Original Paper

Antitumour Activity and Retinotoxicity of Ethyldeshydroxy-sparsomycin in Mice

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The colony formation in agar of human tumour xenografts was used as a test system to study the cytostatic activity of ethyldeshydroxy-sparsomycin (EdSm) at the cellular level. EdSm was additionally studied in vivo in human tumour xenografts and murine tumour models. EdSm showed a clear dose–response effect in vitro. At continuous exposure with 0.01 μg/ml, 2 out of 11 of the tumours responded (a gastric and a small cell lung carcinoma). At 0.1 μg/ml EdSm, the tumour response was 5/11 tumours and at 1 μg/ml the compound was active in all tumours. The maximal tolerable doses of EdSm in vivo have been determined in non-tumour bearing CDF1 mice. In the intraperitoneally (i.p.) given multiple dose schedules the respective LD10 doses indicated that the tolerable cumulative dose increases when lower doses are given more frequently. This also enhances the antitumour activity in L1210 leukaemia to 172% T/C. On the other hand, continuous infusion strongly diminished the tolerable dose as well as the antitumour activity. EdSm was also active against i.p. inoculated P388 leukaemia (150% T/C), B16 melanoma (156% T/C), and RC carcinoma (197% T/C), and the subcutaneously (s.c.) inoculated murine tumours: M5076 sarcoma, osteosarcomas C22LR and CP369, and the LL carcinoma, as well as in the human tumour xenografts: LXFG 529, a non-small cell lung carcinoma; GXF 251, a gastric carcinoma; and FMa, an ovary carcinoma. Possible long-range retinotoxic effects of EdSm were investigated in tumour-bearing mice, cured after surviving treatment with LD50 doses of EdSm, by assaying the protein biosynthetic capacity of the retina by assaying the ocular rhodopsin and opsin levels as parameters. In none of these cases could a significant reduction in either opsin or rhodopsin levels be measured and no changes were seen histologically.

Key words: sparsomycin, protein synthesis inhibitors, retinotoxicity

INTRODUCTION

The natural product sparsomycin (Sm) is an antibiotic produced by Streptomyces sparsogens and Streptomyces cuspidosporus. It was discovered in 1962 by Owen and associates [1] and characterised by Argoudelis and Herr in 1962 [2]. Antitumour activity was observed towards P388 leukaemia only [3]. A clinical phase I study with Sm (NSC-59729) was initiated in 1964 by Close and McFarlane., using a daily schedule for 10–14 days [4]. This study was discontinued because 2 out of 5 patients reported blurred vision», probably caused by degeneration of the retinal pigment epithelium [4, 5].

Despite these disappointing phase I results there is still interest in Sm and its derivatives have attracted our attention. Firstly, an important aspect is that their mode of action differs from classical antitumour agents. These Sm compounds are potent inhibitors of protein synthesis by blocking the ribosomal peptidyl transferase centre [6–11]. Because of this different mode of action, Sm and several derivatives were used in combination chemotherapy studies and were shown to enhance the antitumour activity of cisplatin [12–14]. The structure–activity relationship of Sm derivatives has been explored and several were found to have a therapeutic index broader than that of Sm itself. Overall, ethyldeshydroxy-sparsomycin (EdSm) showed the best biological activities [15, 16]. Secondly, the use of high daily doses of cytotoxic drugs in phase I studies has been abandoned nowadays because of the high risk of accumulation and toxicity. Finally, intensive histopathological and biochemi-
nal analysis of animal models failed to show retinal toxicity of Sm and one analogue, even at repeated toxic doses [17, 18]. Moreover, the synthesis of suitable analogues might overcome the putative ocular toxicity. Therefore, we have extensively studied the antitumour activity and retinotoxicity of EdSm. In this report the results are summarised and discussed.

MATERIALS AND METHODS

Drugs

EdSm was synthesised at the department of Organic Chemistry of the University of Nijmegen, The Netherlands [17], and was acquired in a lyophilised form. The drug was dissolved in phosphate buffered saline (pH 7.4) and stored at 4°C in dark flasks. Solutions with the required drug concentration were prepared just before administration by dilution with isotonic NaCl.

Maximal tolerable dose

The acute toxicity of EdSm has been determined in normal CD2F1 mice. The 50% and 10% lethal doses (LD50 and LD10, respectively) were determined after administration on various routes and schedules. For bolus injections we used five dose levels and six mice per dose level. For continuous administration of EdSm to normal and tumour-bearing mice, we used Alzet osmotic minipumps 1003 D and 1007 D (Alza, Palo Alto, California, U.S.A.). The 1003 D minipump has a volume of 100 μl and a flow capacity of 1.06 ± 0.05 μl/h continuously over 3 days. The 1007 D minipump also has a volume of 100 μl, but a flow capacity of 0.5 μl/h continuously over 7 days. These minipumps were filled with an EdSm solution in isotonic NaCl. Pumps were implanted intraperitoneally (i.p.) or subcutaneously (s.c.) under ether anaesthesia. Three mice were used per dose level in osmotic minipumps.

In vitro xenografts

Experiments with human tumour xenografts were performed by Dr H.H. Fiebig, Department of Internal Medicine, Division of Haematology-Oncology, University of Freiburg, D-7800 Freiburg, Germany. Human tumours, established in serial passage in nude mice, as described by Fiebig and colleagues [19], were used. A single-cell suspension of the solid human tumours was obtained by mechanical disaggregation with scissors and subsequent incubation with enzymes, as described by Fiebig and colleagues [20]. The two-layer soft-agar culture system introduced by Hamburger and Salmon [21] was modified as described by Fiebig and colleagues [19]. In the colony assay, EdSm was applied by continuous exposure until the end of the experiment. Three dose levels were studied in triplicate and in each experiment six untreated cultures were used as controls. 5-Fluorouracil (100 μg/ml, continuous exposure) was added as a positive reference drug. A compound was considered active if it reduced colony formation to 30% or less of the control value.

Antitumour activity

Three human tumours were used to study the antitumour activity of EdSm in vivo: a non-small cell lung carcinoma (LXF2529), a gastric carcinoma (GXF 251) (both studies were tested by Dr H.H. Fiebig), and an ovary carcinoma (FMa), (tested by Dr E. Boven, Free University Hospital, Amsterdam, The Netherlands). The murine leukaemia tumours P388 and L1210 and the solid tumours B16 melanoma and RC renal cell carcinoma were obtained from G. Atassi, Brussels. They were passaged and maintained in animals as recommended by the NCI. The experiments with the osteosarcomas C22LR and LL, the Lewis Lung carcinoma and M5076 sarcoma were performed at the Radiobiological Institute TNO in Rijswijk as reported by Zylciz and associates [18]. The osteosarcoma CP368 was tested at CIVO-TNO, Zeist, The Netherlands, by Dr P. Lelieveld. In the treatment of subcutaneously growing human and murine tumours EdSm was administered to tumour-bearing animals, with tumour sizes between 25 and 50 mm³, at a dose level of 5 and 10 mg/kg on days 1, 5 and 9. Animals were randomised before treatment.

Studies on retinotoxic effects

CD2F1 mice were obtained from Charles River Breeding Laboratories, Inc. Male mice weighing between 18 and 22 g were inoculated intraperitoneally with 10⁵ L1210 cells. Acceptable median control survival times range from 8–11 days for L1210 i.p. tumour. EdSm and cisplatin were administered i.p. at various doses on days 1, 5 and 9 after tumour implantation. Eye toxicity was investigated in tumour-bearing animals which were recorded as cures after drug treatment [22].

Rhodopsin assay. Animals were dark-adapted for 24 h to ensure maximal regeneration of rod visual pigment (opsin to rhodopsin). All procedures were carried out in dim red light (> 610 nm). After pentobarbitan anaesthesia the eyes were extirpated, placed in light-tight containers and directly frozen at −80°C. The animals were sacrificed afterwards. Extraction and assay of rhodopsin have been described previously [23]. Rhodopsin was measured by difference spectroscopy and the rhodopsin concentration was calculated from the difference in absorbance at 500 nm before and after illumination, using a molar absorbance coefficient of 403 000.

Opsi n assay. For quantitative determination of opsin, the earlier described enzyme-linked immunosorbent assay (ELISA) was used [24, 25].

Histopathology. Eyes were fixed in Bouin solution and embedded in paraffin according to standard procedures [26]. Sections of 5 μm were stained with haematoxylin and eosin, and subsequently analysed microscopically.

RESULTS

The cytostatic activity of EdSm was studied in 11 human tumour xenografts in vivo in doses ranging from 0.01 to 1.0 μg/ml (0.0278–2.78 μM) given by continuous exposure (Table 1). A clear dose–response relationship was established. At the dose of 0.01 μg/ml 2 out of 11 human tumours (18%) were sensitive, at 0.1 μg/ml this was 45% and at 1.0 μg/ml all tumours responded, suggesting that if an EdSm concentration of about 2.8 μM can be maintained at the cellular tumour level over a longer period in vivo, this tumour will respond positively to EdSm treatment.

Before determining the antitumour activity of EdSm in different animal tumour models, we investigated the acute toxicity in normal CD2F1 mice. The 50% and 10% lethal doses (LD50 and LD10, respectively) were determined after i.p. EdSm administration on various schedules; the results are summarised in Table 2. For single dose treatment the intravenous (i.v.) route was more tolerable than the i.p. route; the LD10 values are 48.8 and 24.9 mg/kg, respectively. This difference disappeared upon daily administration. Additionally, these data indicate that EdSm is better tolerated when it is given more frequently at lower doses. Daily i.p. administration for 9 days results in a LD10...
of 2 mg/kg. From the s.c. implanted tumours appreciable T/C values (>135%) were only obtained with L1210 and RC. No significant antitumour response was measured in the M5076, C22LR, CP369 and LL tumour models. The two human tumour xenografts most sensitive to EdSm in vitro; i.e. the large cell lung carcinoma LXFG 529 and the gastric carcinoma GXF 251, as well as the ovary carcinoma FMA were tested in vivo. EdSm at a dose of 5 or 10 mg/kg, given i.p. on days 1, 5 (LXFG 529) or 1, 5, 9 (GXF 251 and FMA), did not exert any antineoplastic effect in any of these tumours.

### Retinopathy

Toxic effects of EdSm on the protein biosynthetic capacity of the retina were assessed by assaying the rhodopsin and opsin levels in tumour-bearing mice, cured after treatment with doses of EdSm which were toxic for normal standards (LD50). The photoreceptor cell–RPE (retinal pigment epithelium) complex accomplishes a high turnover rate of visual pigment (ca. 10% per day) and in order to maintain a constant level the photoreceptor cell has a high biosynthetic capacity for the protein opsin (5–10 pmol/10^6 cells per day) [27]. Perturbations like inhibition of this process will, therefore, rapidly lead to a reduction in ocular opsin content, and may eventually effect an almost complete loss in visual pigment protein. For conversion of the apoprotein opsin into the photoactive pigment rhodopsin, the ocular opsin content, and may eventually effect an almost complete loss in visual pigment protein. For conversion of the apoprotein opsin into the photoactive pigment rhodopsin, the photoreceptor cell relies on the production of the required chromophore, 11-cis retinal, by the enzymatic machinery of the retinal pigment epithelium [28]. Selective inhibition of this process could result in impaired ocular opsin levels and reduced rhodopsin levels. In order to incorporate possible additive effects due to tumour- and other disease-related pathogenicity, the toxicity of EdSm was investigated in tumour-bearing mice which survived on relatively high doses of EdSm or Sm, in combination with cisplatin, which at high doses can also cause retinotoxicity [29]. In none of these cases could a significant reduction in either
opsin or rhodopsin levels be measured (Table 4). None of the eyes of mice treated with cisplatin and EdSm showed histological changes.

**DISCUSSION**

The toxicity of Sm analogues in mice is related to their lipophilicity; it decreases with increasing lipophilic character [17]. Therefore, EdSm could be administered at much higher doses than Sm, e.g. LD₅₀ of 8 mg/kg for EdSm compared with 0.26 mg/kg for Sm for the i.p. schedule D1–9. Osmotic minipumps have been very useful in the study of the effects of continuous drug treatment [30–34]. Chronic administration of EdSm using 100 µl osmotic minipumps over 3 days turned out to be less tolerable (LD₅₀ = 2.96 mg/kg) and less therapeutic than repeated bolus injections. Thus, the therapeutic window of EdSm treatment when given chronically seems to be low.

Previous studies have demonstrated that exposure of Chinese hamster fibroblast cells to Sm for 1 h inhibits over 99% of their protein synthetic capacity, but is lethal for only 25% of these cells, due to the fact that after removal of the drug the cells recovered their protein synthesising ability. This reversibility disappeared after longer periods of incubation [35]. Similarly, chronic EdSm treatment might cause an irreversible protein imbalance due to permanent inhibition of protein synthesis, which would enhance its toxicity to normal tissues. When lower doses are given at frequent intervals the cumulative tolerable dose for EdSm increases and the antitumour response is improved. Under those conditions, appreciable antitumour activity for EdSm (> 135%) is obtained when the tumour site as well as the drug treatment is i.p., and in two s.c. implanted tumours (L1210 and RC) when EdSm was given i.v. (139% and 138%, T/C respectively). Worth mentioning is that no antitumour activity of EdSm was noticed when the tumour was implanted s.c. and drug treatment was i.p. It is not clear yet whether this is due to the treatment schedule (D1, 5, 9 instead of D1–9) or mainly due to i.p. instead of i.v. administration. The strongest antitumour responses were obtained upon daily EdSm administration, i.p. when the tumour was implanted i.p. and i.v. when the tumour was implanted s.c. From this we conclude that the application route of choice for EdSm in animal tumour models should implement i.p. administration for i.p. tumours and i.v. administration for s.c. tumours.

**Table 4. Rhodopsin and opsin concentrations in eyes of CD2F1 mice, 6–8 months of age, which were alive and tumour-free 100 days after combined treatment with cisplatin and EdSm in the L1210 tumour model**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Survivors</th>
<th>*Rhodopsin/eye</th>
<th>†Opsin/eye</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>33</td>
<td>0.64 ± 0.05</td>
<td>0.65 ± 0.06</td>
</tr>
<tr>
<td>Group II</td>
<td>67</td>
<td>0.66 ± 0.03</td>
<td>0.65 ± 0.03</td>
</tr>
<tr>
<td>Group III</td>
<td>60</td>
<td>0.67 ± 0.04</td>
<td>0.68 ± 0.02</td>
</tr>
<tr>
<td>Group IV</td>
<td>67</td>
<td>0.66 ± 0.02</td>
<td>0.68 ± 0.06</td>
</tr>
<tr>
<td>Group V</td>
<td>67</td>
<td>0.63 ± 0.05</td>
<td>0.61 ± 0.07</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.71 ± 0.07</td>
<td></td>
</tr>
</tbody>
</table>

Mice from all these groups were histologically checked for degeneration of the neural retina and retinal pigment epithelium. Group I, cisplatin 4 mg/kg; group II, cisplatin 4 mg/kg and EdSm 10 mg/kg; group III, cisplatin 3 mg/kg and EdSm 5 mg/kg; group IV, cisplatin 3 mg/kg and EdSm 10 mg/kg; group V, cisplatin 3 mg/kg and Sm 1.5 mg/kg [14, 22].

* Spectroscopy, n = 4 ± S.D.; † ELISA, n = 4 ± S.D.

Under the auspices of the National Cancer Institute (Bethesda, Maryland, U.S.A.) additional toxicological studies in rats and monkeys have been performed [36]. Retinotoxicity was assessed by electroretinography (ERG) and eye histology. Abnormal electroretinograms were only observed in moribund rats, probably due to their poor general condition. No ERG changes were seen in monkeys. Histopathological changes were not detected in any animal. The plasma membrane of reticulocytes is rather impermeable for Sm [37]. On the basis of these studies, the Sm-related retinopathy is suggestive of a poor general condition, and possibly of inappropriate drug schedules. Since the first reports on retinal toxicity of Sm preparations concern late-stage patients, to a large degree in cachectic condition [4, 5], we extended these analyses to tumour-bearing mice and included the more lipophilic Sm derivative EdSm, which will more easily cross the blood–retina barrier. No evidence was found for any retinotoxic action of Sm or EdSm, either on a supracellular level (retinal morphology) or on a subcellular level (protein biosynthesis, intracellular and intercellular transport). These novel observations are fully in line with our previous results. We have to conclude that Sm-derivatives are not retinotoxic.


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