Eur J Cancer Clin Oncol. Vol. 20, No. 7, pp. 961–966, 1984.
Printed in Great Britain.

0277–5379/84$3.00+0.00

Hemostasis and Mechanism of Action of Selective Antimetastatic Drugs in Mice Bearing Lewis Lung Carcinoma*

TULLIO GIRALDI,†‡ GIANNI SAVA,† ELIO MITRI§ and ROBERTO CHERUBINO†
†*Istituto di Farmacologia e Farmacognosia, Università di Trieste and* §*Centro Immunotrasfusionale, Unità Sanitaria Locale No. 1, I-34100 Trieste, Italy*

Abstract—*The selective antimetastatic agents* p-(3,3-*dimethyl*-1-*triazeno*)*benzoic acid potassium salt* (*DM-COOK*), 5-(3,3-*dimethyl*-1-*triazeno*)*imidazole*-4-*carboxamide* (*DTIC*) *and* (±)1,2-*di*(3,5-*dioxopiperazin*-1-*yl*)*propane* (*ICRF*-159) *have been shown to markedly depress the formation of spontaneous hematogenous metastases in mice bearing s.c. Lewis lung carcinoma, with a mechanism unrelated to cytotoxicity for tumor cells. The effects on hemostasis of DM-COOK, DTIC and ICRF-159 have thus been examined in comparison with those of a purely cytotoxic agent, cyclophosphamide, in mice bearing i.m. Lewis lung carcinoma. The parameters considered are the number of platelets and their aggregability, prothrombin and partial thromboplastin times, plasma fibrinogen concentration and tumor cell procoagulant activity. Slight variations are caused by drug treatment in tumor-bearing mice as compared with untreated tumor-bearing controls; the pattern of effects of the selective antimetastatic agents does not differ from that of the reference cytotoxic compound used, cyclophosphamide. These data thus indicate that the effects on hemostasis of the drugs examined can contribute only marginally to their antimetastatic action, since more pronounced effects on hemostasis have been shown to be required to significantly affect metastasis formation.*

INTRODUCTION

A LARGE number of reports have appeared in the literature during the past years that show that the hemostatic system of the host may play a role in tumor growth and malignancy. The possible influence of platelets as well as the coagulation–fibrinolytic system on tumor cell growth, dissemination and lodgement in distant organs have been the object of review articles (see, for instance, ref. [1]) and a book [2]. Correspondingly, a relatively large number of investigations have also shown that tumor growth, and particularly tumor metastasis, can be influenced (reduced) by substances (i.e. anticoagulants and platelet aggregation inhibitors) acting on the process of blood coagulation [3–5].

Influences on the hemostatic system of the host might be also involved in the mechanism of action of antimetastatic drugs. Drugs such as (±)1,2-di(3,5-dioxopiperazin-1-yl)propane (ICRF-159) and *p*-(3,3-dimethyl-1-triazeno)benzoic acid potassium salt (DM-COOK) have been shown to be highly active in preventing metastasis formation in mice bearing solid tumors producing hematogenous metastases [6–8], with a mechanism unrelated with cytotoxicity for tumor cells [8]. Evidence has been provided that ICRF-159 [9] and DM-COOK [10] reduce the number of tumor cells circulating in the blood-stream of treated tumor-bearing animals. However, with the exception of a report dealing with the effects of ICRF-159 on platelet behavior and thrombogenesis [11], no systematic investigation appears to have been performed in order to examine the effects of ICRF-159 and DM-COOK on hematological parameters which might influence the final steps of the process of metastasis formation, such as tumor cell lodgement into their target organ(s). Consequently, the effects of the *in vivo*

Accepted 29 December 1983.
*Supported by National Research Council, Special Project 'Control of Neoplastic Growth', contract n.82.00320.96.
‡To whom correspondence should be addressed at: Istituto di Farmacologia e Farmacognosia, via A. Valerio 32, 34100-Trieste, Italy.

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treatment with ICRF-159 and DM-COOK have been examined on blood coagulation parameters of mice bearing i.m. Lewis lung carcinoma. Reference drugs such as the purely cytotoxic agent cyclophosphamide [8] and the clinically employed 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) [12] have also been included in the present investigation and are reported herein.

MATERIALS AND METHODS

Drug synthesis and animal treatment

p-(3,3-Dimethyl-1-triazeno)benzoic acid potassium salt (DM-COOK), synthesized following already reported procedures [13], was generously provided by Dr L. Lassiani, Institute of Medicinal Chemistry, University of Trieste, Italy; (±)1,2-di(3,5-dioxopiperazin-1-yl)propane (ICRF-159) and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) were kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A. Cyclophosphamide was a gift of Schering SpA., Milan, Italy.

The treatment was performed i.p. daily for 14 consecutive days following tumor implantation. The animals received the drugs intraperitoneally in volumes of 0.1 ml/10 g of body wt. Cyclophosphamide (37 mg/kg) and DM-COOK (50 mg/kg) were dissolved in distilled water and 0.1 N $NaHCO_3$ respectively; ICRF-159 (25 mg/kg) and DTIC (60 mg/kg) were administered as a suspension in 0.9% NaCl containing 1% sodium carboxymethylcellulose.

Tumor inoculation

Female BDF1 mice weighing approximately 18–20 g, purchased from Charles River (Calco, Como, Italy), were inoculated i.m. in the calf of the left hind leg with 10^6 viable tumor cells. A single-cell suspension of Lewis lung carcinoma was prepared using tumors obtained from C57BL/6 donors similarly inoculated subcutaneously 2 weeks before. The tumor, freed of capsule and necrotic parts, was minced with scissors, gently forced through a 2.00 × 38-mm disposable needle and suspended in 20 vols of Dulbecco phosphate-buffered saline (DPBS). The cell suspension was filtered through a double layer of sterile gauze and washed with DPBS by centrifugation at 500 *g* for 10 min. Cell viability, as determined by trypan blue exclusion, was about 45–50%.

Blood coagulation assay

Blood collection was performed 24 hr after the last drug administration, on day 15 from tumor implantation, by intracardiac puncture in open-chested mice, previously anesthetized with ethyl urethane (1.5 g/kg i.p.); 9 vols of blood were mixed directly in the syringe with 1 vol. of 0.126 M trisodium citrate.

The number of thrombocytes was determined by phase-contrast microscopic counts in a Burker hemocytometer, after dilution of the blood with ammonium oxalate by means of a capillary standardized pipetting system (Unopette, cat. No. 5855F, Becton-Dickinson Italia, Novate Milanese, Italy). For the determination of platelet aggregation, platelet-rich (PRP) and platelet-poor (PPP) plasma, obtained and pooled from groups of 3 donors, were prepared as described by Gasic *et al.* [14]. Platelet concentration in PRP was adjusted with PPP to 4×10^5 platelets/mm^3; platelet aggregation, in the presence of 3.3 μM ADP, was determined at 37°C using an ELVI dual-channel aggregometer with stirring at 1500 rev/min.

The hematological parameters considered (prothrombin time, partial thromboplastin time and procoagulant activity) were measured using a fibrometer electromechanical apparatus (Becton-Dickinson, Italy) and standard procedures. Prothrombin time was measured using commercially available human placenta thromboplastin (Istituto Bohering, Scoppito, Italy). Partial thromboplastin time was determined using activated cephaloplastin reagent (Dade Diagnostic, Aguada, PR, U.S.A.) and fibrinogen was measured as described by Paar [5] using a kit obtained from Boehringer Mannheim, Milano, Italy. The procoagulant activity of tumor cells was determined as one-stage plasma-recalcification time, following the procedures described by Curatolo *et al.* [16], using cell suspensions prepared by gentle mechanical dissociation of *ex vivo* excised tumor samples or of *in vitro* cell cultures.

RESULTS AND DISCUSSION

The data reported here, which are illustrated in the figures, reveal differences in the coagulative status of normal mice as compared with mice bearing Lewis lung carcinoma. In tumor-bearing animals a significant decrease of the number of thrombocytes (Fig. 1a) and a significant increase in plasmatic fibrinogen concentration (Fig. 2c) as compared with untumored controls was observed. These findings are in agreement with those obtained by other authors examining Lewis lung carcinoma [17] and two lines of mFS6 murine fibrosarcoma having different metastatic potential [18]; in the latter case, however, similar blood coagulation changes were caused by both lines, suggesting that primary rather than metastatic tumor growth influences the host's hemostatic system. Prothrombin (Fig. 2a) and partial

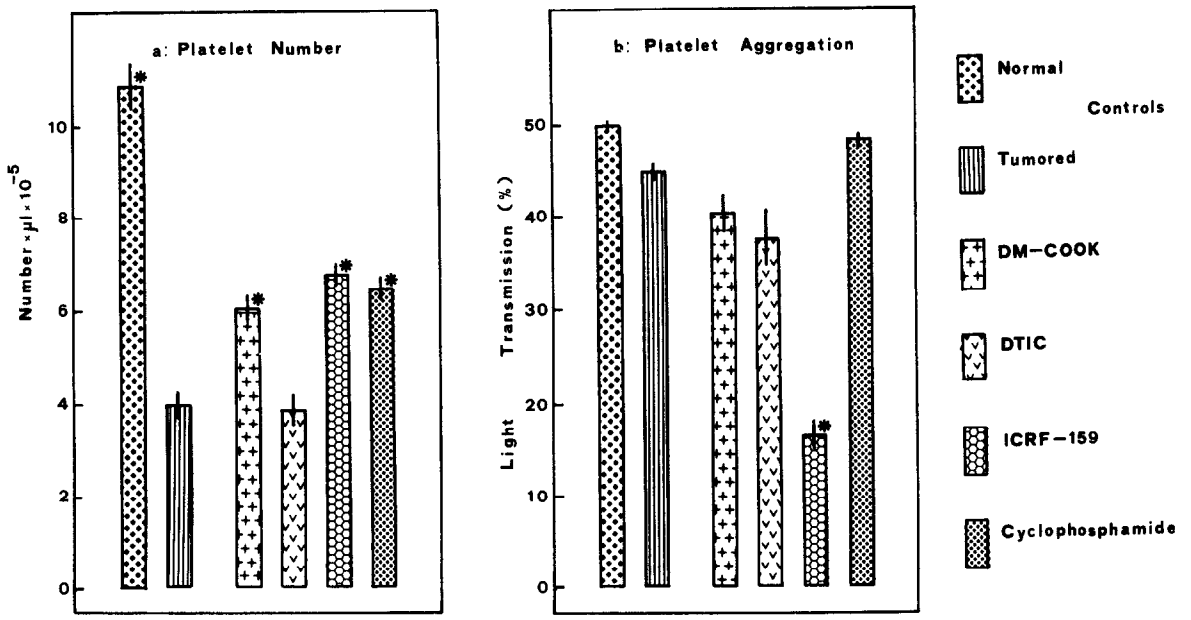

Fig. 1. Effects of in vivo *treatment of mice bearing i.m. Lewis lung carcinoma with the tested drugs on platelet number and aggregation. Each value is the mean ± S.E. obtained using groups of 6 animals (platelet number) and 3 animals (platelet aggregation). The degree of platelet aggregation is quantitated and expressed as described by Donati and deGaetano* [21]. *The statistical analysis performed is the Student–Newmann–Keuls test* [22]; * *mean significantly different from that of the relevant tumored controls,* P = 0.05.

thromboplastin time (Fig. 2b), as well as platelet aggregation (Fig. 1b) by ADP, are not significantly different in the groups of tumored and untumored controls. At the same time, tumor cell suspensions obtained from *in vitro* established cell cultures or from *ex vivo* excised tumor samples showed procoagulant activity (Fig. 3) not differing when normal or factor-VII-deficient plasma is employed for the assay, as already reported by Curatolo *et al.* [16].

When the effects of the treatment with the tested drugs is examined, the plasma or tumor samples were collected at the completion of treatment, which is similarly and markedly active for all the tested drugs in inhibiting the development of spontaneous pulmonary metastases [8, 12]. Moreover, the dosages employed for each drug are equitoxic and thus allow comparison of the effects obtained with each drug [8, 12].

The effects of the treatment of tumor-bearing animals with the tested compounds in comparison with untreated tumor-bearing controls consist, with the exception of DTIC, of a significant increase in the number of thrombocytes; platelet aggregation is reduced only by the treatment with ICRF-159 (Fig. 1), as already reported by Atherton *et al.* [11]. On the other hand, the effects of the tested drugs on the plasma coagulative status show a tendency towards a reduction, consisting of a significant prolongation of prothrombin time for all the drugs or limited to ICRF-159 and cyclophosphamide for partial thromboplastin time; fibrinogen is significantly reduced by the tested agents, with the exception of DM-COOK (Fig. 2). The procoagulant activity of tumor cells is not modified by the treatment with the drugs examined here (Fig. 3). Similarly unaffected by DM-COOK and ICRF-159 is the plasminogen-dependent fibrinolytic activity of tumor cells, since both drugs do not inhibit tumor cell plasminogen activator assayed fluorimetrically; cathepsin B activity is similarly not modified [19].

It thus appears that different degrees of alterations of the parameters considered are caused by each individual drug, and that DM-COOK in general causes the smallest variations from tumor-bearing controls. No indication on the mechanism by which the tested compounds induce hypocoagulability has been presently obtained. Alterations in absorption and utilization of vitamin K might be involved: the possibility of the involvement of hepatic toxicity is possible for cyclophosphamide which, unlike DM-COOK, causes severe morphological toxic damage to the liver [20]. Furthermore, it is difficult to determine for each drug the balance between the hypocoagulability indicated by the prolongation of prothrombin and partial thromboplastin times, reduced plasma fibrinogen concentration and platelet aggregation from one hand, and the hypercoagulability indicated by the increased platelet counts from the other. However, since no unequivocal pattern is recognizable in the effects on the hematological parameters considered for the selective antimetastatic agents DM-COOK, DTIC and ICRF-159, and since their

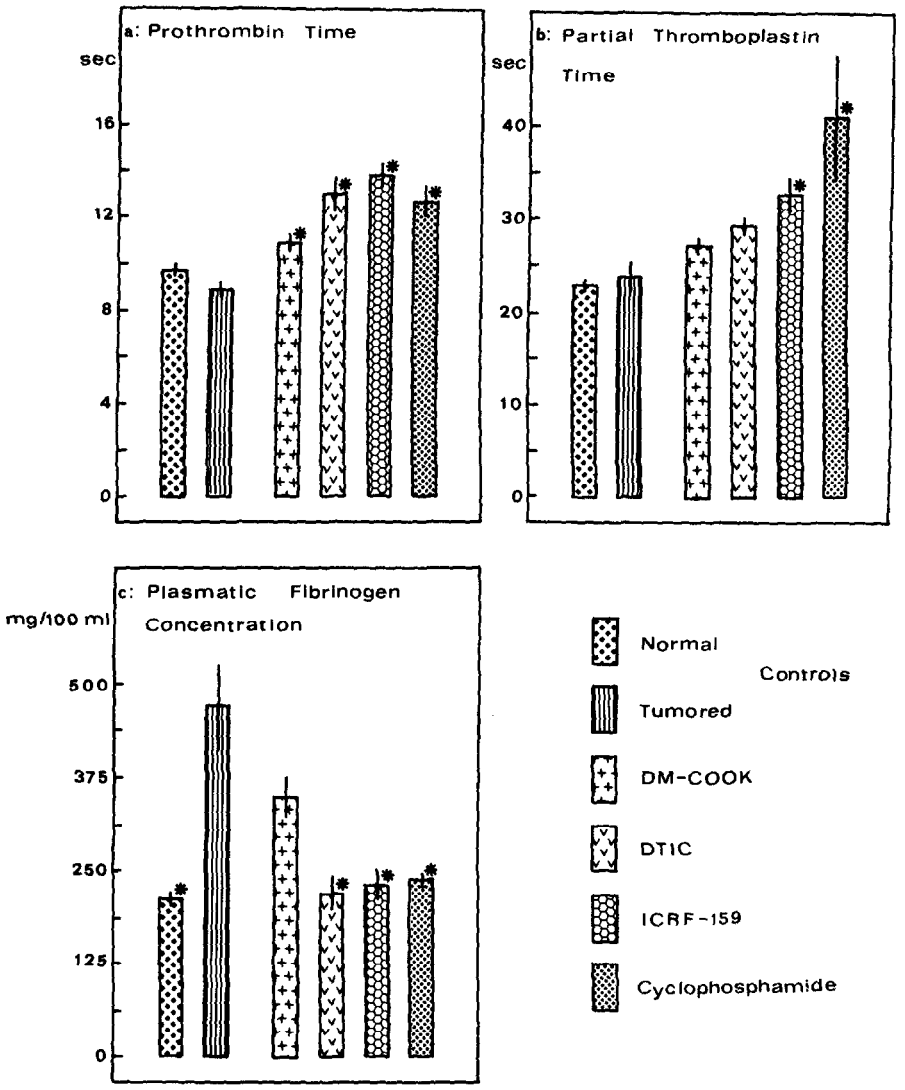

Fig. 2. Effects of in vivo *treatment of mice bearing i.m. Lewis lung carcinoma with the tested drugs on plasma coagulative status and fibrinogen concentration. Each value is the mean ± S.E. obtained using groups of at least 6 animals. The statistical analysis performed is the Student-Newmann-Keuls test* [22]; * *mean significantly different from that of the relevant tumored controls,* $P = 0.05$.

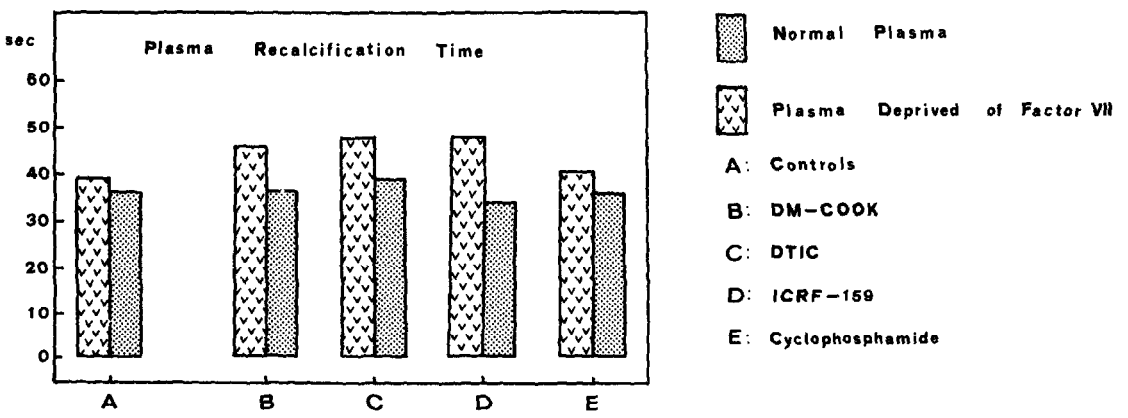

Fig. 3. Effects of in vivo *treatment of mice bearing i.m. Lewis lung carcinoma with the tested drugs on the procoagulant activity of tumor cells. The procoagulant activity of* in vivo *treated tumor cells* (10^5 *trypan blue excluding cells per assay) is expressed as the plasma-recalcification time obtained using normal mouse plasma and human plasma deficient in Factor VII; the results of a representative experiment obtained pooling the tumor cells from 3 different donors for each treatment are presented.*

effects do not markedly differ from those of a purely cytotoxic antineoplastic drug such as cyclophosphamide, it appears unlikely that the interactions with blood coagulation are responsible for the prevention of metastasis development caused by the selective antimetastatic drugs tested. This conclusion is further supported by data reported in the relevant literature showing that a markedly more pronounced depression of the coagulative status of the host (for example, decreased fibrinogenemia or thrombocyte number) is required in order to observe antimetastatic effects of a magnitude approaching that of DM-COOK and ICRF-159 [3–5].

These data thus support the view, originating from the experimental evidence already presented, that the antimetastatic effects of ICRF-159 and DM-COOK (and presumably also those of DTIC) should be mainly attributed to inhibition of tumor cell entrance into the blood-stream caused by the drugs [8–10].

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