

Enhancement of antitumor activity of the oxazaphosphorine cytostatic SUM-IAP by *N*-methylformamide

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Abstract

Purpose SUM-IAP has been developed with the aim to optimize therapeutic response and minimize toxic reactions of oxazaphosphorine cytostatics. In therapy tests in mice, the primary tumor was successfully eradicated, but animals died due to formation of lethal metastases. We supposed that high activities of SUM-IAP detoxifying enzymes caused metastasis formation in the liver. Therefore, therapy tests with SUM-IAP in combination with cisplatin and *N*-methylformamide (NMF), which were not detoxified in the liver, were carried out.

Method Antitumor activity was assayed in female CD2F1 mice with advanced subcutaneously growing P388 mice leukemia cells.

Result The results of the therapy tests with SUM-IAP plus cisplatin were as expected: No formation of metastases and long-time survival of more than 100 days were observed; however, the toxicity was increased as measured by decrease in body weight and the number in leukocytes. The results of the tests in combination with NMF were surprising: Applying only half the dose of SUM-IAP used in the experiments with cisplatin, no metastases were found and long-time survivors did not show signs of additional toxicity.

Conclusion NMF strongly enhances the antitumor activity of the oxazaphosphorine cytostatic SUM-IAP in mice

with subcutaneously growing P388 mice leukemia cells by an unknown mechanism of action.

Keywords Oxazaphosphorine cytostatics · SUM-IAP · *N*-methylformamide · Cisplatin

Introduction

SUM-IAP (1, Fig. 1) is an oxazaphosphorine analogue. Under physiological conditions, it spontaneously hydrolyzes to mesyl-I-aldophosphamide (2, Fig. 1), which is in equilibrium with 4-hydroxy-mesyl-I-phosphamide (2a, Fig. 1). The half-life of hydrolysis is 84 h. The long half-life of hydrolysis ensures long lasting, but low concentrations of the aldophosphamide derivative. According to Hohorst et al., low but long lasting concentrations are necessary for low toxicity chemotherapy with oxazaphosphorine cytostatics (Hohorst et al. 1988). The principal idea for the use of oxazaphosphorine cytostatics for low toxicity chemotherapy is based on the discovery of enzymatic toxification of 4-hydroxycyclophosphamide (Voelcker et al. 1981). Bielicki et al. showed that aldophosphamide (2, Fig. 1 $R_1=R_2=-CH_2CH_2Cl$, $R_3=H$) is cleaved by exonucleases releasing acrolein (10, Fig. 1) and the alkylating moiety phosphoramidate mustard (8, Fig. 1 with $R_1=R_2=-CH_2CH_2Cl$, $R_3=H$) (Bielicki et al. 1983). Detailed investigations demonstrated highest cleavage activity by 3'-5' exonucleases with proof reading activity such as DNA polymerase I (Klenow fragment) from *E. coli* or pol δ prepared from rabbit bone marrow. The cleavage activity of enzymes like phosphodiesterase I from snake venom or phosphodiesterases found in rat- and human serum was determined to be 2 to 3 orders of magnitude lower (Bielicki et al. 1983). Based on these findings, Hohorst et al. concluded that the

Dedicated to Prof. Dr. Hans Jürgen Hohorst (March 14, 1924–January 10, 2015).

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bearing mice, i.e., SUM-IAP (*1*, Fig. 1), was chosen for subsequent experiments.

Although the primary tumors were successfully eradicated and the life span increased by about 370 %, the main problem in therapy tests with SUM-IAP was the mortality of the animals due to formation of liver metastases. It was assumed that the reason for formation of liver metastases was the high activity of aldehyde- or/and alcohol dehydrogenases in the liver which detoxified the active metabolite of SUM-IAP to the corresponding not cytotoxic 4-keto-derivative and/or carboxylic acid (6,7, Fig. 1). This suggestion prompted us to perform therapy tests with SUM-IAP in combination with cytostatics which were not detoxified by liver enzymes. We choose cisplatin and N-Methylformamide (NMF). The results of these experimental chemotherapy tests in female CD2F1 mice bearing advanced sc. transplanted P388 mouse leukemia cells are described.

Materials and methods

SUM-IAP (*N*-(2-Chloroethyl)-*N'*-(methanesulphonylethyl)-phosphorodiamide-2-(2'-[4'-carboxy-1',3'-perhydrothiazinyl])-ethylester), (*1*, Fig. 1), was synthesized according to Zimmermann (Zimmermann et al. 2000).

Animals

Female CD2F1 mice from Zentralinstitut für Versuchstierkunde, Hannover, Germany, were housed under the following conditions: room temperature 18–22 °C, dark- and light periods of 12 h, free access to laboratory chow (Altromin 1324, Altromin GmbH, Lage, Germany) and water. Animals were randomly divided into experimental groups.

Antitumor activity was assayed in female CD2F1 mice: 10⁶ P388 mice leukemia cells were transplanted sc. under the skin of the flank. Therapy was started on day 7 after tumor transplantation when tumor area was approx. 0.5 cm². Drugs were administered sc. into the flank opposite to the tumor: The median survival time of untreated controls was 11–12 days.

For determination of leukocytes

10 µl of blood samples were drawn from the retrobulbar plexus and diluted with 10 ml “Isoton” (Coulter electronics limited, England). After lysis of erythrocytes with “Zap Oglobin” (Coulter electronics limited, England), number of leukocytes were measured with a cell counter (Casy 1, Schärfe, Reutlingen, Germany).

Aldophosphamide (2, Fig. 1 $R_1=R_2=-CH_2CH_2Cl$, $R_3=H$), hydroxypropanale (9) and acroleine (10) liberated from 4-hydroxycyclophosphamide (2a, Fig. 1

$R_1=R_2=-CH_2CH_2Cl$, $R_3=H$) were transferred into the 2,4-dinitrophenylhydrazone derivatives by the following procedure: Samples of 40 µl were diluted with 200 µl saturated 2,4-dinitrophenylhydrazine solution (solvent 1 N HCl) and vigorously shaken with 1 ml of a mixture of 20 % toluene and 80 % isooctane at room temperature for 30 min (Eppendorf mixer 5432 from Netheler + Hinz, Hamburg, Germany). 100 µl of the organic layer was used for HPLC after centrifugation. HPLC conditions: C-18 column, elution with a solution of increasing methanol concentration in water (65 to 80 % methanol in water within 2.5 min) monitoring at 340 nm, 2,5 ml/min, automated gradient controller and pumps from Waters. Protein-containing samples were deproteinized by addition of equal volumes of 0.15 M solutions of Ba(OH)₂ and ZnSO₄ × 7H₂O. Rat serum ultrafiltrate was kept at pH7 by addition of NaOH.

Results

Therapy tests with SUM-IAP in combination with cisplatin

Table 1 shows the results of therapy tests with SUM-IAP and SUM-IAP in combination with cisplatin in female CD2F1 mice bearing solid growing P388 mouse leukemia tumors. Therapy was started on day 7 or day 8 after sc. transplantation of 10⁶ mouse leukemia cells when tumor area was grown to approx. 0.25 cm². SUM-IAP was administered twice per day on day 7–11 and day 21–25. The single dose administered sc. was 133 mg/kg the total dose 2660 mg/kg.

One of the four animals survived; the observation period of 100 days and was considered to be cured. The remaining three animals died between day 52 and day 70 (increase in lifespan 370 %). In one animal, regrowth of the primary tumor was observed at day 41. One animal developed an ascites tumor on day 70, and one suffered from metastases in the region of the lymph nodes of forelegs and hind legs. In all the three not cured animals, metastases in the liver were observed.

In summary: One of the four animals was cured; formation of metastases was observed in all therapy-resistant animals.

In the same experiment (with the exception that therapy was started on day 8 after tumor transplantation instead on day 7) with two daily additional ip. applications of cisplatin (1.8 mg/kg on day 13 and day 27), however, four of five animals were cured. In the therapy-resistant animal, regrowth of the primary tumor was observed and postmortem examination revealed metastases in the liver.

The therapeutic effect of cisplatin alone was only marginal in this tumor model. No animal was cured even after

Table 1 Therapy tests with SUM-IAP, cisplatin and a combination of SUM-IAP and cisplatin in female CD2F1 mice bearing sc. transplanted P388 mice leukemia cells

Dose (mg/kg)/schedule		Long-time survivors ^c	Increase in life span ^d (%)	Change in no. of leukocytes ^e (%)	Body weight change ^e (%)	Remarks
SUM-IAP ^a	Cisplatin ^b					
10(2 × 133) d7-11, 21–25	–	1/4	370	–88 (d25)	<–5	Death of three mice due to metastases between d52 and d70
	2 × 1.8 d13, 27	4/5		–75 (d28)	–17	Death of one animal due to regrowth of tumor and liver metastases (day 69)
	2 × 3.6 d13, 27	4/5		–84 (d28)	–18	Death of one mice on day 72 due to drug toxicity (bwc >–25 %)
–	2 × 3.6 d7,10	0/4	76	–21 (d11)	<–5	Regrowth of transplanted tumor in all animals

^a One sc. injection in the morning and evening

^b Intraperitoneal (ip.) injection in the morning

^c Surviving time >100 days

^d Surviving time of untreated P388 tumor bearing control mice 11–12 days

^e Calculated using the mean before and the lowest mean after treatment
long time survivors are indicated in bold

therapy with two daily applications of twice the dosage of cisplatin (3.6 mg/kg ip. on day 7 and 10). The increase in life span in this experiment was 76 %. All animals finally died due to regrowth of primary tumors.

Therapy tests with SUM-IAP in combination with NMF

Table 2 shows the results of therapy tests with SUM-IAP (133 mg/kg sc. administered twice daily on day 7–11). Three of four animals developed visible metastases in the region of lymph nodes of forelegs until day 29. In the remaining animal metastases were observed until day 56. Attempts to cure metastases bearing animals by further therapy with two daily applications of 133 m/kg SUM-IAP failed. Animals were killed between day 46 and day 84. Postmortem examination showed liver metastases in all animals.

A further therapy test with the same dose and schedule of SUM-IAP application but with additional applications of 200 mg/kg NMF (12 single injections on day 13 to 24) was carried out in five mice with sc. transplanted P388 mouse leukemia cells. Four mice were cured. One animal was killed on day 49 because of regrowth of the primary tumor. In the animal, neither visible external metastases nor liver metastases or metastases anywhere else in the abdominal range were found after postmortem examination.

The most remarkable result in this experiment is lack of any sign of toxicity except a short, but reversible decrease

in the number of leukocytes after SUM-IAP injection. The body weight of animal increased steadily (Fig. 2).

In control experiments with NMF, no or only marginal antitumor activity (ILS 12 %) was detected (Table 2).

Discussion

The results of therapy tests in combination with cisplatin clearly demonstrated long-time survival and prevention of metastases but an increase in toxicity resulting in a decrease in body weight of more than 25 % in one animal after injection of the drug combination (Table 1). Most surprising, however, the combination with NMF showed rather promising results. Applying only half the dose of SUM-IAP used in the experiments with cisplatin followed by long-time therapy with an absolutely non-toxic dose of NMF, four of five animals were cured, whereas in the same experiment without NMF all animals developed metastases. The underlying mechanism of the “NMF phenomenon,” so far is currently unclear, but in the following some possible explanations will be discussed.

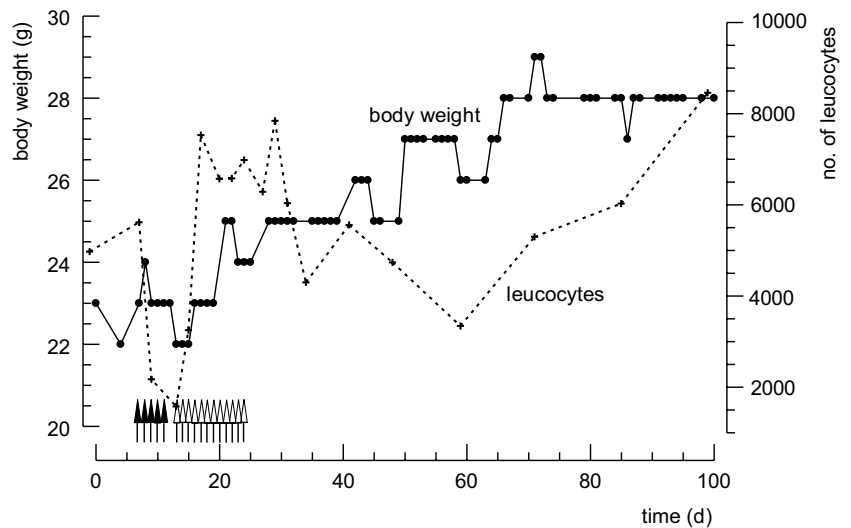
NMF is a polar substance for which the cell membrane represents no barrier. It has been synthesized in 1953 as a solvent for the parenteral administration of water-insoluble substances in experimental chemotherapy. Since NMF was found to be active in chemotherapy tests against the

Table 2 Therapy tests with SUM-IAP, NMF and a combination of SUM-IAP and NMF in female CD2F1 mice bearing sc. transplanted P388 mice leukemia cells

Dose (mg/kg)/schedule		Long-time survivors ^c	Increase in life span (%) ^d	Change in no of leukocytes ^e (%)	Body weight change (%) ^e	Remarks
SUM-IAP ^a	NMF ^b					
5(2 × 133) d 7-11	–	0/5	170	–48 (d11)	–9	Death due to regrowth of transpl. tumor and metastases in all animals
	12 × 65 d13-24	0/5	150	–80 (d11)	<–5	5/5 primary tumor, 1/5 metastases
	12 × 130 d13-24	0/5	130	–63 (d14)	+25	5/5 primary tumor, 2/5 metastases
	12 × 200 d13-24	4/5	–	–68 (d14)	+22	Death of one animal d 50 due to regrowth of primary tumor
–	5 × 200 d8-12	0/5	12	+58 (d9)	<–5	

^a One sc. injection in the morning and evening
^b ip. injection in the morning
^c Surviving time >100 days
^d Surviving time of untreated P388 tumor bearing control mice 11–12 days
^e Calculated using the mean before and the lowest mean after treatment
 long time survivors are indicated in bold

Fig. 2 Body weight and no. of leukocytes in female CD2F1 mice bearing sc. transplanted P388 mice leukemia cells after therapy with 5(2 × 133) mg/kg SUM-IAP sc. d7-11 (black arrows) and 12 × 200 mg NMF ip. d13-24 (open arrows), mean values from 4 or 5 animals are shown



sc. transplanted S-180-Sarcoma in mice, clinical trials have been carried out but were canceled because signs of hepatotoxicity were observed. Characteristic of all known in vitro and in vivo effects of NMF is that they are reversible and last only as long as the cell is exposed to NMF (Clarke et al. 1953; Meyers et al. 1956).

NMF was shown to be active against various human tumors in nude mice (Gescher et al. 1982; Iwakawa et al. 1987). The effect of NMF is not cytoreductive but cytostatic and depends on tumor mass. Small tumors respond better to NMF than large tumors (Gescher et al. 1982;

Tofilon et al. 1987). Furthermore an antimetastatic activity of NMF was demonstrated (Iwakawa et al. 1987). Postmortem examination of mice which received 300 mg/kg NMF (daily for 18 days) after surgical removal of a metastatic mamma carcinoma showed that only 17 % of mice in the NMF group suffered from metastases as compared to 55 % in the control group.

In combination with other anticancer agents, the timing of the additional NMF treatment is important. NMF pretreatment in mice with sc. transplanted mamma carcinoma cells reduced the efficacy of a subsequent treatment

with cisplatin, while the treatment with NMF following the cisplatin therapy showed a supra-additive therapeutic success. These results are explained by the fact that due to previous treatment with NMF the cells were in growth arrest which makes them insensitive for cytostatics (Tofilon et al. 1987).

NMF is considered to be a differentiating agent. In rats suffering from (1,2-dimethylhydrazine induced) colon cancer, the tumor cells were less dedifferentiated and the onset of tumor growth was significantly delayed, if animals were given NMF via the drinking water for the duration of the 1-week carcinogen application (O'Dwyer et al. 1988).

In vitro studies on NMF-treated human carcinoma cells demonstrated a glutathion depletion. The transplantation of these cells treated in the latter way showed a lack of tumorigenicity in nude mice. Tumorigenicity was fully restored by adding of progenitors of glutathion biosynthesis like L-cystein to NMF-treated cells. From these findings, the mechanism of antitumor action of NMF was linked to glutathion depletion (Codeiro and Savarese 1986). Recent findings however suggest that depletion of the glutathion pool is a side effect of other cytopathological effects of NMF (Malorny et al. 1992). Such cytopathological events are: time and dose-dependent surface blebbing, retraction of the cell body, modification of the cytoskeletal components (Malorny et al. 1992). Other cytopathological events caused by NMF are induction of apoptosis, loss of expression of the c-myc protein (Chatterjee et al. 1989; Wood and Elvin 1995) and G1 arrest of colon cancer cells by NMF due to p27 induction (Pagnotta et al. 2005). But the critical point in in vitro experiments with NMF is that cytopathological events are only achieved with NMF concentrations approximately 50 times higher than those which would be reached after injection of 200 mg/kg in mice. Therefore, the results of cell culture experiments with NMF cannot be transferred without limitation to experimental chemotherapy tests in mice. Kalyani et al. investigated the interaction of NMF with commercially available superoxide dismutase 1 (SOD 1) by spectroscopic and molecular modeling studies (Kalyani et al. 2014). Their findings show that NMF causes loss of enzymatic activity due to perturbation of secondary structure of SOD 1. SOD 1 deficiency induces apoptosis associated with O_2^- -accumulation in the cytoplasm and mitochondria which increases loss of mitochondrial membrane potential and DNA-damage-mediated p53 activation (Watanabe et al. 2013). Perhaps, this and/or interaction of NMF with enzymes involved in cell cycle control is the reason of the NMF phenomenon i.e., the cure of tumor bearing mice with a low toxicity combination therapy with SUM-IAP and NMF.

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Compliance with ethical standards

Conflict of interest The author declares no conflict of interest.

Ethical approval All applicable international, national and institutional guidelines for the care and use of animals were followed.

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