Title

Anti-Tumor Activity of 1-ethyl-3-(3'-Dimethylaminopropyl) Carbodiimide. HCl Against Mouse Neuroblastoma C1300

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Running Title:

Carbodiimide Chemotherapy of Neuroblastoma C1300 Tumors

SUMMARY

The anti-tumor activity of the hydrochloride salt of 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI·HCl) has been studied with transplanted neuroblastoma C1300 tumors in syngeneic A/HeJ hosts. The action of this drug is strongly dependent on tumor age, with the maximum effect being observed at three weeks following transplantation. The relationship between drug effectiveness and tumor age suggests that EDCI·HCl may facilitate the interaction of host immune agents with their tumor-specific antigen targets. Experimental results are presented that argue against other possible mechanisms of drug action, such as a vasoconstrictive effect, anti-metabolite action, and dependence of drug effectiveness on tumor anoxia. Evidence that EDCI·HCl potentiates a lipid-dependent host immune reaction is discussed.

INTRODUCTION

A large body of experimental evidence indicates that all classes of tumors -- spontaneous, transplanted, and chemically- or virally-induced -- possess antigens that elicit a host immune response. Despite the immunogenicity of tumor cells, the rejection of large tumors seldom occurs in autochthonous or syngeneic hosts. Inability of the host to reject established tumors has been attributed to deficiencies in the afferent, central, and efferent processes involved in the immune response.[1,2,3] With regard to inadequacy of the efferent response, a number of theories have been proposed. The inability of cell-bound or humoral antibodies to interact effectively with tumor-specific antigen targets may result, for example, from inhibition by blocking antibodies,[4] antigen masking by a surface mucoid coat,[5] or steric hindrance resulting from an abnormally high electric charge density at the tumor cell surface.[6] It is conceivable, therefore, that drugs reactive with chemical groups at the cellular surface could serve to remove restrictions preventing the cytotoxic action of antibodies directed against tumor-specific antigens. Several recent studies have demonstrated that neuraminidase, an enzyme that cleaves sialic acid groups linked by O-glycosidic bonds to carbohydrate at the cellular surface, facilitates an immune response against transplanted tumors in mice.[5,7]

By virtue of their reactivity with carboxylic acids and other nucleophilic groups, carbodiimides constitute a class of compounds that could serve to potentiate the efferent host immune response by modifying ionogenic moieties at the tumor cell surface. Reduction of the negative surface charge of erythrocytes through reaction of a

carbodiimide with anionic groups at the cell periphery,[8] and the alteration of blood group antigens by a carbodiimide agent,[9] have previously been established. In this paper, we report studies on the anti-tumor activity of the hydrochloride salt of 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI·HCl) against neuroblastoma C1300 tumors in syngeneic A/HeJ hosts. The effectiveness of EDCI·HCl is shown to be greatest against well-established tumors, and evidence that this drug potentiates the cytotoxic action of host antibodies is discussed.

MATERIALS AND METHODS

Chemicals. The hydrochloride salt of 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI·HCl) was obtained from the Ott Chemical Company (Muskegon, Michigan). The quaternized derivative 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide methiodide (EDCIMI) was prepared from freshly distilled 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide base (EDCI) by reaction with methyl iodide.[10] Both EDCI·HCl and EDCIMI are hygroscopic, and were stored in a vacuum desiccator. The anti-metabolite 2-amino-6-mercaptopurine (6-thioguanine: 6-TG) was obtained from Calbiochem (Los Angeles, California). Other reagents used in these studies were ^{3}H-labelled thymidine (0.5 mCi/ml; Schwartz/Mann, Orangeburg, New York), and ^{131}I-labelled human serum albumin (0.5 mCi/ml; Squibb, New York, New York).

Transplantation. Chemotherapy trials were performed with the transplanted neuroblastoma C1300 tumor maintained in syngeneic A/HeJ hosts (Jackson Laboratories, Bar Harbor, Maine). Transplantation procedures followed Cancer Chemotherapy National Service Center specifications.[11] Toxicity studies were performed with normal A/HeJ mice.

Chemotherapy trials were initiated at 4, 11, and 21 days following tumor implantation. The size of the transplant inoculum was adjusted to give uniform tumor growth throughout the trial period. Chemotherapy trials undertaken at 11 or 21 days following transplantation were performed only with mice bearing tumors of mean diameter 1.2 ± 0.2 cm. Tumors of this size on the 11th and 21st day after transplantation were obtained with inocula of 2.5 and 0.3 mm diameter, respectively.

Drug Injection. EDCI·HCl and EDCIMI were administered by s.c.,

i.p., and i.v. routes in an injection volume of 0.2 ml. The s.c. injections were made at the midline of the back, and i.v. injections were administered in the tail vein as a bolus. Individual solutions of drug at a specified dose level (mg drug per kg body weight) were prepared for each mouse body weight in grams (typically 20 to 28 g). A calculated amount of drug was weighed with an accuracy of ± 0.1 mg and dissolved in 10 ml of sterile saline immediately prior to injection. For consistency, injections were based on mouse body weight rounded off to the lower gram. Control animals were injected with saline.

Identical procedures were used with 6-TG, except that a few drops of 1 N NaOH were added to completely dissolve the drug. The resulting pH of the injected solution was approximately 11.7. The 6-TG was administered i.p. in three doses at hourly intervals. Control mice were injected i.p. with saline adjusted to pH 11.7.

Chemotherapy Trials. When drug injections were made on the fourth day following transplantation (prior to palpable tumor growth), mice were randomized into drug-treated and control groups of equal size. Tumors were excised from drug-injected and control mice on the seventh day after injection. Effectiveness of chemotherapeutic treatment was judged from the average tumor weight of drug-injected mice divided by the average tumor weight of saline-injected control mice: the T/C ratio. In experiments initiated on the 11th or 21st day following transplantation, the mean diameter of each tumor was determined from caliper measurements of the long and short tumor axes. Mice were then closely pair-matched on the basis of their tumor axial ratios and mean diameters. Each pair of mice was randomized with respect to drug or saline injection. Progress of chemotherapy was followed by caliper measure-

ments for seven days after the initial drug injection, at which time the tumors were excised and weighed for calculation of a T/C ratio.

Plasma Volumes. The isotope dilution technique was used to determine plasma volumes of drug-injected and control mice. For this purpose, 0.5 μCi of ^{131}I-labelled human serum albumin was injected i.v. Blood samples obtained by cardiac puncture 15 min after injection of the isotope were counted in a well-type scintillation counter (Nuclear-Chicago, Des Plaines, Illinois).

RESULTS

Drug Chemistry. Chart 1 depicts the structural formulas of carbodiimide agents used in the chemotherapy trials reported here. Spectroscopic studies have established that both the free base 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI) and the quaternized derivative 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide methiodide (EDCIMI) exist only as open chain carbodiimide structures.[12] The hydrochloride salt of EDCI base (EDCI·HCl) exists in aqueous solution at physiological pH as an equilibrium tautomeric mixture of two species: 7.4% as 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride and 92.6% as 2-ethylamino-3,3-dimethyl-3,4,5,6-tetrahydropyrimidine chloride.[12]

Toxicity Studies. Toxicity data for EDCI·HCl, EDCIMI, and 6-TG administered to normal A/HeJ mice are summarized in Table 1. Animals shipped during the winter months were frequently in poor health, and exhibited a low tolerance for EDCI·HCl and EDCIMI. For this reason, averages of toxicity data accumulated over an eight month period are presented in Table 1.

Regardless of the route of administration, the toxic levels of EDCI·HCl fell within a very narrow dose range. EDCI·HCl was better tolerated by the i.p. than by the s.c. route. A lower toxicity by the i.p. route can be attributed either to a more rapid absorption of drug from the peritoneal cavity followed by rapid clearance from the circulation, or to a substantial drug clearance on the first pass of portal venous blood through the liver. Death resulting from s.c. or i.p. doses of EDCI·HCl generally occurred within one week following injection. Surviving animals appeared completely normal throughout

a four month observation period, and maintained stable body weights. As judged by microscopic pathology, all tissues except for the liver retained a normal appearance following injection of EDCI·HCl. Within one day following administration of EDCI·HCl, substantial glycogen depletion and cytoplasmic vacuolation of parenchymal cells were observed in the liver. This damage is probably reversible since the livers of drug-injected mice regained a normal appearance by the seventh day post-injection. The significance of the changes in liver cells following administration of EDCI·HCl is not clear at present. The observed glycogen depletion may result from stress or a lack of food intake. Changes in cell morphology, however, may be due to a specific effect of EDCI·HCl on parenchymal liver cells.

A second adverse effect of EDCI·HCl administered by either the i.p. or s.c. route was a marked decrease in plasma volume. As early as one hour following injection of EDCI·HCl, a rise in hematocrit was noted. At four hours post-injection, the increase in hematocrit reached a maximum value of approximately 20%, and remained at this level during the following four hours. By 12 hours post-injection, the hematocrit returned to normal. This hemoconcentrating effect of EDCI·HCl was found to be rather insensitive to the administered dose, since an identical rise in hematocrit was noted with s.c. doses of 20 mg/kg and 30 mg/kg, and with i.p. doses of 40 mg/kg and 50 mg/kg. Based on an isotope dilution study with ^{131}I-labelled human serum albumin, the plasma volumes of mice administered either 20 mg/kg or 30 mg/kg s.c. doses of EDCI·HCl decreased by 30% during the four hour period following injection. The total red cell volume, however, remained constant. One possible explanation for this decrease in plasma volume is a loss of plasma at the site of drug injection. EDCI·HCl

administered s.c. in the back was noted to produce an inflammatory reaction. Despite the substantial loss of blood plasma following EDCI·HCl injection, this factor does not appear to play a primary role in drug toxicity since a quantitatively similar rise in hematocrit occurs following administration of both lethal and sublethal doses.

The i.v. injection of EDCI·HCl resulted in a completely different symptomatology and pattern of death than that observed following s.c. or i.p. drug administration. At dose levels in excess of 15 mg/kg, all mice were found to develop convulsions within 5 sec post-injection. With doses below 40 mg/kg, these convulsions were followed by stupor with subsequent complete recovery within 4 to 5 min. Dose levels above 45 mg/kg were lethal. Surviving mice had normal hematocrits, and showed no adverse effects of drug injection during a four month observation period.

The i.v. administration of EDCIMI resulted in a symptomatology and pattern of death comparable to those observed following i.v. injection of EDCI·HCl, except that significantly smaller doses were required to produce these effects. Doses of EDCIMI ranging from 1 to 3 mg/kg resulted in convulsions, followed by stupor with subsequent complete recovery. At higher doses, significant mortality occurred. Surviving mice appeared completely normal throughout a four month observation period. The difference in i.v. dose levels of EDCI·HCl and EDCIMI required to produce immediate symptomatology correlates with the difference in the number of moles of open chain carbodiimide present per unit weight of these two compounds. This fact suggests that the convulsions which follow an i.v. injection of EDCI·HCl are a consequence of the carbodiimide hydrochloride isomer, and are not

directly related to the reduced pyrimidine species.

When EDCIMI was administered s.c., the symptomatology and pattern of death were similar to those observed following injection by the i.v. route, except that a much larger dose was required to produce convulsions. In addition, the onset of convulsions was delayed by approximately 5 min relative to an i.v. dose. The fact that administration of EDCIMI by the s.c. route leads to immediate symptomatology is in sharp contrast to the observed behavior of A/HeJ mice following s.c. injection of EDCI·HCl. It is probable that s.c. injection of EDCIMI produces transiently a high concentration of open chain carbodiimide in the circulation.

The i.p. administration of 6-TG in three 20 mg/kg doses at hourly intervals was well tolerated by A/HeJ mice. No weight loss or chronic effects of 6-TG injection were noted over a four month observation period. A number of single injection protocols with 6-TG dose levels in excess of 60 mg/kg were also attempted, but these led to a substantial weight loss and chronic effects resulting from bone marrow toxicity.

The toxicity of EDCI·HCl, EDCIMI, and 6-TG in tumor-bearing mice was generally comparable to that observed in normal mice. An exception occurred, however, in several experiments where EDCI·HCl was administered s.c. to mice with 4-day-old C1300 tumors. In these experiments, the mortality level was substantially higher than normal (cf. Tables 1 and 2). At present, we have no explanation for this observation, other than ascribing the elevated toxicity to a combined stress resulting from transplantation and subsequent drug injection.

Chemotherapy Trials. The effects of EDCI·HCl, EDCIMI, and 6-TG

on the growth of neuroblastoma C1300 tumors are summarized in Table 2 and Chart 2. Extensive studies on the anti-tumor activity of EDCI·HCl administered by the s.c. route were carried out with a single 30 mg/kg dose, since less than 10% mortality was observed at this dose level in mice bearing well-established tumors and in good health at the initiation of chemotherapy testing. Following a single 30 mg/kg dose to A/HeJ mice bearing 4-, 11-, and 21-day-old C1300 tumors, the respective T/C ratios at 7 days post-injection were 0.82, 0.69, and 0.58. Based on a Student's "t" test, the T/C ratios observed with 11- and 21-day-old C1300 tumors had a high level of statistical significance ($P<0.001$). The difference in T/C ratios between mice bearing 11- and 21-day-old tumors at the time of injection was only weakly significant ($P\approx 0.2$). The rate of tumor growth following injection of EDCI·HCl, however, showed a different pattern for these two groups. As shown in panel b of Chart 2, the s.c. administration of a 30 mg/kg dose of EDCI·HCl resulted in complete arrest of 21-day-old C1300 tumors for a period of 4 days post-injection. In contrast, EDCI·HCl administration failed to arrest the growth of 11-day-old C1300 tumors (panel a, Chart 2).

The response of established C1300 tumors to EDCI·HCl was observed to be critically dependent on the dose of administered drug. A 27 mg/kg s.c. dose of EDCI·HCl was found to produce a weaker response in 21-day-old C1300 tumors than a 30 mg/kg s.c. dose, as judged by both the rate of tumor growth and the T/C ratio at 7 days post-injection (Table 2, panel c of Chart 2).

Microscopic pathology of 21-day-old C1300 tumors from A/HeJ hosts injected with a 30 mg/kg s.c. dose of EDCI·HCl showed significant

destruction of tumor cells as early as 12 hours after administration of the drug. By 24 hours post-injection a blackening was consistently noted in the tumors of drug-treated mice, and pathology studies demonstrated extensive regions of hemorrhagic tissue. The peripheral regions of tumors from drug-treated and saline control mice are compared in Figs. 1 to 4. Based on the uptake of ^{3}H-labelled thymidine, no DNA synthesis was observed in tumors from drug-injected mice.

An attempt was made to prolong the carcinostatic effect observed following a 30 mg/kg s.c. dose of EDCI·HCl to mice bearing 21-day-old C1300 tumors. A second s.c. injection at this dose level on the fourth day following the initial injection was ineffective in arresting subsequent tumor growth.

When injected i.v. at the maximum tolerated dose of 45 mg/kg, EDCI·HCl exhibited no anti-tumor action (panel d, Chart 2). This result suggests that EDCI·HCl is rapidly cleared once it enters the circulation, and that its effectiveness against established tumors when injected by the s.c. or i.p. route is a consequence of its slow release into the blood.

The effect on 4- and 21-day-old C1300 tumors of a 50 mg/kg i.p. dose of EDCI·HCl is comparable to that observed with a 30 mg/kg s.c. dose (see Table 2 and panel f of Chart 2). The response of 21-day-old tumors to drug administered by the i.p. route is also extremely dose dependent, as demonstrated by the ineffectiveness of a 40 mg/kg dose (panel e, Chart 2).

When EDCIMI was administered s.c. at the maximum tolerated dose of 37 mg/kg, no effect was observed on 21-day-old C1300 tumors (panel g, Chart 2). Since EDCIMI exists exclusively as an open chain carbo-

diimide, this result suggests that the reduced pyrimidine isomer plays an essential role in the action of EDCI·HCl against established tumors.

Administration of 6-TG in three 20 mg/kg i.p. doses at hourly intervals to mice bearing 4- and 21-day-old C1300 tumors resulted in respective T/C ratios of 0.20 and 0.57 at 7 days post-injection (Table 2 and panel h of Chart 2). From this study, it is clear that the anti-metabolite 6-TG is most effective during initial stages of tumor growth, in contrast to EDCI·HCl. The strong response of 4-day-old tumors to 6-TG also indicates that the access of drug to recently transplanted tumors is not limited by the possible lack of a fully developed vasculature.

DISCUSSION

In preliminary trials, the anti-tumor activities of the hydrochloride salt of 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI·HCl), 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide methiodide (EDCIMI), and 1-cyclohexyl-3-(2-morpholinylethyl)carbodiimide metho-p-toluenesulfonate were studied using five lines of transplanted tumors: neuroblastoma C1300, carcinoma 15091A, and sarcoma SaI in A/HeJ hosts; sarcoma S180 in Swiss/Webster hosts; and carcinoma Ca755 in C57BL/6J hosts. These experiments established that only EDCI·HCl was effective as a chemotherapeutic agent, and that the response of all five tumor lines to this drug was greatest at three weeks following transplantation. Previous studies have also established that carbodiimide bases (e.g. dicyclohexylcarbodiimide) are ineffective as chemotherapeutic agents when administered during the first week following transplantation.[13]

In extending our chemotherapy trials to encompass a large number of injection protocols in mice bearing 4-, 11-, and 21-day-old tumors, we restricted our attention to defining the effects of EDCI·HCl on the growth properties of the neuroblastoma C1300 in syngeneic A/HeJ hosts. Choice of the C1300 tumor line was motivated by three factors. First, a uniform growth rate could be attained with the C1300 tumor over a period of four weeks following transplantation. Second, the C1300 tumor is encapsulated and does not metastasize.[14] Third, the neuroblastoma C1300 tumor can be explanted directly from an A/HeJ host into cell culture, thereby permitting the performance of *in vitro* studies on drug mechanism in parallel with *in vivo* clinical studies.

The results presented here demonstrate that a strong response to EDCI·HCl occurs only with older, well-established C1300 tumors, suggesting that this drug may be enhancing the host immune response. The timing of the immune response against transplanted tumors in syngeneic hosts has not been determined with precision, primarily because the small amount of cytotoxic antibody present in the sera of these animals is difficult to detect.[15] Some experimental observations indicate that inhibitory humoral and cellular antibody responses may not reach maximal levels for a period in excess of 10 days following tumor transplantation.[16,17] In the case of tumor homographs, maximum levels of isoantibodies have been observed at 10 to 14 days after tumor inoculation.[18] The dependence of EDCI·HCl activity on tumor age therefore appears to correlate well with the timing of the host immune response. In addition, the fact that a massive destruction of tumor cells results within 24 hours following drug injection indicates that an immunologic action by EDCI·HCl must occur in the efferent limb of the immune arc.

While the pattern of response suggests that EDCI·HCl may act through an immunologic mechanism, other possible modes of drug action must be considered. The administration of EDCI·HCl by either the s.c. or i.p. route to normal or tumor-bearing mice results in a significant decrease in plasma volume, presumably due to the inflammatory reaction at the site of drug injection. As a result of the decrease in plasma volume, these animals have a marked peripheral vasoconstriction. In large tumors containing necrotic regions, the blood supply is already compromised. It is probable that a further decrease in blood supply will occur as a result of the vasoconstricting effect

of EDCI·HCl, and this reduction could adversely affect tumor growth. An argument that vasoconstriction does not lead to the observed anti-tumor action is provided by the experiment in which EDCI·HCl was administered i.p. at a dose level of 40 mg/kg. This dose was found to have no effect on the growth of 21-day-old C1300 tumors, but did produce a reduction in plasma volume comparable to that observed with effective chemotherapeutic doses (e.g. 50 mg/kg i.p. or 30 mg/kg s.c.). Moreover, in mice bearing 21-day-old tumors, acute bleeding (25% of the blood volume) did not cause a reduction in tumor growth rate.

The action of EDCI·HCl may also result from an anti-metabolite effect.[19,20] The possibility exists that in the case of 4-day-old tumors, the supply of drug may be limited since a complete vascular network is not acquired by tumors for a period of 3 to 6 days following transplantation.[21] The access of EDCI·HCl to a 4-day-old tumor might then be limited to diffusion from neighboring tissues, while a well-established tumor with a fully developed circulatory system would receive a substantially greater supply of drug. The data presented here cannot rule out the possibility that EDCI·HCl exerts an anti-metabolite effect which depends upon the age of tumors as a consequence of the developmental stage of their blood supply. However, the experiments with 6-TG suggest that this is probably not the case, since the action of this anti-metabolite is considerably greater against 4-day-old than 21-day-old C1300 tumors. Barring significant differences in the diffusion rates of 6-TG and EDCI·HCl through tissues, the effectiveness of the former during initial stages of tumor growth suggests that 4-day-old C1300 tumors have an adequate access to other injected drugs such as EDCI·HCl.

The effectiveness of EDCI·HCl against established tumors may also result from a more potent drug action against anoxic tissue. We have observed, however, that the anti-tumor activity of a 30 mg/kg s.c. dose of EDCI·HCl is greater against 21-day-old than 11-day-old C1300 tumors of identical size. Since the extent of anoxia in these two groups of tumors is comparable, it is improbable that the action of EDCI·HCl is dependent upon the presence of anoxic tissue.

On the basis of these arguments, a non-immunologic mechanism of EDCI·HCl action appears unlikely. In preliminary studies with C1300 tumor cells maintained in tissue culture, we have found that physiological concentrations of EDCI·HCl serve to potentiate a cytotoxic action by immune serum from syngeneic A/HeJ tumor hosts. This effect has been observed only when the immune serum was lipemic, or when lipemic heterologous serum was added to the culture medium. Lipemic normal serum in the presence of EDCI·HCl did not have a comparable cytotoxic effect. These observations indicate that EDCI·HCl may be enhancing an immunologic response against C1300 tumor cells mediated by humoral antibody and dependent upon the presence of auxiliary lipids. The existence of lipid dependent immune reactions is well-established, including the interaction of antibodies with the tumor antigen cytolipin H.[22] We have also obtained preliminary evidence that the activity of EDCI·HCl against 21-day-old C1300 tumors in fasted mice is significantly less than the response observed with non-fasted mice. In conjunction with this study, it has been found that the level of low density serum lipoproteins is elevated in mice bearing 21-day-old C1300 tumors, but drops below normal during a one day fasting period.

Using a cell culture system, we have also observed that physiological concentrations of EDCI·HCl reduce the net negative surface charge of C1300 tumor cells by 20% within a period of two hours. This fact lends credence to our original speculation that carbodiimide reactions at the tumor cell surface could serve to remove charge-determined restrictions on antibody interaction. In the absence of detailed knowledge concerning the chemical nature and ultrastructural localization of tumor-specific antigens,[23] it is difficult at present to visualize the mechanism by which a reduction in the net negative charge could facilitate antigen-antibody interactions at the cell periphery. We speculate that the anti-tumor action of EDCI·HCl may result from one of three possible effects on the tumor cell surface. First, reaction of carbodiimide with sialic acid carboxyls and other nucleophilic groups may serve to reduce the electric charge density near tumor-specific antigen sites. Second, carbodiimide reactions may promote the dissociation of an extraneous sialomucin coat from the cell surface, thereby unmasking tumor-specific antigen sites. Third, carbodiimide may serve as an intermediate in coupling reactions between membrane proteins,[24] thereby forcing a conformational change that exposes "cryptic" antigen sites at the tumor cell surface. All three of these alternatives are more attractive if tumor-specific antigens are asialyated, as in the case of cytolipin H.[23] In this event, the probability is greater for preservation of the specificity of tumor-specific antigens following introduction of a carbodiimide agent.

A final point that merits discussion is the role of the reduced pyrimidine isomer in the anti-tumor action exerted by EDCI·HCl. On

the basis of experimental results presented here, it is not possible to assess whether one or both of the EDCI·HCl tautomeric species is the effective anti-tumor agent. The fact that the quaternized derivative EDCIMI has no effect on tumors suggests that the reduced pyrimidine isomer may be essential for the activity of EDCI·HCl. Two possible mechanisms can be proposed. First, the reduced pyrimidine species may be metabolized and/or excreted less rapidly than the carbodiimide hydrochloride isomer, and thereby serve to prolong the presence of carbodiimide in the circulation. Second, the reduced pyrimidine species may exert an anti-metabolite effect that promotes tumor cell destruction in the wake of an immunologic attack potentiated by reaction of the carbodiimide isomer with ionogenic groups at the cell surface. Further cell culture experiments with EDCI·HCl and EDCIMI, as well as isotope studies of drug kinetics and biotransformation, should serve to clarify the role of the reduced pyrimidine isomer in the anti-tumor action of EDCI·HCl.

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Table 1

Toxicity data accumulated over an 8 month period for a single dose of the hydrochloride salt of 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI·HCl) and 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide methiodide (EDCIMI) in normal A/HeJ mice, and for a fractionated dose of 6-thioguanine (6-TG).

Drug	Route of Administration	Dose[a] (mg/kg body wt)	Survivors[b]	% Mortality
EDCI·HCl	s.c.	27	15/16	7
		30	53/70	24
		33	21/34	38
		35	12/23	48
		40	3/10	70
EDCI·HCl	i.p.	35	15/16	7
		40	18/22	18
		45	26/32	19
		50	21/32	34
		55	9/26	65
EDCI·HCl	i.v.	35	6/6	0
		45	10/12	17
		55	7/12	42
		60	0/6	100
EDCIMI	s.c.	35	12/12	0
		37	12/12	0
		40	12/20	40
		45	0/12	100
EDCIMI	i.v.	3	6/6	0
		4	2/6	67
6-TG	i.p. (Hourly doses)	3 x 20	10/10	0

[a] Animal weights were stable for a period of 4 months post-injection with the drugs and dose schedules used in these studies.

[b] Death resulting from s.c. or i.p. doses of EDCI·HCl usually occurred within 1 to 7 days post-injection. Death resulting from an i.v. dose of EDCI·HCl or an i.v. or s.c. dose of EDCIMI occurred within 10 min post-injection. No chronic effects were noted in survivors over a 4 month period.

Table 2

Response of the neuroblastoma C1300 tumor to a single dose of the hydrochloride salt of 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI·HCl) and 1-ethyl-3-(3'-dimethyl-aminopropyl)carbodiimide methiodide (EDCIMI), and a fractionated dose of 6-thioguanine (6-TG).

Drug	Route of Administration	Dose (mg/kg body wt)	Day of Injection[a]	Number of Drug-Injected Mice[b]	% Mortality	Host Weight Change at 7 Days Post-Injection[c]	(T/C)[d]	P[e]
EDCI·HCl	s.c.	30	4	133	57	- 1.8 (- 7.4%)	0.82	0.07
		30	11	65	8	- 1.5 (- 6.1%)	0.69	<0.001
		30	21	42	33	- 0.7 (- 2.7%)	0.58	<0.001
		27	21	8	0	- 0.3 (- 1.2%)	0.67	0.05
EDCI·HCl	i.p.	50	4	40	15	- 2.4 (- 9.6%)	0.95	0.58
		50	21	10	10	- 3.5 (- 14.8%)	0.51	<0.001
		40	21	10	10	- 1.8 (- 7.4%)	0.98	0.92
EDCI·HCl	i.v.	45	21	6	17	- 0.5 (- 2.0%)	0.92	0.61
EDCIMI	s.c.	37	21	12	8	- 0.7 (- 2.7%)	0.99	1.00
6-TG	i.p.	3 X 20	4	36	3	- 2.0 (- 7.5%)	0.20	<0.001
	(Hourly doses)	3 X 20	21	10	0	- 1.5 (- 6.4%)	0.57	0.005

[a] The day of tumor implantation is counted as Day 0.

[b] In chemotherapy trials initiated at 4 days following tumor implantation, the mice were randomized and divided into a drug-injected group and a saline-injected control group of equal size. For drug injections performed at 11 and 21 days following tumor implantation, mice were pair-matched on the basis of tumor size.

[c] Numbers in parentheses indicate percentage weight loss relative to the day of drug injection.

[d] T/C ratios were determined from the average tumor weight of drug-injected mice divided by the average tumor weight of saline-injected control mice at 7 days post-injection. In experiments initiated at 11 and 21 days following tumor implantation, the weights of tumors from saline control mice pair-matched with mice that expired following drug injection were not included in the calculation of T/C ratios.

[e] Based on a Student's "t" test for the significance of the difference in tumor weights of drug-injected and saline-injected control mice at 7 days post-injection.

Legends for Illustrations

Chart 1. Chemical structures are shown for the carbodiimide agents used in chemotherapy trials with the neuroblastoma C1300 tumor line. EDCI is the carbodiimide free base 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide. EDCI·HCl is the hydrochloride salt of EDCI, and exists in aqueous solution at neutral pH as a tautomeric mixture of two species: 7.4% as the carbodiimide hydrochloride (I) and 92.6% as the reduced pyrimidine isomer 2-ethylamino-3,3-dimethyl-3,4,5,6-tetrahydropyrimidine chloride (II). EDCIMI is the quaternized derivative 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide methiodide produced by reaction of EDCI with methyl iodide.

Chart 2. The mean diameters of C1300 neuroblastoma tumors are plotted for drug-injected mice relative to saline-injected control mice over a period of 7 days post-injection. Data are presented for single doses of the hydrochloride salt of 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI·HCl) and 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide methiodide (EDCIMI), and for a fractionated dose of 6-thioguanine (6-TG). Drug-injected and control mice were closely pair-matched on the basis of tumor size at the time of injection. The number of pairs of mice used in these trials, the mortality levels observed following drug injection, and the T/C ratios at 7 days post-injection are summarized in Table 2.

Figure 1. The peripheral region of a 21-day-old C1300 tumor is shown at 25 hours following administration of a 30 mg/kg s.c. dose of the hydrochloride salt of 1-ethyl-3-(3'-dimethylaminopropyl)

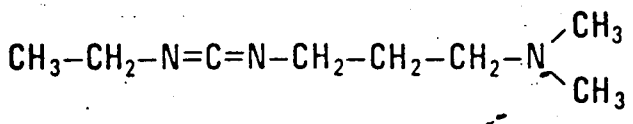
carbodiimide (EDCI·HCl). The interior region of this tumor (not shown) exhibited substantial necrosis, with few remaining areas of confluent cells. H. and E., X 300.

Figure 2. Radioautograph of tumor region shown in Fig. 1, demonstrating essentially no uptake of ^{3}H-labelled thymidine. The tumor host was administered an i.v. pulse of ^{3}H-labelled thymidine (1 mCi/kg body wt) at 24 hours following drug injection, and sacrificed one hour later. Oil immersion, X 2000.

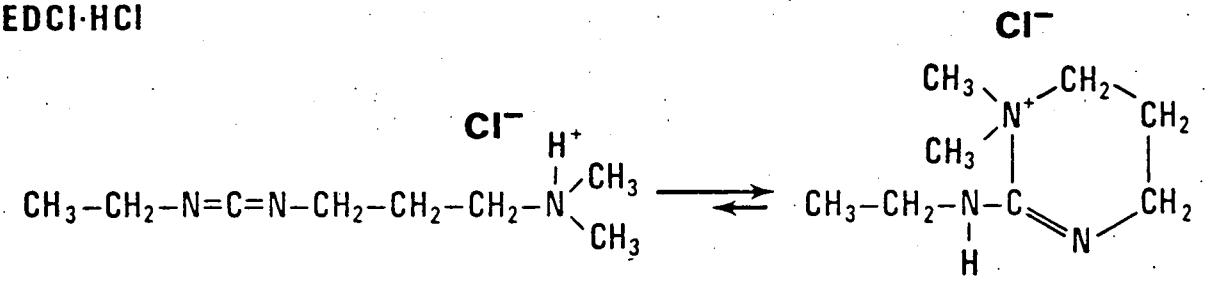
Figure 3. Peripheral region of a 21-day-old C1300 tumor from a saline-injected control mouse sacrificed at 25 hours post-injection. The central region of this tumor (not shown) exhibited little necrosis. This tumor had a mean diameter of 1.22 cm at the time of injection, and was pair-matched with the tumor shown in Figs. 1 and 2. H. and E., X 300.

Figure 4. Radioautograph of the tumor region shown in Fig. 3. A large number of cells are synthesizing DNA, as evidenced by uptake of ^{3}H-labelled thymidine. Oil immersion, X 2000.

EDCI

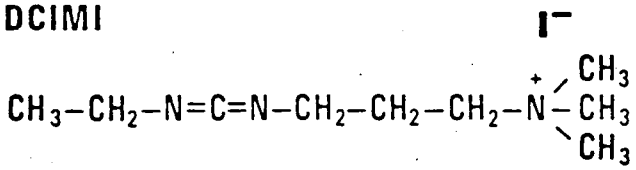


EDCI·HCl



(I) (II)

EDCIMI



DBL 725 5310

CHART 1

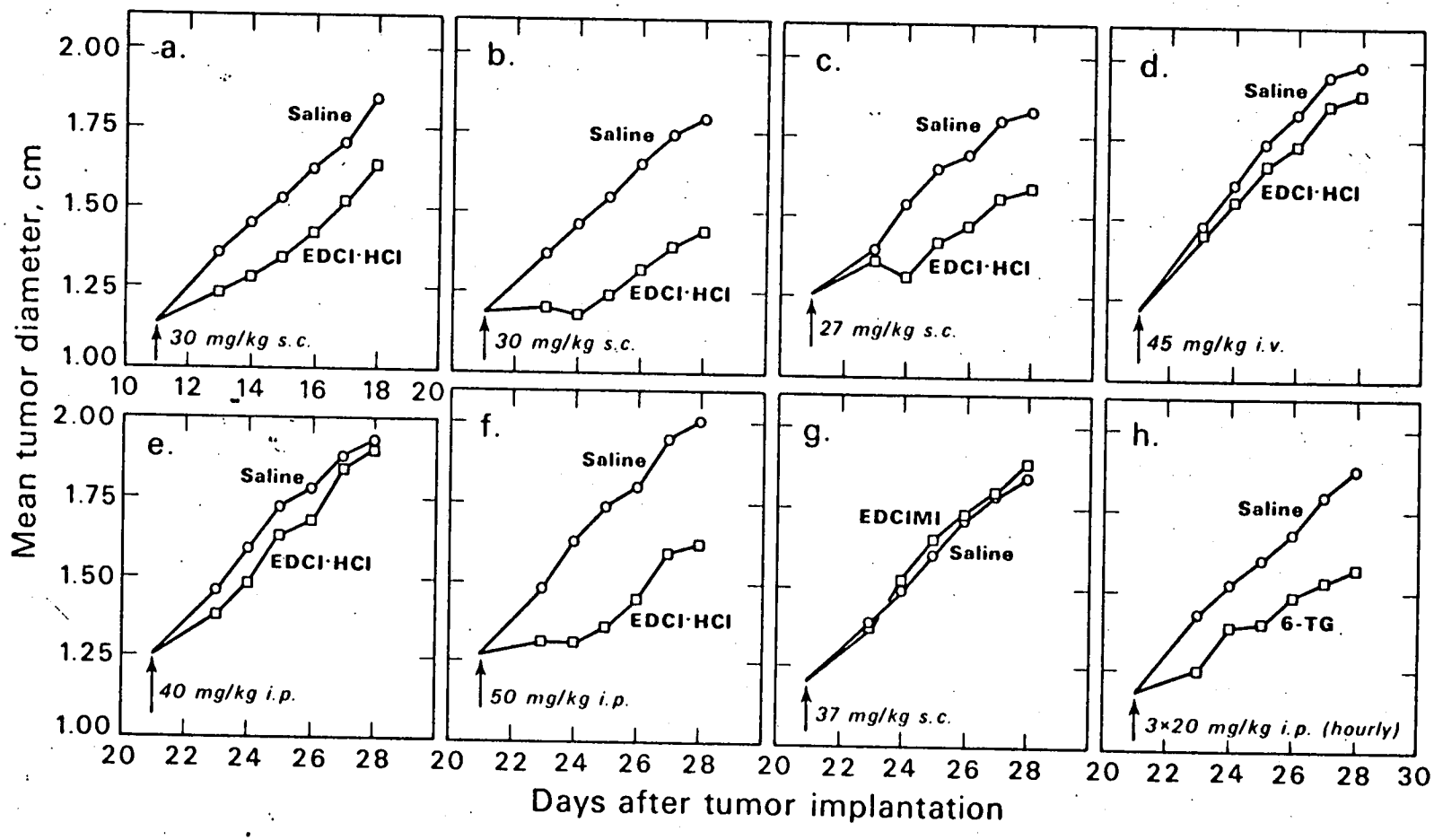
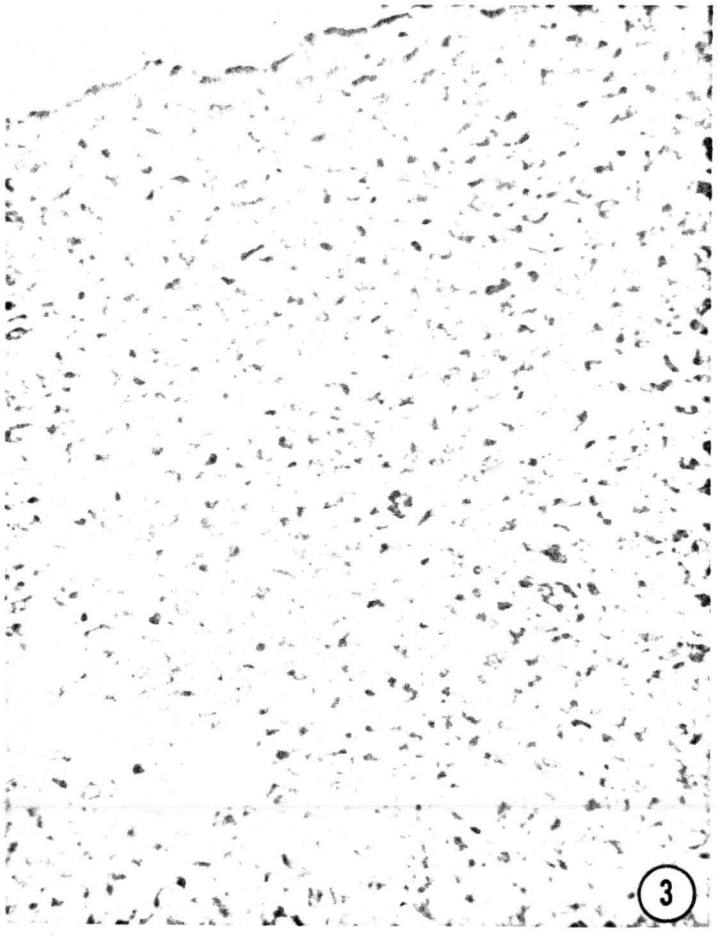
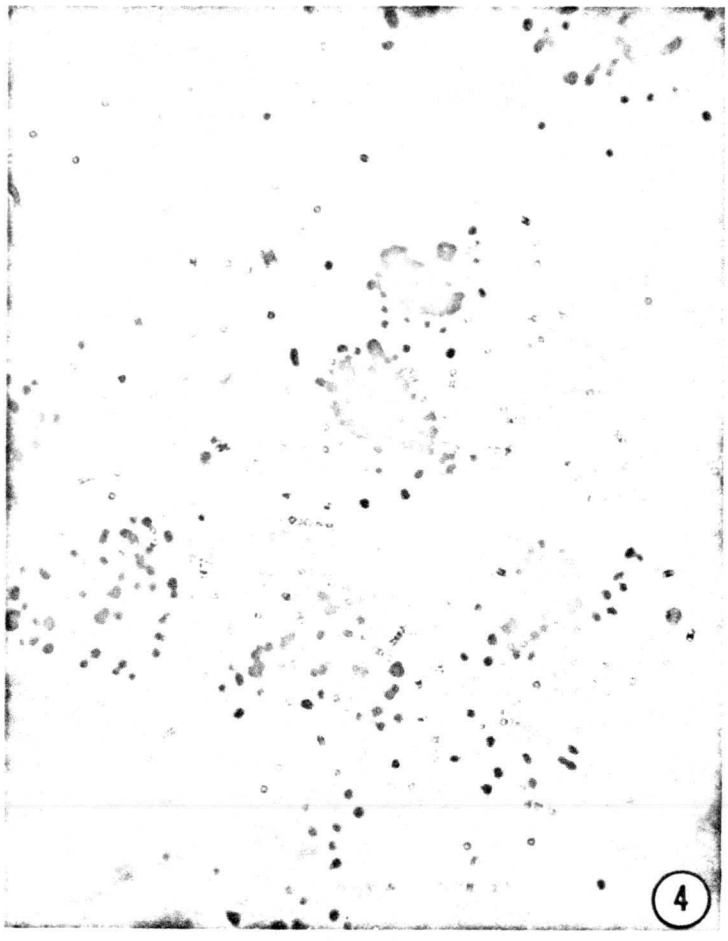


CHART 2

DBL 725 5311





FIGURES 1 - 4