Cancer Chemother Pharmacol (1993) 32: 90–95

Cancer Chemotherapy and Pharmacology

Review

Alkylphosphocholines: a new class of membrane-active anticancer agents

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Received 5 November 1992/Accepted 19 November 1992

Introduction

Since it was shown that lysolecithin stimulated macrophage phagocytosis, the hypothesis was put forward that lysophospholipids might be physiological mediators of immunological reactions. This assumption was the rationale for the chemical synthesis of compounds with a metabolism different from that of the naturally occurring lysophospholipids. Because of their slower degradation in the circulation they were thought to be potent immunomodulators. The lead structure of this whole class of compounds became 1-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine (ET18 OCH3; Fig. 1), which indeed had some interesting immunopharmacological properties [1].

Throughout the investigations into the pharmacology of alkyllysophospholipids it turned out that they exerted concentration-dependent cytotoxic effects in various tissue-culture systems. Later, Fleer and co-workers [5] demonstrated that their cytotoxicity correlated with their substrate specificity for phospholipase C and/or D. From these studies it was finally concluded that the glycerol structure was not an essential structural element for the antitumor activity of the molecule [4], and hexadecylphosphocholine (INN, miltefosine; Fig. 1) was identified as a prototype of the alkylphosphocholines, a new generation of anticancer agents.

In vitro and in vivo activity of miltefosine against screening tumors

The in vitro spectrum of activity of miltefosine was established in a selection of rodent and human cell lines. The test assay used was a soft-agar tumor stem-cell assay, with the period of exposure to the compound being between 7 and 12 days. The concentrations of miltefosine that inhibited the colony formation of various cell lines by 90% (IC_{90}) are given in Table 1. The human KB and HL-60 cell lines as well as the mouse leukemia L1210 line were, in our hands, the lines most sensitive to miltefosine. In contrast, NK-mouse lymphoma and normal mouse bone marrow were completely resistant to the cytotoxic action of miltefosine. A rat-hepatoma cell line and a human colon-carcinoma line were moderately sensitive.

In contrast to the in vitro data summarized above, it turned out that none of the conventional rodent tumors were sensitive to miltefosine treatment in vivo (Table 2). Only marginal activity was observed in intravenously transplanted Lewis lung carcinoma and in the solid MOP-C plasmocytoma. In view of its good in vitro activity, the lack of in vivo effects of miltefosine in L1210 leukemia is intriguing. Even sublethal doses of miltefosine were incapable of prolonging the survival of leukemia-bearing animals. Pharmacokinetic studies have demonstrated that cytotoxic plasma levels following a single oral dose were maintained for 3–4 days [28]. For the time being, the discrepancy between the in vitro and in vivo effects observed in the L1210 line remains enigmatic. Recently, Yanapirut et al. [32] also drew attention to a discrepancy between the in vitro and in vivo activity of miltefosine. The authors speculated that the compound's mode of action might differ, depending on the test system used.

The correlation between in vitro and in vivo sensitivity to miltefosine, however, was excellent in the KB tumor. This tumor is an established human squamous-cell carcinoma line that after transplantation of a 1-mm^3 fragment grows as a solid tumor in nude mice. A single oral administration of 215 mg/kg miltefosine completely inhibited tumor growth. Even large established KB tumors could be brought to remission by two treatments with miltefosine spaced 7 days apart (Fig. 2). This sensitivity of established KB tumors to miltefosine is highly significant since according to our experience this transplantable human tumor shows a relatively poor response to conventional cytotoxic drugs such as cyclophosphamide and cisplatin.

Paper presented at the 4th International ARTAC Workshop on Therapeutic Trials in Cancer and AIDS

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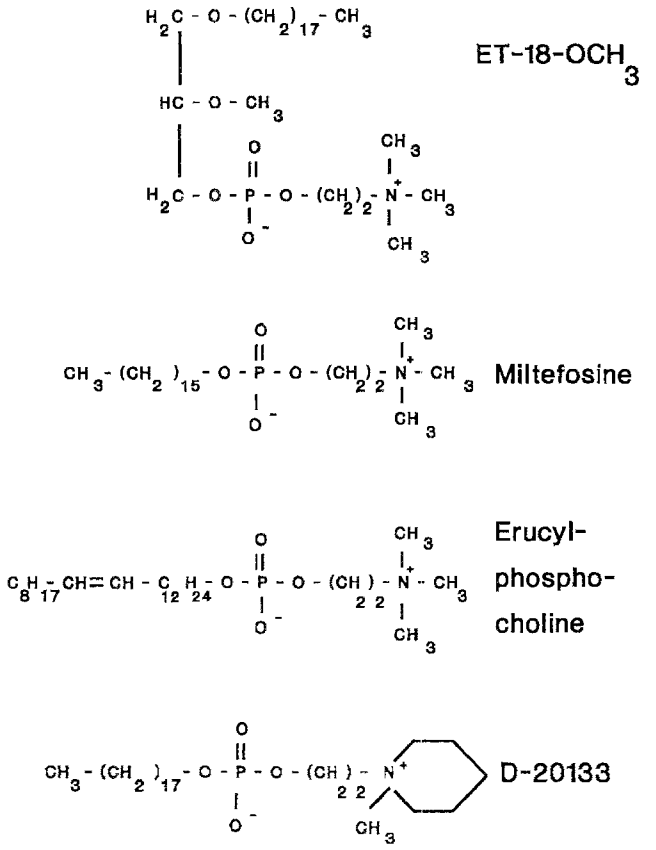

Fig. 1. Chemical structures of Et-18-OCH_3 and selected alkylphosphocholine derivatives

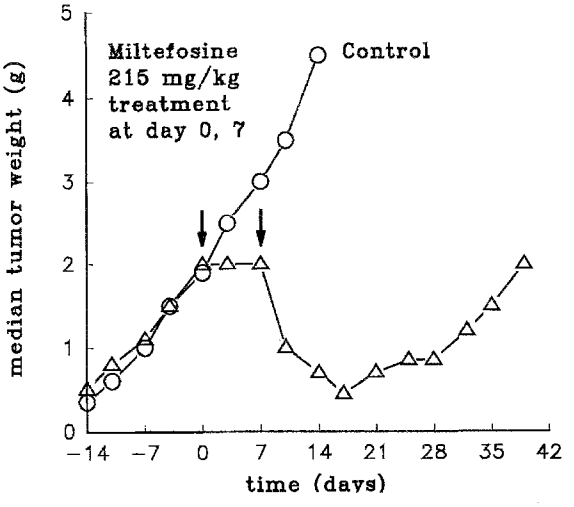

Fig. 2. Growth curves generated for advanced (ca. 2 g) KB tumors in nu/nu mice treated with two oral doses of miltefosine

Activity of miltefosine against autochthonous rat tumors

Muschiol and collaborators [19] have shown that miltefosine and other phospholipid derivatives exhibit significant anticancer activity in animals bearing the autochthonous methylnitrosourea (MNU)-induced rat mammary carcinoma. This suggested to us that there might also be activity against the classic hormone-dependent dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma. This tumor is usually induced by gavage of 20 mg DMBA into 50-day-old female Sprague-Dawley rats. Various miltefosine schedules were capable of completely suppressing tumor growth and, similar to the KB tumor, large DMBA tumors could also be brought into remission by oral miltefosine treatment (Fig. 3). Again, in our hands, the DMBA-induced rat mammary carcinoma is rather insensitive to cytotoxic agents such as cisplatin and cyclophosphamide, and this is particularly true for larger tumors.

If oral miltefosine treatment was initiated directly after the administration of the carcinogen DMBA, the appearance of tumors could be delayed for as long as the animals were treated [13]. However, after the cessation of therapy the tumors appeared in treated animals with the same distribution and growth kinetics observed in previously untreated controls. This finding suggested that the alkylphosphocholine effectively inhibited cell proliferation and did not interfere with the carcinogenic action of DMBA. Extensive studies into the schedule dependency of miltefosine were carried out in DMBA-tumor-bearing rats. The result of these studies was that the antitumor effect appeared to be completely independent of the schedule, provided that the total dose remained constant over the treatment period. This observation led us to conclude that the plasma half-life of the compound in rats was rather long. As mentioned above, subsequent pharmacokinetic studies have confirmed this assumption [28].

Table 1. In vitro activity of miltefosine in the clonogenic assay using human- and rodent-tumor cell lines

Clonogenic assay	IC_{90} (μg/ml)
L1210 mouse leukemia	3.16
NK-mouse lymphoma	>100
Ah 13s rat hepatoma	10
KB human carcinoma	0.7
HL60 human leukemia	2.8
CO 115 human colonic carcinoma	10
Mouse bone marrow	>100

Table 2. Summary of the in vivo activity of miltefosine in various transplantable tumors

Tumor	Injection site	Animal	Result
DS carcinosarcoma	s.c.	Rat	Negative
Leukemia L5222	i.p.	Rat	Negative
Leukemia L1210	i.p.	Mouse	Negative
Leukemia P388	i.p.	Mouse	Negative
B-16 melanoma	s.c.	Mouse	Negative
Lewis lung carcinoma	s.c.	Mouse	Negative
Lewis lung carcinoma	i.v.	Mouse	Marginal
MOP-C plasmocytoma	s.c.	Mouse	Marginal
KB (human)	s.c.	Nu/nu mouse	Active

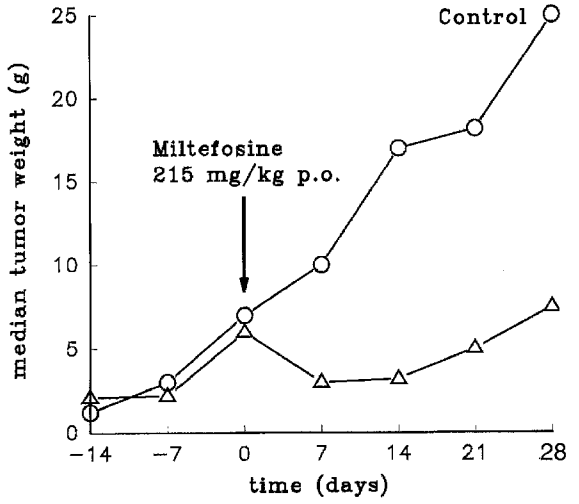

Fig. 3. Growth curves generated for advanced (ca. 6 g) DMBA tumors in rats treated with a single oral dose of miltefosine

Activity of protein kinase C and phospholipase C

In vitro studies conducted by Überall and co-workers [26] using the NIH-3T3 cell system revealed that miltefosine inhibited protein kinase C (PKC) activity in cell-free extracts as well as in intact cells, and this also seems to be true for other cell systems [9]. Miltefosine also antagonizes phorbol ester-stimulated proliferation of epithelial cells [6]. Apparently the mechanism of inhibition was similar to that of other alkyllysophospholipids and consisted of competition with the biological activator phosphatidylserine. An extension of these studies suggested that PKC was not the only target of miltefosine. As one would have assumed from previous biochemical studies [5], there was some indirect evidence that miltefosine also acted as a competitive inhibitor for phosphoinositidase C [26]. In addition, miltefosine suppressed the translocation of phosphocholine cytidylyltransferase from the membrane to the cytosol [7]. Therefore, this compound may be considered as a multifunctional inhibitor of phosphoinositide metabolism. The breakdown of membrane-bound inositol lipids generates a variety of second messengers such as diacylglycerol and inositol phosphates, which are of great importance for cellular functions, cell growth, and cell proliferation. It is likely that the inhibition of enzymes within this signal cascade ultimately results in a blockade of cell division.

Induction of differentiation by miltefosine

Apart from signal transduction, PKC may have other functions in cellular processes. In two recent publications, Minana and co-workers [18] and Parodi and co-workers [21] proposed that PKC plays a key role in the control of cellular differentiation and that inhibition of this enzyme induces differentiation in mouse and human neuroblastomas. In vitro, miltefosine was shown to be a potent inducer of tumor-cell differentiation [11]. In vivo, the miltefosine-induced remission of DMBA mammary carcinomas went along with histological signs of tumor differentiation: throughout the therapy the typical structure of an adenocarcinoma gradually adopted the features of normal milk-producing breast tissue [13]. Furthermore, the promonocytic leukemia cell line U-937 was inhibited by miltefosine and it was shown that the compound induced histone H1°, a basic chromosomal protein, which is frequently associated with cell differentiation. The synthesis of this protein preceeds the expression of several parameters of monocytic differentiation in the cells [14].

Another aspect of the differentiation-inducing capacity of miltefosine is the repeatedly observed induction of leukocytosis following systemic treatment. During its toxicological evaluation, this compound was found to induce an increase in white blood cells associated with a predominance of mature granulocytes [10]. Later, during initial clinical studies this phenomenon was also observed in many patients on miltefosine treatment [31]. The significance of this observation is not yet fully understood. Studies conducted by Vehmeyer and collaborators [30] indicated that miltefosine enhanced CSF-dependent progenitor-cell colony growth. Studies carried out by Nooter et al. [20] essentially confirmed this finding and suggested that miltefosine was synergistic with hemopoietic growth factors. Since some of these growth factors represent T-cell products, the question as to whether or not miltefosine might directly affect T-cell responses in vitro was also investigated. In the presence of interleukin 2 (IL-2), miltefosine treatment dependently enhanced the production of interferon gamma up to 20 times as compared with control values. Immunofluorescence studies demonstrated that miltefosine also increased IL-2-receptor and HLA-DR-antigen expression [29]. Pignol and co-workers [22] reported that lipopolysaccharide-stimulated human monocytes could be further stimulated by alkyllysophospholipids to increase their tumor-necrosis-factor (TNF) production. If substantiated, the effects of miltefosine on lymphokine production may open a new avenue to our understanding of the mode of antitumor action of this class of compounds.

Immunological effects of miltefosine

Although the immune system was the original target for alkyllysophospholipid derivatives, miltefosine does not appear to be a major immunomodulator. Its substantial antitumor activity in human xenotransplants in nude mice indicates that the presence of T-cells, which are largely absent in these animals, is not a prerequisite for the drug's action. Studies into the role of natural killer (NK) cells gave negative results, and miltefosine was equally incapable of inducing cytotoxic spleen cells against relevant tumor cells. There was no significant effect on antibody production following sensitization with sheep red blood cells. Finally, the phagocytosis of mouse bone-marrow macrophages was not stimulated but rather inhibited by miltefosine. Therefore, it was concluded that the antitumor effect of miltefosine must be largely independent of immunological factors [12]. However, this statement is entirely based on the results of our screening methods, and weak or more sophisticated changes in the immune system may have

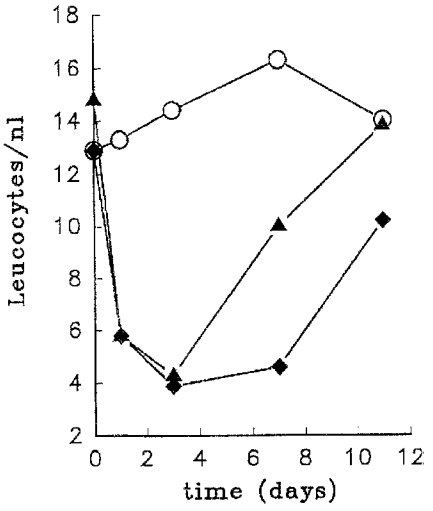

Fig. 4. Leukocyte count in rats treated intravenously with 50 mg/kg cyclophosphamide with and without concomitant oral miltefosine treatment (50 mg/kg, days 0–4) ○, controls; ▲ Cyclophosphamide + miltefosine; ◆ cyclophosphamide alone

escaped detection. As outlined in the previous paragraph, the activation of certain lymphokines could well contribute to the mode of action of miltefosine.

Antiinvasive properties of miltefosine

Studies into the potential antiinvasive properties of miltefosine revealed that it inhibited the directional migration of MO4 cells as well as their invasiveness in vitro. These effects were seen at nontoxic miltefosine concentrations and were compatible with the postulated action of this compound at the level of the cell membrane [23]. Recently, it was also shown that miltefosine inhibited the migration of granulocytes upon a chemotactic stimulus, and this response was associated with an inhibition of intracellular actin formation (Wolff et al., in preparation). Again, it is known that actin formation is under the control of PKC [17].

Combination of miltefosine with conventional chemotherapy

Inhibitors of PKC such as quercetin, tamoxifen, or staurosporine have been shown to enhance the antiproliferative effects of cisplatin [15]. In a study of an ether lipid analog as an inhibitor of PKC and cisplatin, Hofmann and coworkers [16] have reconfirmed previous data. Using an in vivo mouse model, Spruß and co-workers [24] were capable of showing that there was also considerable therapeutic synergy between miltefosine and a new cisplatin derivative. This study was particularly interesting because in the tumor model chosen, miltefosine had no effect at the doses given, whereas in combination with a platinum complex it greatly enhanced the antitumor effect. Miltefosine acted under these experimental conditions as a pharmacological response modifier for the platinum compound. Studies using the DMBA-induced mammary carcinoma of Sprague-Dawley rats revealed therapeutic synergy of miltefosine with cyclophosphamide as well.

Furthermore, in rats injected with a single dose of cyclophosphamide (50 mg/kg) alone or in combination with miltefosine, the leukocytes fell progressively in both groups for 3 days (Fig. 4). In the miltefosine-treated group, recovery occurred considerably earlier. It appears that the stimulating effect of miltefosine on hemopoietic cell growth can be exploited therapeutically (Stekar and Hilgard, in preparation). In an in vitro study on the cross-resistant pattern of membrane-active lipids, Berdel and coworkers [2] demonstrated that miltefosine had substantial cytotoxic activity in a Chinese hamster ovarian cell line as well as in its multidrug-resistant (MDR) subline, which was selected for colchicine resistance. In contrast, two other cell lines selected for Adriamycin resistance expressed a modest cross-resistance to miltefosine and other phospholipid derivatives.

MDR is characterized by a rapid drug efflux due to the activity of an energy-dependent membrane-bound drug pump, the *mdr1*-gene-dependent P-glycoprotein. MDR-reversal agents restore the lowered drug accumulation within the cell through effects on the cell membrane. Since the membrane is also the obvious target structure of miltefosine's action, it is justifiable to assume that this compound exerts certain effects on MDR. MDR-mediated drug resistance and PKC activity are correlated with respect to P-glycoprotein, which is stimulated by this enzyme. As several chemosensitizers, e.g., verapamil, are PKC inhibitors, it can be expected that inhibitors such as the alkylphosphocholines are also capable of modulating MDR [8]. This potentially interesting pharmacological effect is currently under study. Alkyllysophospholipids such as Ilmofosine have been shown to reverse MDR in appropriate experimental systems [16].

Topical treatment of human cutaneous breast-cancer metastases

Although topical treatment of cutaneous breast-cancer metastases has no curative intent, it may be of considerable palliative value to the patient. In various clinical trials throughout Europe, more than 200 patients were treated with a 6% miltefosine solution applied twice daily on cutaneous lesions. The overall cumulative objective-response rate obtained in the different studies was around 30%. Some prognostic factors such as the depth of the lesion and the presence or absence of ulcerations influenced the results to a certain degree. The side effects were generally tolerable and consisted of occasional reddening, itching, and burning at the local site of drug application. Systemic absorption of miltefosine through the skin could be ruled out, and it is therefore likely that the observed effects were indeed related to the topical treatment. To enhance the penetration depth of miltefosine into the skin, the drug was formulated in a mixture of alkylglycerols [27].

Second-generation alkylphospholipids

Clinical trials have been initiated with oral miltefosine; however, the major problem was the gastrointestinal toxicity of the compound and, as a result, the drug could not be appropriately dosed and the response rates remained low. Therefore, it was the aim of analogue research to identify new alkylphosphocholines with an improved therapeutic index. One of the second-generation compounds that fulfilled this requirement was an alkylphosphocholine analogue without the choline moiety (D-20133, Fig. 1). This analogue was more active in vitro and in vivo than miltefosine, and it also showed lower gastrointestinal-tract toxicity in preclinical studies [25]. A detailed report about this new compound is in preparation. Another derivative under development is Erucylphosphocholine (Fig. 1), the first alkylphosphocholine that could be given intravenously without causing intravascular hemolysis [3]. Further studies are in progress to characterize these second-generation compounds so as to introduce them into clinical phase I trials.

Conclusion

From the foregoing it is evident that the experimental profile of miltefosine and alkylphosphocholines is different from that of conventional anticancer agents. Much evidence indicated that the mode of action of alkylphosphocholines was related to plasma-membrane-associated phosphoinositide metabolism. Inhibition of phospholipase C and/or PKC is currently thought to be the main biochemical target of alkylphosphocholines. Considering the great importance of both phosphoinositide metabolism and the regulation of a broad spectrum of cellular functions, including differentiation and invasion, miltefosine could become an interesting new drug. In addition, it may be a tool for further studies into the biochemical pathway of signal transduction in malignant cells.

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