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The stable prostacyclin analogue Cicaprost inhibits metastasis to lungs and lymph nodes in the 13762NF MTLn3 rat mammary carcinoma

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Prostacyclin and its stable analogues have been shown to interfere specifically with certain steps of the metastatic cascade. The antimetastatic activity of the stable prostacyclin analogue Cicaprost (Schering AG) on haematogenous metastasis in a series of tumours in rats and mice has been well established. In order to test the effect of Cicaprost on lymphogenous metastasis we chose the metastatic cell clone MTLn3 derived from the 13762NF rat mammary carcinoma. The effect of Cicaprost on prevention of lung metastasis, lymph node metastasis and primary tumour growth was investigated. Cicaprost given in daily doses of 0.01, 0.03 and 0.1 mg/kg orally, reduced the number of lung metastases in a dose-dependent manner. Whereas the median number of lung metastases in the controls was greater than 1000, Cicaprost at a dose of 0.1 mg/kg reduced the number of lung metastases to between 11 and 100. The weight of the ipsilateral axillary lymph nodes was diminished by Cicaprost to 30–50% of controls. Moreover, metastasis to the contralateral axillary lymph node was completely inhibited by Cicaprost at all three doses tested. Cicaprost did not influence the growth rate of the MTLn3 cell clone implanted into the mammary fat pad or the weight of the primary tumour at the end of treatment. In conclusion, in addition to its dose-dependent effect on haematogenous metastasis, Cicaprost strongly inhibits lymph node metastasis.

Keywords: Cicaprost, lung metastasis, lymph node metastasis, MTLn3 cell clone

Introduction

Cicaprost, a metabolically fully stable and orally active analogue of endogenous prostacyclin (PGI_2) (Figure 1), was originally developed for therapy of diseases in which platelet aggregation is involved [1, 2]. Cicaprost exhibits strong anti-aggregatory effects on platelets, comparable to natural PGI_2. First clinical studies in human volunteers have shown a long-lasting inhibition of platelet aggregation after oral administration [3].

Honn *et al.* [4] first reported the antimetastatic activity of prostacyclin on lung colony formation after intravenous inoculation of B16a melanoma.

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During the past 10 years, prostacyclin has been described as a potent compound for antimetastatic therapy [5]. While the majority of experiments using the artificial metastasis assay were performed with prostacyclin itself, there is also some information on the effectiveness of prostacyclin analogues in these models [6]. In contrast, there have been few investigations on the antimetastatic activity of stable prostacyclin analogues in animals bearing spontaneously metastasizing tumours in which either the primary tumour was removed or allowed to remain *in situ* [7].

We have recently demonstrated the strong antimetastatic effect of Cicaprost on the subcutaneously implanted, haematogenously metastasizing

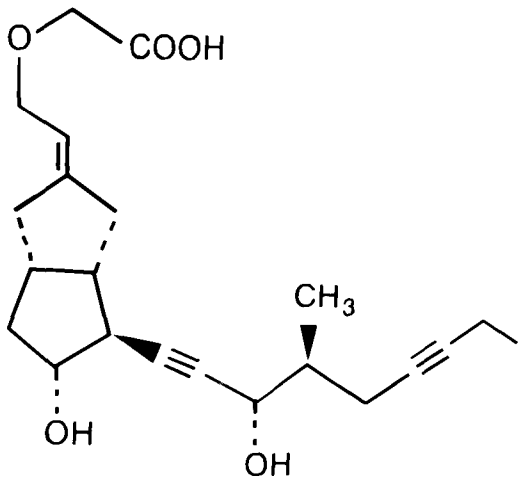

Figure 1. Chemical structure of Cicaprost.

R3327 MAT Lu rat prostate carcinoma as well as the M5076 mouse reticulum sarcoma [8, 9]. Cicaprost was also tested for its antimetastatic potency in the SMT 2A rat mammary carcinoma, which metastasizes following subcutaneous implantation via the haematogenous as well as the lymphogenous route [10]. In this model we found besides a strong dose-dependent effect on the number of blood-borne lung metastases, an inhibition of the weight of axillary lymph nodes [10]. Since this had not previously been reported for prostacyclin analogues, we were interested to determine the effect of Cicaprost in a metastasizing tumour exhibiting a more prominent lymphogenous metastasis.

We report here experiments carried out using the MTLn3 cell clone, derived from the 13762NF carcinoma [11], a tumour which colonizes regional lymph nodes and subsequently spreads to more distant nodes and therefore provides a useful model for the study of lymphogenous metastasis.

Materials and methods

Cell line and culture condition

The tumour cell clone used in this study was established from the 13762NF mammary adenocarcinoma implanted into syngeneic F344 rats. Tumour cell clone MTLn3 (culture passages 15–20) was grown in α-modified minimal essential medium (AMEM) supplemented with 5% foetal bovine serum (FBS) (Gibco, Karlsruhe, Germany) without antibiotics [12]. The tumour cells were harvested at 60–80% cell confluence, using 0.125% trypsin in 2 mM EDTA in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (trypsin–EDTA).

Spontaneous metastasis model

Tumour cells grown *in vitro* to approximately 60% confluence were harvested using trypsin–EDTA and washed twice in AMEM without serum. Cell viability was >95% as determined by Trypan Blue exclusion. For tumour implantation, female F344 rats (Charles River, Wiga, Germany) were anaesthesized with metofane (methoxyflurane; Janssen, Pitman-Moore Inc., USA) and 10^6 tumour cells in 0.1 ml AMEM were injected into the mammary fat pad. Animals were allocated to control ($n = 13$) or three different groups of treatment ($n = 10$).

The growth of the primary tumour was determined weekly by caliper measurements and was calculated as the product of the largest diameter and its perpendicular dimension. Animals were sacrificed 25 days after tumour implantation. Primary tumour, lungs, ipsilateral and contralateral axillary lymph nodes and inguinal lymph nodes of each animal were removed and weighed.

The number of metastatic lung foci was counted with the aid of a dissecting microscope after fixation in Bouin's solution for one day. At least nine animals were used per treatment group.

Cicaprost treatment

Female F344 rats were treated daily with Cicaprost until day 25 (end of the experiment), starting 2 h before tumour implantation.

The LD_{50} of a single administration of Cicaprost, determined in Wistar-Han rats, is around 10 mg/kg p.o. Cicaprost given once orally at a dose of 1.0 mg/kg p.o. to Wistar-Han rats lowers the blood pressure for 2–3 h [13, 14]. As female F344 rats are more sensitive to Cicaprost at a dose range of 0.5–1.0 mg/kg p.o., the compound was given at doses of 0.01, 0.03 and 0.1 mg/kg p.o. daily. Control animals received saline.

Statistics

Weight of lymph nodes, lungs and primary tumours was analysed by the Dunnett t-test. p-values <0.05 were considered significant.

Results

The number of lung metastases, the weight of regional and distant lymph nodes and the weight

of the primary tumour at the end of the experiment were determined. Table 1 demonstrates the effect of Cicaprost at a dose of 0.01–0.1 mg/kg p.o. on the number of lung metastases. It was technically difficult to estimate the precise number of lung metastases since 25 days after implantation of MTLn3 cells into the mammary fat pad, seven of 13 animals (54%) had >1000 lung metastases. Numbers of metastases were therefore arranged in groups, as shown in Table 1.

Following treatment with 0.1 mg/kg Cicaprost, 30% of animals had >1000 metastatic foci, compared to 54% of controls. Moreover, whereas none of the control animals was completely free of metastases, 40% (four of 10) in the group treated with 0.03 mg/kg Cicaprost and 43% (four of nine) in the group treated with 0.1 mg/kg Cicaprost had no macroscopically visible lung metastases. Both treatment groups were statistically significantly different from controls. An exact mathematical determination of the median number of lung metastases is not possible, as the numbers are arranged in groups of logarithmic decades. Nonetheless, the data demonstrate that 0.1 mg/kg Cicaprost reduces the median number of metastases to 11–100, compared to >1000 in the controls.

The antimetastatic effect of Cicaprost is further shown by a decrease in the mean lung weight of tumour-bearing animals (Figure 2). The mean lung weight of control animals was 2100 mg: Cicaprost at doses of 0.03 and 0.1 mg/kg caused a statistically significant reduction in mean lung weight (1350 mg). The mean lung weight of non-tumour-bearing female F344 rats is around 950 mg (data not shown).

Figure 3 demonstrates the effect of 0.01–0.1 mg/kg Cicaprost on the weight of the ipsilateral (site of implantation of the primary tumour) axillary lymph node. At all doses used Cicaprost significantly reduced the weight of the ipsilateral axillary lymph node. Histological examination demonstrated that nodes weighing more than 120 mg were completely colonized by tumour cells. The structure of the 'normal' node had disappeared.

The effect of Cicaprost on the weight of the axillary lymph node on the side contralateral to tumour implantation is shown in Figure 4. Cicaprost at all doses tested led to an almost complete inhibition of lymph node metastasis into the contralateral axillary lymph node. The mean weight of

Table 1. Effect of Cicaprost on the number of lung metastases in animals bearing mammary fat pad implanted MTLn3 cell clone

Number of lung metastases[a]	Number of animals			
	Control	Cicaprost (mg/kg)		
		0.01	0.03	0.1
>1000	7	5	3	3
101–1000	1	1	2	1
11–100	2	0	1	1
1–10	3	2	0	0
0	0	2	4	4
Total number of animals	13	10	10	9

[a]Female F344 rats were injected with 10^6 tumour cells into the mammary fat pad. 25 days after tumour implantation the animals were assessed for metastasis to the lung. One animal of the group (0.1 mg/kg Cicaprost) was excluded because of no tumour take. The number of metastatic lung foci was counted after fixation in Bouin solution for one day. The numbers are arranged in groups of decades. The 0.03 mg/kg dose ($p < 0.05$) and the 0.1 mg/kg dose ($p < 0.05$) are statistically significantly different compared to the control.

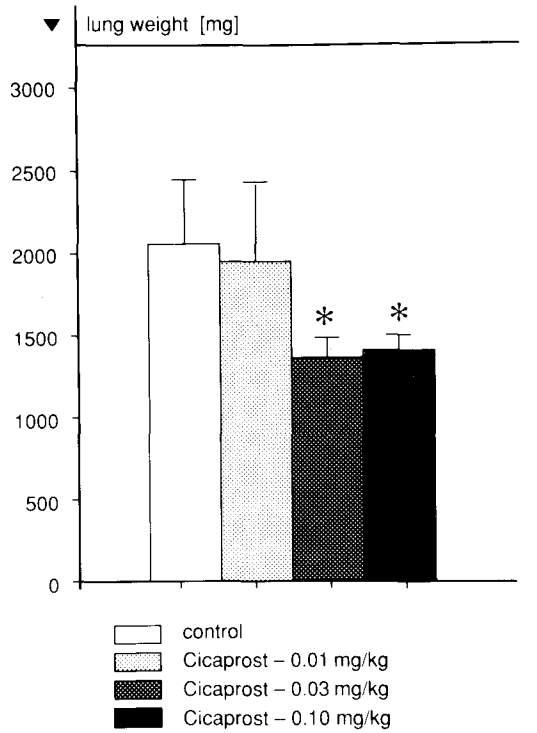

Figure 2. Effect of Cicaprost (0.01, 0.03 and 0.1 mg/kg p.o. daily) on the mean lung weight (± SD). The 0.03 mg/kg ($p < 0.05$) and the 0.1 mg/kg ($p < 0.05$) doses are statistically significantly different to the control.

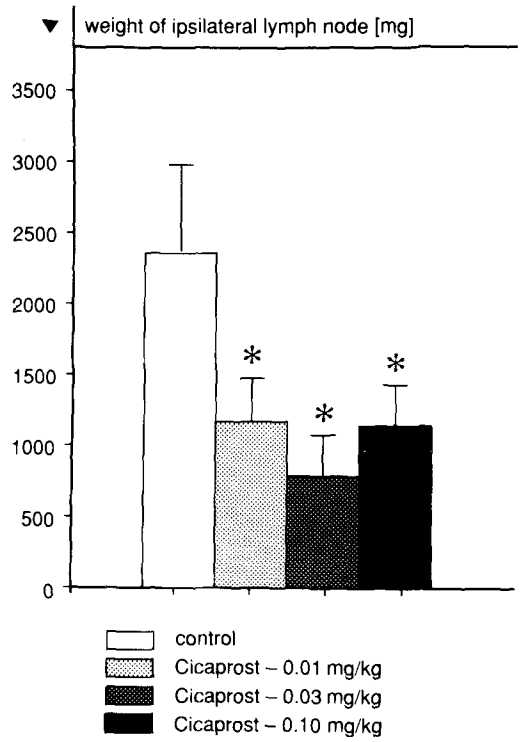

Figure 3. Effect of Cicaprost (0.01, 0.03 and 0.1 mg/kg p.o. daily) on the mean weight of the ipsilateral axillary lymph node (± SD). The 0.01 mg/kg ($p < 0.05$), the 0.03 mg/kg ($p < 0.05$) and the 0.1 mg/kg ($p < 0.05$) doses are statistically significantly different compared to the control.

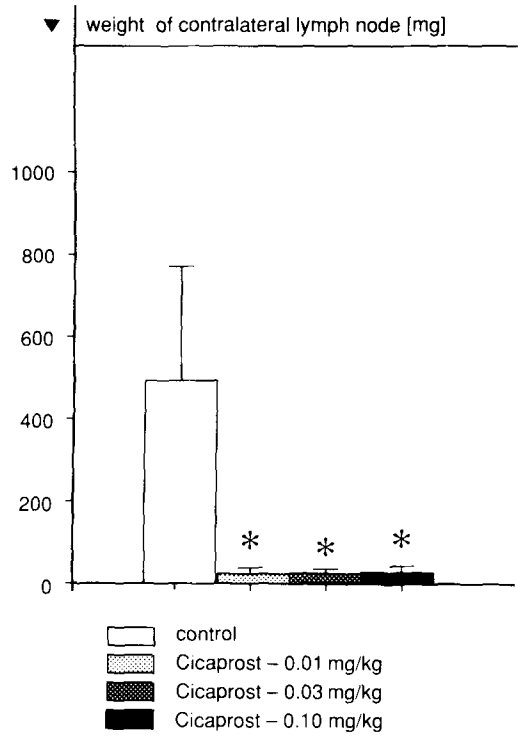

Figure 4. Effect of Cicaprost (0.01, 0.03 and 0.1 mg/kg p.o. daily) on the mean weight of the contralateral axillary lymph node (± SD). The 0.01 mg/kg ($p < 0.001$), the 0.03 mg/kg ($p < 0.001$) and the 0.1 mg/kg ($p < 0.001$) doses are statistically significantly different compared to the control.

contralateral axillary lymph nodes of animals treated with Cicaprost was identical to the mean weight of axillary lymph nodes of non-tumour-bearing F344 rats (30 mg).

In order to distinguish between a non-specific antimetastatic effect arising from an inhibitory effect on the growth of the primary tumour and a specific antimetastatic effect without alteration of tumour growth at the primary site, the mean tumour weight at day 25 was determined (Figure 5), as well as the growth rate. Cicaprost at all three doses tested did not statistically significantly alter the mean weight of the primary tumour and tumour area (data not shown). The experimental data obtained from this experiment were reproduced in a second experiment which was carried out in a comparable procedure (data not shown).

Discussion

Although a relatively high proportion of cancer cases are diagnosed in the early stages and treatment with surgery, chemotherapy and radiotherapy often effectively controls tumour growth at the primary site, the majority of patients die from metastases, and therefore, the number of patients cured is very low [15]. Development of drugs focusing on the prevention of further metastatic tumour spread could lead to compounds able to reduce cancer mortality [16]. Therefore, many experimental compounds acting at different levels of the metastatic cascade have been tested for their potential beneficial effects on metastasis.

Strong evidence has been obtained in many investigations suggesting a role for the haemostatic system in the process of tumour metastasis [17]. Current perceptions view the participation of cellular and non-cellular blood constituents, or both, as a process in which circulating tumour cells interacting with components support their capabilities to form distant metastases. The fact that anti-aggregatory and anti-coagulatory compounds are able to interfere with the process of tumour cell–platelet aggregation encouraged the testing of various compounds in metastasis models [18].

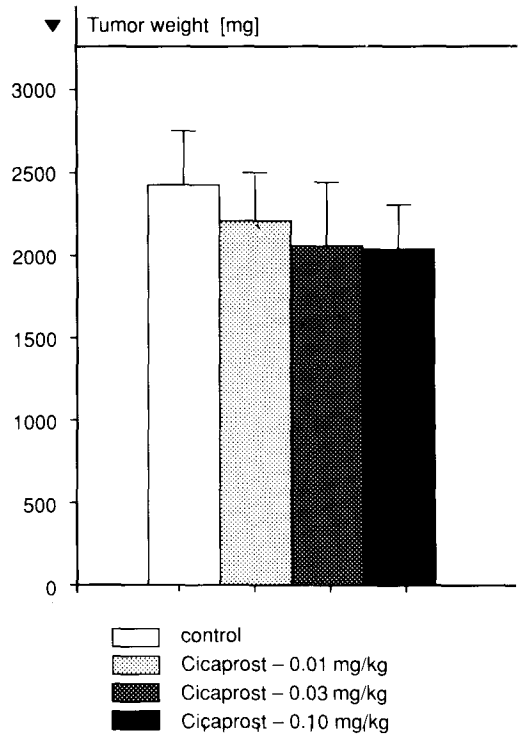

Figure 5. Effect of Cicaprost (0.01, 0.03 and 0.1 mg/kg p.o. daily) on the mean weight of the mammary fat pad implanted MTLn3 tumour.

Since Honn *et al.* [4] found a strong decrease in lung colony formation of B16a melanoma in mice pretreated with prostacyclin, many efforts have been undertaken to characterize the potential of this new class of components in 'artificial' metastasis models [19–21]. Most recently, stable prostacyclin analogues have been investigated in animals bearing spontaneously metastasizing tumours, in which either the primary tumour was removed or remained within the animal. Sava *et al.* [7] have demonstrated an antimetastatic effect in spontaneously metastasizing Lewis lung carcinoma of Iloprost treatment following surgical removal of the primary tumour. Recently, we found that the stable, orally active prostacyclin analogue Cicaprost is able to decrease the number of spontaneous metastases in several rodent tumours [8, 9]. These models, the lung metastasizing R3327 MAT Lu prostate carcinoma of the Cop rat and the liver metastasizing M5076 reticulum sarcoma of the C57BI/6 mouse reflect metastasis of blood-borne tumour cells.

Most recently, using the spontaneously metastasizing SMT 2A mammary carcinoma of the rat, we found a 40% reduction of the metastasis-induced increase in lymph node weight by a dose of Cicaprost which almost completely inhibited lung metastasis. A dose of Cicaprost which only decreased the number of lung metastasis to about 50% did not, however, alter the mean lymph node weight [10]. Therefore, we were interested in testing the effect of Cicaprost in an animal tumour model exhibiting more pronounced lymphogenous metastasis.

The 13762NF mammary adenocarcinoma was induced in female F344 rats with 7,12-dimethylbenzanthracene by Segaloff [22], and its metastatic behaviour was well characterized by Bogden and colleagues [23]. In this study we used the highly metastatic cell clone MTLn3 isolated from the 13762NF tumour system by Neri *et al.* [11]. This cell clone injected into the blood circulation did not alter platelet count, indicating failure to induce platelet aggregation [24]. Nicolson *et al.* [25, 26] have demonstrated that the MTLn3 cells adhere to and invade lung tissue at significantly higher rates than poorly metastatic cell clones isolated from the 13762NF tumour.

Cicaprost caused a dose-dependent decrease of the number of lung metastases, leading to a significant proportion of animals without grossly obvious lung metastases and a reduction in the metastasis-induced increase of mean lung weight. Tumour metastasis into regional lymph nodes was strongly inhibited by Cicaprost. A significant involvement of the axillary lymph node contralateral to the site of tumour implantation was observed in control animals. This lymph node metastasis was almost completely inhibited by all doses of Cicaprost tested (0.01–0.1 mg/kg). No effects of Cicaprost could be identified on growth and cell cycle distribution of MTLn3 cells exposed to various concentrations of Cicaprost (10^{-10}–10^{-6} M) in tissue culture (data not shown). This is in line with the notion that the weight of the primary tumour was unaffected by Cicaprost.

No metastases were found in other organs, such as liver, spleen and kidney, in either the control or in Cicaprost-treated animals.

The therapeutic effectiveness of compounds acting at the level of blood coagulation and platelet aggregation was shown by McCulloch and George using the same tumour model [27]. They demonstrated an antimetastatic effect of warfarin, a coumarin derivative, on lung metastasis from MTLn3 cell clones implanted into the mammary fat pad. This effect was not accompanied by any effect on the growth of the primary tumour. In addition, they found a significant participation of the clotting factors II, IX, and X in the antimetastatic effect of warfarin. Alteration of fibrin level does

not contribute to either the antimetastatic drug effect of warfarin or to the metastasis of the MTLn3 cell clone [28]. Unfortunately, there is no information on possible effects of warfarin on lymph node metastasis. This fact might be explained by the short duration of the animal experiment (23 days) as well as the low rate of lung metastasis in the control (median: 1–10 metastases/lung) [27].

Another therapeutic effect was achieved by the use of the phosphodiesterase inhibitor RA 233. As Lichtner *et al.* [29] have demonstrated, the median number of lung metastases in animals bearing mammary fat pad implanted MTLn3 cell clone was decreased by the use of RA 233, although this decrease was not statistically significant. Since the primary tumour was, however, removed 14 days after implantation (mean tumour weight about 50 mg), it is difficult to compare the effects of RA 233 with those of Cicaprost reported in this study. There was, however, a significant increase in the number of lung colonies when MTLn3 cells were injected intravenously into rats pretreated with RA 233.

Biochemical studies demonstrated that prostacyclin and its stable analogues bind to G protein-linked PGI_2 receptors and elicit their effects by changing intracellular concentrations of second messengers (cyclic AMP, diacylglycerol, Ca^{2+}) [30, 31]. Cicaprost acts as a common pathway inhibitor of platelet activation by different stimuli [14]. Several investigators also demonstrated an inhibitory effect of prostacyclin on tumour cell–platelet activation as well as lung colony formation after systemic tumour cell inoculation [20, 21]. Furthermore, prostacyclin and its stable analogues are able to increase the anti-adhesive properties of the endothelium [32]. This observation leads to another interesting aspect of a possible mode of antimetastatic action of Cicaprost which was suggested by Honn *et al.* [33]. They demonstrated that prostacyclin and its stable analogues (i.e. Cicaprost) are potent inhibitors of 12-S-HETE (as well as phorbol acetate) and stimulated adhesion of W256-cells to both endothelial cells and their extracellular matrix. Thus, a direct action of Cicaprost on the adherence mechanism of tumour cells should be considered [33]. Further studies on the mechanism(s) of the profound inhibition of lymphogenous as well as haematogenous metastasis are in progress.

Taken together, our results show a dose-dependent decrease of the lung metastasizing potential of the mammary fat pad implanted MTLn3 cell clone by the stable prostacyclin analogue Cicaprost. Moreover, we demonstrate for the first time that Cicaprost largely prevented spontaneous lymph node metastasis.

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