Toxicity, Antitumor and Chemosensitizing Effects of 3-Chloroprocainamide

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3-chloroprocainamide (3-CPA), an analog of metoclopramide (MCA), dose-dependently inhibited tumor growth in scid mice xenografted with a human brain astrocytoma (T24) when given intramuscularly to mice every third day for 14–20 days. 3-CPA was shown to have the same efficacy on tumor growth inhibition as neutral metoclopramide (neutral MCA) at the doses of 10–40 mg/kg when evaluated by tumor doubling time, tumor growth time for tumor volumes to reach 1 000 mm^3 and area under growth curve. 3-CPA at the dose of 3×40 mg/kg was also shown to enhance the cytotoxicity induced by a single dose of cisplatin at 7.5 mg/kg. A dose of ≤ 160 mg/kg of 3-CPA did not show any notable extrapyramidal symptoms which was observed for neutral MCA treated mice at the dose of 20 mg/kg. The lethal response dose of 3-CPA for scid mice was 320 mg/kg which is 4 times higher than that determined for neutral MCA (80 mg/kg). These results support 3-CPA as a good candidate drug representing a new generation of benzamides for further clinical development as a cancer therapy drug.

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The benzamide and nicotinamide analogs are a well recognized class of drugs known to possess the pharmacological properties of radio- and chemosensitization (1). Recently, metoclopramide (MCA), an N-substituted benzamide analog which has been widely used as an effective antiemetic in the clinic for over 30 years (2), has been shown to possess antitumor and radio/chemosensitizing effects in nude or scid mice xenografted with human squamous cell carcinomas of the head and neck (3–6) or a human lung adenocarcinoma (7, 8), and in rats transplanted with rat glioma cells (9). Moreover, a phase I/II clinical trial has confirmed the potential of MCA to radiosensitize tumor cells to cytotoxicity in patients with inoperable non small cell lung cancer (10).

3-chloropracainamide (3-CPA) is a uniquely synthesized N-substituted benzamide (Oxigene Inc.) whose chemical structure differs from MCA only by lacking an orthomethoxy benzamide ring substitution (Fig. 1). This ring substitution is thought to be responsible for binding dopamine D_2 receptors (11), which in turn mediate undesirable extrapyramidal effects of MCA (2). Thus, this modification of the MCA structure gave the expectation that 3-CPA could reduce or even avoid the extrapyramidal effects while maintaining antitumor and radiosensitizing properties. In vitro experiments have already shown that 3-CPA was susceptible to radiolysis, able to inhibit cell proliferation and induced cytotoxicity by apoptosis (12–14), supporting that this analog of MCA has not lost its cancer therapy benefit. We report in the present paper the side effects, antitumor activity and chemosensitization of 3-CPA in scid mice xenografted with a human brain astrocytoma and in comparison to MCA.

MATERIAL AND METHODS

Mice and tumor line

Male/female scid mice at 6–8 weeks and weighing 18–30 g were maintained under sterile but non specific pathogen-free conditions. They were used for xenograft inoculation with tumor line T24. The tumor graft was initially inoculated by tumor tissue suspension directly from a human brain astrocytoma, and later grafts were serially transplanted by subcutaneous inoculation of tumor tissue suspension into the right flank of mice for 20–26 passages. When the tumor volume reached an average size of 100 mm^3 (50–150 mm^3), the animals were randomly divided into several groups and treated with the drugs at doses between 10–320 mg/kg. The histology of the astrocytoma in this study was confirmed by a hematoxylin-eosin staining and by an avidin/biotin-complex technique, i.e. a

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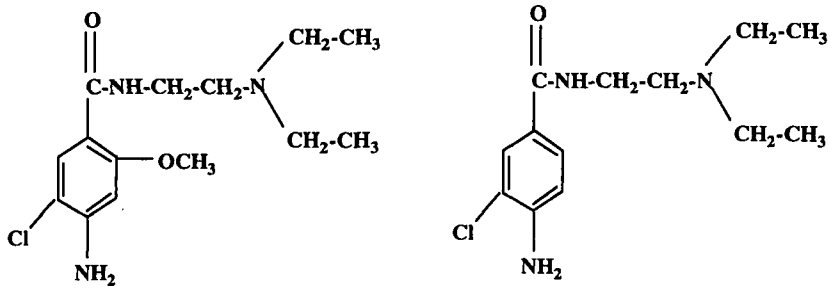

Fig. 1. Molecule structure of 3-chloroprocainamide and metoclopramide.

bovine polyclonal antibody was used against human glial fibrillary acidic protein (GFAP). The animals were treated according to the Swedish guidelines for humane treatment of laboratory animals and the experiments were approved by the Ethical Committee for Animal Research in Malmö/Lund, Sweden.

Drugs

Neutral MCA (Neu-Sensamide™) and 3-CPA were supplied by Oxigene Europe AB (Lund, Sweden). Neutral MCA was provided as a solution in 2 ml sealed ampoules according to the formulation: MCA hydrochloride monohydrate 112.2 mg/ml and sodium metabisulphite 2 mg/ml dissolved in 50 mM sodium phosphate buffer at pH 6.5–7.0. 3-CPA was supplied as a powder in 100 ml sealed ampoules. Five grams of 3-CPA monohydrochloride were used to make a solution of 100 mg/ml (pH 6.7) using the same formulation as neutral MCA, and then autoclaved at 121°C for 20 min. Cisplatin was commercially obtained from Bristol Laboratories, Syracuse, NY, USA and diluted with physiological saline before use.

Drug treatment

All the drugs were adiministered by intramuscular (i.m.) injection to the animals in a final injection volume of 100 μl according to following designs: (a) The study comparing the antitumor effects of 3-CPA and neutral MCA was performed by treating mice with 3-CPA at doses of 10, 20, 40, 80, 160 and 320 or neutral MCA at 10, 20, 40 and 80 mg/kg every third day for 6 repeated doses. 3-CPA at 320 mg/kg and neutral MCA at 80 mg/kg were only given once because of their lethal toxic effect. For consideration of local tissue toxicity, the injected concentrations respectively corresponded to 2.5, 5, 10, 20, 40, 80 mg/ml for 3-CPA and 2.5, 5, 10, 20 mg/ml for MCA. (b) The study of chemosensitization by 3-CPA was carried out by randomly dividing the tumor bearing mice into four groups. The first group consisted of untreated controls and the second was given 3-CPA at the dose of 40 mg/kg which was repeated after 24 and 48 h (3 × 40 mg/kg). The third group was given a single dose of cisplatin at 7.5 mg/kg. The repeat dose schedule and cisplatin dose were chosen based on the previous results for MCA described by Lybak et al. (15). The fourth group was given a combination of 3-CPA and cisplatin, i.e. a single dose of cisplatin at 7.5 mg/kg followed 1 h by 3-CPA at 40 mg/kg and this 3-CPA dose was repeated after 24 and 48 h.

Toxic side effects

Acute toxicity was observed within 3 h after 3-CPA and neutral MCA injection mainly as local skin reaction, motionlessness, drowsiness, restlessness and death. The re-

Tab

Toxicity observed in scid mice xenografted with a human brain astrocytoma (T24)

Dose	Mouse number		Local skin reaction		Motionless	
mg/kg (mg/ml)[a]	3-CPA	Neutral MCA	3-CPA	Neutral MCA	3-CPA	Neutral MCA
0 (0)	21	11	0	0	0	0
20 (5)	10	11	0	0	0	4*
40 (10)	21	11	0	0	0	11*
80 (20)	10	5	0	0	0	5*
160 (40)	11	—	0	—	0	—
320 (80)	9	—	0	—	0	—

3-CPA at 20–160 mg/kg and neutral MCA at 20–40 mg/kg were given i.m. every third day for 14–20 experiment was performed for neutral MCA at doses of 160–320 mg/kg. Data are expressed as the
[b] Observation of motionless, drowsiness and restlessness. *: $p < 0.05$ vs. 3-CPA.

sponse duration was based on the observation of motionlessness and drowsiness, which are the known extrapyramidal effect-related behaviors of MCA (16). Body weights were recorded every week throughout the experiment. The animals were individually sacrificed when the tumor volume reached 1 350 mm^3.

Tumor volume measurement and response evaluation

All tumors were measured every second day with a caliper since they were palatable about four weeks after inoculation. The tumor volumes were calculated as: Volume = $(L \times W^2) \times 0.4$ (17), where L is the length of tumor and W is the width of tumor. Log relative tumor size (RTS) was used to describe the tumor growth. RTS was calculated as the tumor volume at the time of measurement divided by the tumor volume at the start of treatment, and log RTS produce a normal or near normal distribution of RTS values. The area under the curve (AUC), tumor doubling time and tumor growth time were used to evaluate treatment efficacy. AUC was calculated directly from the curve. It gives an overall index of tumor size and accounts for both degree and duration of inhibition and daily fluctuations will be smoothed out by this calculation (18). Tumor growth time was refered to the time for the tumor volumes to reach 1 000 mm^3. Both tumor doubling time and tumor growth time were individually estimated from the growth curve of Log RTS fitted by a polynomial regression and they were used for evaluation of tumor growth delay in different stage (19).

Statistics

Data analysis was carried out using the SPSS program (SPSS Inc.). Difference among groups was tested by one-way analysis of variance (ANOVA). Difference between two groups was further analyzed by the Duncan test at the significance level of 0.05. The toxic response data were analyzed by χ^2-test.

RESULTS

Toxicity

The acute toxicity observed after 3-CPA and neutral MCA injection is summarized in Table 1. Four out of 11 mice given neutral MCA at 20 mg/kg were observed motionless within 60 min after injection. The incidence was increased to 100% at 40 mg/kg with prolonged response duration. However, no obvious acute toxicity was observed in the mice treated with 3-CPA with a dose below or equal to 160 mg/kg, which was significantly different from that of neutral MCA (χ^2-test, $p < 0.05$). A restlessness sign was observed when 3-CPA dose was administered at 320 mg/kg but it disappeared in 30 min if the mice survived. The lethal toxicity was noticed when mice were given 3-CPA at 320 mg/kg (4/9) or neutral MCA at 80 mg/kg (2/5) with 4 times difference. Body weight gains were observed for all groups during the experiment (Table 2). However, no statistical difference was observed between different treatments for body weight gain, nor for primary organ weights.

Antitumor activity of 3-CPA and neutral MCA

By analysis of the AUC value at day 15 after treatment, a significant inhibition of tumor growth was noticed in the mice treated with either 3-CPA or neutral MCA at 10 mg/kg compared with that of the control (Fig. 2). This inhibition increased with the treatment dose ($r = 0.96$ for neutral MCA curve and 0.99 for 3-CPA curve, $p < 0.05$ for both), but it tended to saturate at the higher doses. No statistically significant difference was observed between the treatments of 3-CPA and neutral MCA at any dose treatment between 10–40 mg/kg. These results were supported by evaluation of tumor doubling time and tumor growth time (Table 3). The same efficacy in retardation of tumor growth was found between neutral MCA and 3-CPA.

Sensitization of the cytotoxicity of cisplatin by 3-CPA

3-CPA is a close structure analog of MCA (Fig. 1) and in vitro evaluation of radiosensitization has indicated that

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and treated with 3-chloroprocainamide (3-CPA) and neutral metoclopramide (MCA)

Drowsiness		Restlessness		Death		Response duration[b] (hour)	
3-CPA	Neutral MCA	3-CPA	Neutral MCA	3-CPA	Neutral MCA	3-CPA	Neutral MCA
0	0	0	0	0	0	0	0
0	5*	0	4*	0	0	0	0 1
0	11*	0	11*	0	0	0	1 2
0	5*	0	0	0	2	0	2 3
0	—	0	—	0		0	—
0	—	9	—	4		0–0.5	—

days. 3-CPA at 320 and neutral MCA at 80 mg/kg were given once because of lethal toxic effect. No number of mice showing toxic signs. [a] The concentrations of drugs in a final injection solution.

Table 2

Body and organs in grams weights from scid mice xenografted with a human brain astrocytoma (T24) and treated with 3-chloroprocainamide at different doses

Treatment	Number	Liver	Spleen	Kidney	Brain	Body weight
Control	11	1.22 ± 0.25	0.062 ± 0.018	0.16 ± 0.04	0.38 ± 0.02	26.5 3.7
10 mg/kg	10	1.24 ± 0.27	0.061 ± 0.019	0.16 ± 0.04	0.38 ± 0.02	26.2 3.7
20 mg/kg	10	1.18 ± 0.25	0.063 ± 0.021	0.15 ± 0.06	0.41 ± 0.07	25.2 3.6
40 mg/kg	11	1.16 ± 0.18	0.072 ± 0.019	0.16 ± 0.03	0.37 ± 0.02	24.3 2.3

Data are expressed as means ± SD. No statistical differences were observed between groups.

they were equipotent (12). MCA has also been shown to chemosensitize cisplatin in vivo (6, 15). Therefore, we have tried to demonstrate whether or not 3-CPA can also chemosensitize cisplatin under similar in vivo conditions. To this purpose, we have used a human glioma xenografted into scid mice to study the sensitizing properties of 3-CPA. Regardless of the treatment all the tumor growth curves displayed exponential increases indicating tumor regrowth not tumor shrinkage (Fig. 3). However, a slower tumor growth rate was shown in the mice treated with either 3-CPA at a dose of 3 × 40 mg/kg or cispaltin at 1 × 7.5 mg/kg, and the greatest tumor growth retardation was shown for mice treated with the combination of 3-CPA and cisplatin. The inhibition of tumor growth curves tended to parallel the untreated controls after one week of treatment whether treated with cisplatin or 3-CPA or the combination, suggesting an early mediated tumor cytotoxicity during and just after the drug treatment phase.

The differences among different treatment groups were also confirmed to be statistically significant by analysis of tumor doubling time, the tumor growth time and AUC (Table 4). The combination of 3-CPA and cisplatin gave longer tumor doubling times, longer tumor growth times and lower AUC when compared with those for controls

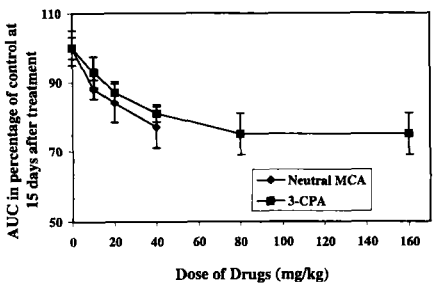

Fig. 2. Dose response of 3-chloroprocainamide (3-CPA) and neutral metoclopramide (MCA) in retardation of tumor growth by evaluation of area under the tumor growth curve (AUC). Scid mice xenografted with a human brain astrocytoma (T24) were intramuscularly injected with neutral MCA or 3-CPA every third day for 14–20 days. The growth curve was plotted as log relative tumor size (RTS) over treatment time and AUC was calculated according to the growth curve over 15 days of treatment. Data are means of 10–21 animals ± SE. No significant differences were observed between the treatment of 3-CPA and neutral MCA at any dose of treatment between 10–40 mg/kg.

($p < 0.05$). Moreover, they are also significantly different from those treated with 3-CPA or cisplatin alone. Taken together our data (Fig. 3 and Table 4) confirm that 3-CPA, like its close analog MCA, can sensitize cisplatin induced tumor cytotoxicity.

DISCUSSION

A number of benzamide derivatives have been shown to possess the properties of antitumor activity or sensitization of cytotoxicity induced by chemo- or radiotherapies both in vitro and in vivo (7, 15, 20–31). The mechanism of action that has so far been attributed to this class of agents can be conveniently divided into two pharmacological categories: namely (a) the non-N-substituted benzamides and nicotinamides which can alter tumor blood flow and thus increase DNA damage and sensitizes (32) and (b) the N-substituted benzamides and nicotinamides which can inhibit DNA repair, increase DNA damage, induce apoptosis and thereby sensitize (12, 13, 30, 33).

3-chloroprocainamide (3-CPA) like MCA belongs to the N-substituted subclass of benzamides, and it has been shown in this study to dose-dependently inhibit tumor growth and sensitize the cytotoxicity of cisplatin when tested against a xenografted human brain astrocytoma (T24) in scid mice (Figs. 2 and 3). The fact that 3-CPA exhibits the same efficacy of preventing tumor growth as

Table 3

Tumor doubling time and tumor growth time in scid mice

Dose	Tumor doubling time[a]		Tumor growth time[b]	
mg/kg	3-CPA	Neutral MCA	3-CPA	Neutral MCA
0	4.3 ± 0.28	4.0 ± 0.47	15.8 ± 0.41	16.6 ± 0.56
10	4.9 ± 0.42	5.0 ± 0.31	16.5 ± 0.86	17.2 ± 0.78
20	5.2 ± 0.27	5.5 ± 0.45	17.9 ± 1.28	18.0 ± 1.01
40	6.0 ± 0.26	6.3 ± 0.43	18.7 ± 1.53	18.9 ± 0.74

[a] The animals were intramuscularly injected with 3-chloroprocainamide and neutral MCA every third day for 6 repeated doses.
[b] The time for tumor volumes to reach 1 000 mm^3. Data are means of 10–11 animals ± S.E.
* $p < 0.05$ compared with control. No significant differences between 3-CPA and neutral MCA.

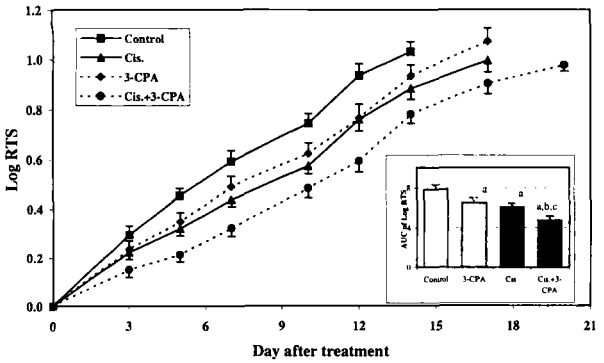

Fig. 3. Tumor growth curve of log relative tumor size for a human brain astrocytoma (T24) xenografted into scid mice. Animals were intramuscularly injected with 3-chloroprocainamide (3-CPA) at 40 mg/kg every 24 hours for 3 days, a single dose of cisplatin (Cis.) at 7.5 mg/kg or both in combination (Cis. + 3-CPA). Data are expressed as average of 10–11 animals ± SE. The insert figure is area under the curve (AUC) calculated at day 14 after treatment. a: $p < 0.05$ vs. control; b: $p < 0.05$ vs. 3-CPA; c: $p < 0.05$ vs. Cis.

neutral MCA clearly supports the biochemical considerations that (a) the methoxy ring substitution present in MCA but absent in 3-CPA is not a key feature for establishing sensitizing or antitumor activities since both drugs are equipotent (Fig. 2), and (b) that the tumor growth inhibition by 3-CPA is not mediated by binding to dopamine (D_2) receptors because 3-CPA did not induce extrapyramidal side effects (Table 1) yet it had comparable antitumor activity to MCA (Fig. 2).

Metoclopramide has long been offered in clinic in an acidic formulation (34). There are two known hydrogen bonds in the MCA molecule of such a formulation. One is between the carboxamide hydrogen and the orthomethoxy benzamide substitution and the other is between the carbonyl oxygen of the carboxamide and the tertiary N-substituted diethylaminoethyl side chain of the benzamide moiety which has been shown to be pH sensitive (11, 35). These hydrogen bonds convert MCA into a condensed conformation and hence there is a greater affinity to D_2 receptors. By adjusting the pH from 2.5–3.5 to 6.7–7.0, the neutralized MCA has been shown to have the same radiosensitizing efficacy as acidic MCA in enhancement of cytotoxicity in vitro (12–14) or in vivo induced by radiation in scid mice xenografted with a human lung adenocarcinoma (7, 8) while having reduced sedation in the rat (12, 13) and reduced extrapyramidal side effects in humans (36). The reduced side effect of neutral MCA is believed to be associated with a reduced affinity for the D_2 receptors due to the hydrolysis of the pH sensitive hydrogen bond and a more extended MCA molecule conformation (Schwartz & Pero, unpublished data, (12, 36)). Compared with MCA, 3-CPA (pH 6.7–7.0) lacks the orthomethoxy benzamide ring substitution which is believed to be responsible for binding dopamine D_2 receptors and mediating sedation and extrapyramidal effects and hence it has even more extended conformation (Fig. 1). These data may explain why 3-CPA administered mice did not show sedative symptoms even at doses up to 160 mg/kg whereas these side effects were readily observed with MCA at 20 mg/kg. The fact that 3-CPA did not induce any significant extrapyramidal side effects, but yet has shown antitumor and chemosensitizing properties comparable in potency to MCA, supports continuing the preclinical and clinical development for this new N-substituted analog. Moreover, because it is a safe analog to MCA, it can be administered at higher doses to achieve even greater effective tumor cytotoxic responses.

Another interesting observation was that MCA i.m. injections of either MCA or 3-CPA did not cause any notable local tissue reaction even at injected concentrations as high as 20 to 80 mg/ml. This was clearly unexpected since 4–5 mg/ml i.m. injected MCA in the rat caused severe local tissue reactions (12, 37). The reason for this discrepancy is currently unknown, but it should be pointed out that scid mice are immunodeficient and there may be less of an inflammatory reaction at the injection site for these animals.

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Table 4

Tumor doubling time and tumor growth time in scid mice xenografted with a human brain astrocytoma and treated with 3-chloroprocainamide (3-CPA), cisplatin (cis.) or both in combination

Group	Number	Tumor doubling time		Tumor growth time*	
		(Day)	Ratio[d]	(Day)	Ratio
Control	11	3.20 ± 0.40	–	13.1 ± 0.47	–
3-CPA	11	4.42 ± 0.55[a]	1.38	15.22 ± .53[a]	1.16
Cis.	11	4.99 ± 0.46[a]	1.56	17.13 ± 0.83[a]	1.31
Cis. + 3-CPA	10	6.77 ± 0.65[a,b,c]	2.11	19.04 ± 1.08[a,b,c]	1.45

Animals were treated with 3-CPA at 3 × 40 mg/kg, cis. at 1 × 7.5 mg/kg or both in combination (Cis. + 3-CPA). Data are mean ± SE. a: $p < 0.05$ vs. control; b: $p < 0.05$ vs. cisplatin alone; c: $p < 0.05$ vs. 3 CPA alone. d: ratio divided by the control. *: the time taken for tumor volumes to reach 1 000 mm^3.

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