate or alpha ketoglutarate.

Discussion. The stimulating effect of ALA on nitrate reductase activity and nitrogen assimilation is reported for the 1st time. The assimilated nitrogen is partitioned more in the non-proteinaceous fraction in the presence of ALA, as the increase in protein was often insignificant. The stimulation was more apparent when the source of inorganic nitrogen was nitrate than when it was ammonium. Apparently, the increase in inorganic nitrogen assimilation is through nitrate reductase activity, as it is believed to be the rate limiting enzyme in nitrate assimilation pathway[17]. Further, the increase in nitrate reductase activity appears to be specific, as another important enzyme of the pathway, the NADH glutamate dehydrogenase, was little affected. Effect on peroxidase, an enzyme unrelated to nitrogen metabolism pathway but containing tetrapyrroles, was also generally insignificant.

Nitrate reductase is a sensitive enzyme and its activity is regulated by several nutritional and environmental factors[18]. Amino levulinic acid may increase its activity either by some direct activation process or through some indirect products. As a precursor of tetrapyrroles, it may increase nitrate reductase level by inducing the cytochrome b component of the enzyme complex. In our experiments, ALA did not increase nitrate reductase activity in the dark, when