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Original Paper

Combination of the New Minor Groove Alkylator Tallimustine and Melphalan

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The benzoyl nitrogen mustard derivative of distamycin A, tallimustine, belongs to a new class of alkylating agents, known as DNA minor groove alkylating agents. It alkylates adenine N3 with high sequence specificity, causing no alkylation of guanine N7, the main site of alkylation of clinically used nitrogen mustards such as L-PAM. The present study investigated the *in vivo* antitumour activity of a combination of tallimustine and melphalan (L-PAM). Two murine tumours were used: i.p. (intraperitoneally) transplanted L1210 leukaemia and i.m. (intramuscularly) transplanted M5076 ovarian reticulum cell sarcoma (M5). In L1210, which is only marginally sensitive to tallimustine, the combination of tallimustine 3 mg/kg i.p. with L-PAM 10 mg/kg i.p. was as effective as 20 mg/kg L-PAM, which is the maximum tolerated dose. In M5, which is sensitive to both drugs, the combination was superior to either drug alone. The results suggest that the combination of tallimustine and L-PAM—or possibly in general, minor groove alkylators and major groove alkylators—may be therapeutically advantageous and therefore should be investigated clinically.

Key words: antineoplastic agent, alkylating drug, *in vivo*, mouse tumours, melphalan, tallimustine

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INTRODUCTION

ALKYLATING AGENTS are still among the most active drugs for the therapy of several solid and haematological malignancies. Those currently in clinical use bind mainly in the major groove of DNA to GC-rich sequences, forming a number of DNA adducts. Most of these alkylators are bifunctional and can induce the formation of DNA–protein and DNA–DNA cross-links, the latter lesion considered as being particularly cytotoxic [1–3].

A new class of alkylating agents which bind to the minor groove of the DNA helix has recently been found to be effective in preclinical tests, and initial clinical investigations are now in progress to test their toxicity and activity. One of these, at the moment in phase I–II in Europe and U.S.A., is tallimustine (FCE 24517, TAL), the benzoyl mustard derivative of distamycin. A preliminary indication is that haematological toxicity may strongly limit its dose escalation [4]. It therefore seemed worth exploring alternative dosage schedules and combination therapies, using subtoxic doses of tallimustine with other effective drugs with different mechanisms of action.

Tallimustine binds DNA, forming only a few, highly sequence-specific alkylations on adenines located in an adenine–thymidine-rich sequence. It does not alkylate on guanine N7 when incubated with DNA *in vitro* [5, 6]. Other classical nitrogen mustards, for example, melphalan (L-phenylalanine mustard, L-PAM), alkylates mainly guanine N7 *in vitro* [7] or in living cells growing in culture [8]. The different modes of action of tallimustine and L-PAM are also suggested by the observation that mouse leukaemic cells resistant to L-PAM are just as susceptible as the parental cell line to tallimustine [5].

Recent *in vitro* studies have shown that pretreatment of DNA with distamycin A [9] or with tallimustine [10] strongly modified the pattern of alkylation of L-PAM to guanine N7. This finding and the evidence that tallimustine and L-PAM act by different mechanisms prompted us to evaluate the effects of a combination of these two drugs on murine tumours. The present study reports the findings obtained in L1210 leukaemia and ovarian reticulum cell

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sarcoma growing in mice, using different dosage schedules with the combination of the two drugs.

MATERIALS AND METHODS

Drugs

L-PAM was supplied by Sigma (St Louis, Missouri, U.S.A.); tallimustine (FCE24517) was kindly provided by Pharmacia Farmitalia-Carlo Erba (Milan, Italy). Drugs were freshly suspended, diluted in saline and injected i.p. in a volume of 0.3 ml/mouse. Control mice received 0.3 ml of the vehicle.

Animals

Inbred male CD2F1/CrlBR and female C57Bl/6NCrlBR mice were supplied by Charles River Italy (Calco, Italy). The animals were housed under conditions of controlled temperature, light cycles and humidity and were fed an open formula diet (Altromin Mt, Rieper, Bolzano, Italy) with tap water *ad libitum*. All mice weighed 20 ± 2 g at the start of experiments.

Procedures involving animals and their care conformed with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council directive 86/609, OJ L358, 1 Dec. 12, 1987; NIH guide for Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

Antitumour activity studies

L1210 leukaemia and M5076 (M5) ovarian reticular cell sarcoma were originally supplied by Mason Research Institute (DCT Animal and Human Tumor Bank, Worcester, Massachusetts, U.S.A.). M5 was serially transplanted in syngeneic female C57Bl/6 mice by i.m. passage every three weeks. L1210 leukaemia was maintained i.p. in ascitic form by weekly passages in CD2F1 mice.

Drugs were administered i.p. at different schedules and doses, starting on day 1 after L1210 implantation, or in two cycles starting on day 1 and day 13 after M5 implantation. Seven to ten animals were used in each treatment group in each experiment with L1210 bearing mice. The survival curves of control group and of the reference single-dose treatments were obtained plotting the data of at least three independent experiments (22–36 mice). Each treatment group with M5 tumour bearing mice consisted of 10 mice. Confirmatory experiments were performed with groups of seven mice.

Control L1210 tumour bearing mice died between days 7 and 11, while control M5 bearing mice died between days 23 and 27.

RESULTS

In pilot experiments, we established the doses of L-PAM and tallimustine that could be combined without lethal toxicity. The dose of 3 mg/kg tallimustine could be combined with L-PAM 10 mg/kg, with a maximal weight loss of 20%. It was also possible to split the dose of tallimustine with a 1 h or 24 h interval, reducing the toxicity. In fact, 6 mg/kg caused 100% lethality in the first 10 days, when given i.p. as a single injection, whereas two doses of 3 mg/kg were lethal in 50% of the animals, deaths occurring between 20 and 40 days.

The antitumour activity of several combination schedules of tallimustine and L-PAM was tested, using sublethal doses of the drugs which were similarly active against L1210 leukaemia as a single agent: 3 mg tallimustine and 10 mg L-PAM were used and the $100 \times T/C$ was close to 180 in both cases.

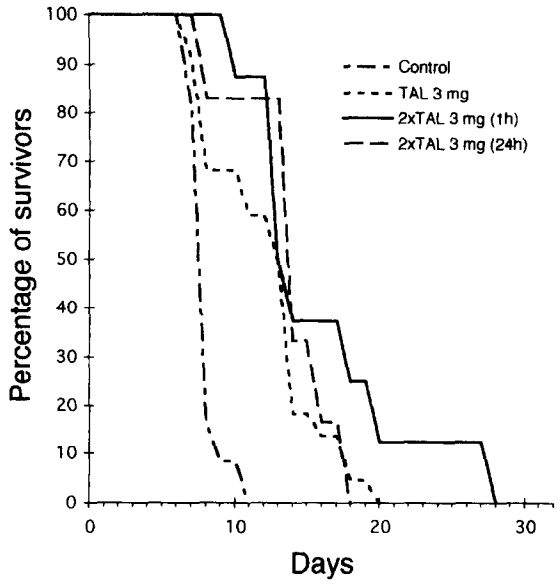

Figure 1. Survival of L1210 tumour bearing mice after tallimustine, given as a single dose or two doses. Each group consisted of 7–10 mice except for groups of control and reference single-dose treatments which consisted of 36 and 22 mice, respectively.

Survival was slightly longer with the repeated 3 mg + 3 mg treatment than that with a single 3 mg dose (Figure 1) and the 1 h interval was superior to 24 h. A major increase in survival was observed when L-PAM was given in combination with tallimustine (Figure 2). The order of administration of the two drugs was not important, but the interval was, since survival was much longer with the 1 h interval than with 24 h (Figure 3). Five out of 36 mice were cured with the association of tallimustine and L-PAM at the 1 h interval but none when the drugs were spaced 24 h apart. Tallimustine and L-PAM given with an interval of 24 h showed the same antileukaemic activity as the single doses

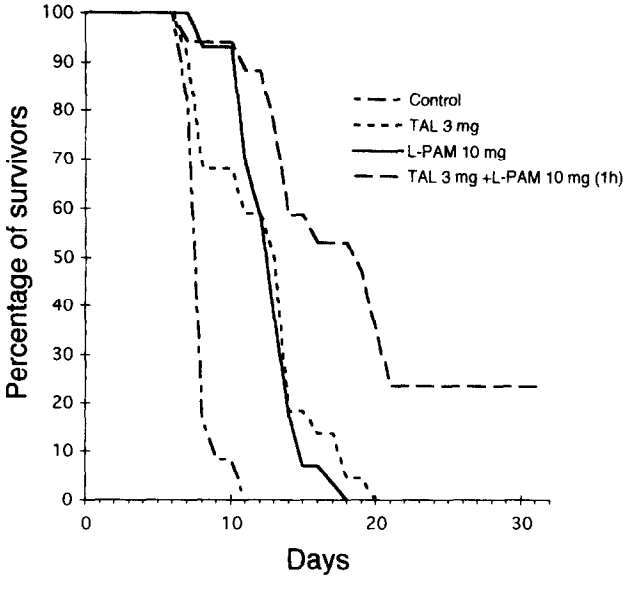

Figure 2. Survival of L1210 tumour bearing mice after tallimustine, L-PAM and the combination of the two drugs. Results of two experiments are presented. In each experiment, 7–10 mice were used in the treatment groups except for groups of control and reference single-dose treatments which consisted of 36 and 22 mice, respectively.

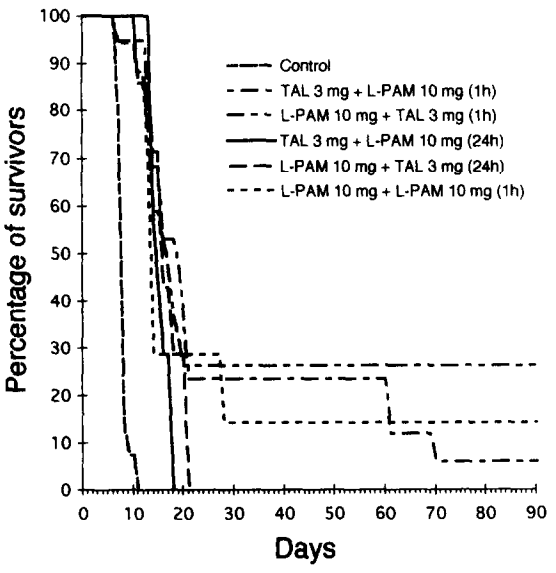

Figure 3. Survival of L1210 tumour bearing mice after combined tallimustine + L-PAM treatment in different schedules. The L-PAM + L-PAM combination is also shown for comparison. Each treatment group consisted of 7–10 mice except for groups of control and reference (TAL + L-PAM, 1 h interval) treatments which consisted of 36 and 17 mice, respectively.

of tallimustine or L-PAM. One mouse out of seven was cured with the two doses of L-PAM at a 1 h interval, but this treatment too was no more active than the L-PAM + tallimustine combination (Figure 3).

These schedules were also tested in mice bearing a solid tumour, the ovarian reticular cell sarcoma M5. Mice were injected with a single drug or the combination at a 1 h interval, in two cycles, on day 1 and day 13 after the i.m. tumour inoculum.

The first death was observed in the control group on day 23, and the control tumour weight was 1.9 ± 0.6 g. On the same day, only palpable tumours were observed in 4/10 mice treated with 3 mg/kg tallimustine alone and 3/10 mice treated with 10 mg/kg L-PAM alone, while the tumour was not detectable at all in animals which had received the two-dose combinations (tallimustine + tallimustine, L-PAM + L-PAM, L-PAM + tallimustine, tallimustine + L-PAM, with a 1 h interval between the doses).

Figure 4 shows the survival curves for all treatment groups. Tallimustine alone was active on M5 tumours ($100 \times T/C = 144$), and 3 mg tallimustine was slightly less active than 10 mg L-PAM alone. A second 3 mg tallimustine dose did not improve survival compared with a single 3 mg dose (except for one mouse out of 10), while causing the greatest weight loss (9%, after the second cycle or treatment). Antitumour activity was higher on combining tallimustine with L-PAM (Figure 4(a)). Like the L1210 tumour bearing mice, the addition of a second drug (L-PAM or tallimustine) 1 h after the first L-PAM injection increased activity. Unlike L1210, however, in M5 bearing mice, the combination of L-PAM and tallimustine gave higher antitumour activity than L-PAM + L-PAM (Figure 4(b)).

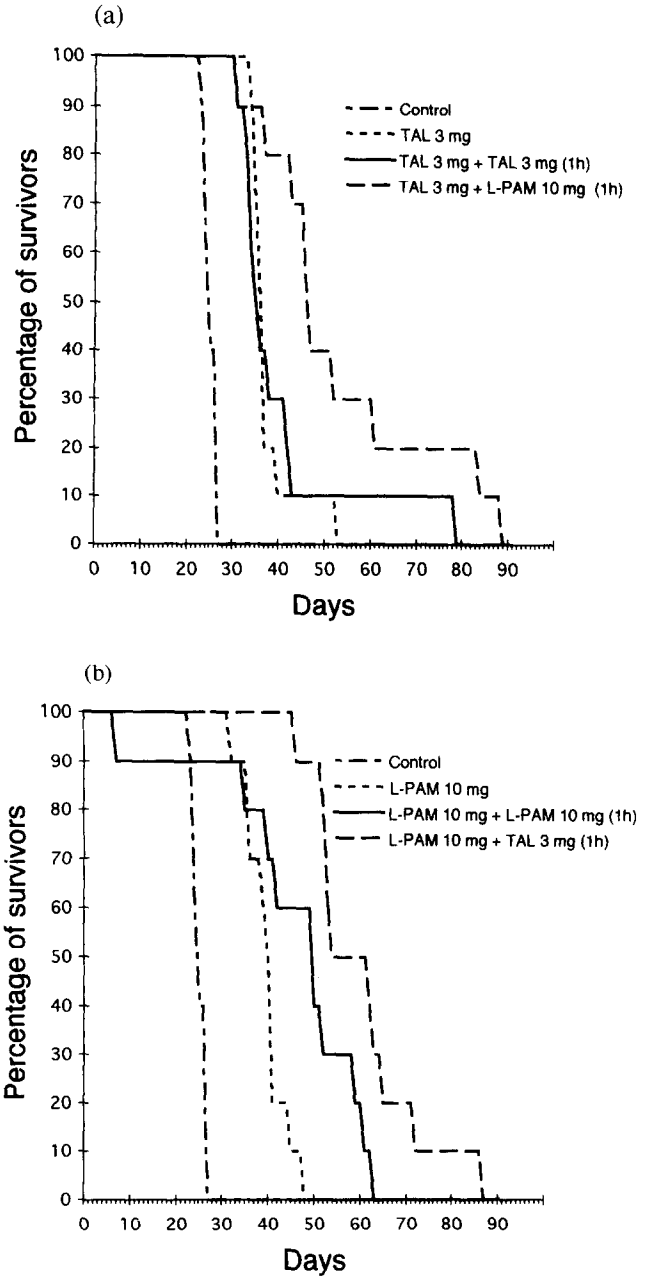

Figure 4. (a) Survival of M5 tumour bearing mice after treatments based on tallimustine as a single or the first drug, as two doses or in combination with L-PAM. Each group consisted of 10 mice. (b) Survival of M5 tumour bearing mice after treatments based on L-PAM as a single or the first drug, as two doses or in combination with tallimustine. Each group consisted of 10 mice.

DISCUSSION

The present study provides the first evidence that a DNA minor groove alkylating agent can be combined with a conventional alkylating agent which alkylates DNA in the major groove producing good antitumour effects *in vivo* at a tolerable dose.

In principle, drugs which are combined in polychemotherapy regimens should act by different mechanisms and not be possibly cross-resistant. There have been studies on combinations of alkylating agents that interact differently with DNA [11, 12]. In the case of nitrogen mustards and nitrosoureas, there are well-documented differences related to the alkylation of guanine O6, which is an important site of alkylation for nitrosoureas but not for nitrogen mustards. The cellular activity of O6-alkylguanine-DNA alkyltransferase appears to be important in the effect of nitrosoureas, but not of nitrogen mustards [13, 14]. The different patterns of activity

of nitrosoureas and nitrogen mustards and their lack of cross-resistance in some experimental animal models have led to them being combined in some clinical protocols.

The differences in the mode of action of minor groove alkylators and nitrogen mustards are much more marked. Minor groove alkylators which have shown antitumour activity, distamycin or CC-1065 derivatives, do not alkylate guanine at all [6, 15], but produce a limited number of highly sequence-specific alkylations at the N3 adenine position. In the specific case of the distamycin derivative tallimustine, the alkylations are directed towards adenine N3, mainly in the sequence TTTTGA, although other AT-rich hexamers have been recently found to be targets of alkylation [6]. With nitrogen mustards such as L-PAM, the major site of alkylation is certainly guanine N7, in the major groove of DNA, and some preference has been reported for alkylations of guanine in sequences with several contiguous guanines [7, 8, 16].

Although both tallimustine and the CC-1065 derivatives are at an early stage of development and it is too early to envisage whether they will have any real impact in cancer chemotherapy, their promising preclinical activity suggests some potential for their clinical use.

The present findings suggest that minor groove alkylators can be effectively combined with conventional nitrogen mustards, as no antagonistic effect has been detected. The combination can prove advantageous as there are preclinical indications that these drugs are not cross-resistant [5].

A specific point concerning these drugs regards the sequence and timing of the combinations. The importance of this was indicated by Hartley and associates [6] who showed that when DNA is reacted *in vitro* with distamycin and subsequently with nitrogen mustards, the pattern of alkylation of the latter compounds was different from control DNA. The changes in DNA structure induced by tallimustine were even more dramatic according to Coley and associates [9]. The reaction of DNA with tallimustine significantly modified and reduced the alkylation sites of several nitrogen mustards including L-PAM.

If this dramatic effect also occurs *in vivo*, one might expect a marked difference in the biological effects of the combination of tallimustine and L-PAM using different sequences. Some difference was in fact observed; administering L-PAM 1 h before tallimustine resulted in reduced toxicity and appeared to be therapeutically advantageous in L1210 tumour bearing mice. However, in M5 reticulum cell sarcoma bearing mice, the advantage of giving L-PAM first was apparent on day 50, but all animals had died by day 90. The *in vitro* studies suggest that the interaction between tallimustine and L-PAM at the DNA level requires very high concentrations, which may perhaps be achieved in i.p. transplanted L1210, when drugs are given i.p., but not in i.m. growing M5.

When there was a 24 h interval between the doses of the two drugs, results were much worse. It is possible if the interval between the two drugs is long that there is enough time for the tumour cell to repair the damage produced by the first drug before the other drug arrives to cause further damage. When the interval is shorter, the accumulation of different DNA lesions will be lethal.

In terms of antitumour activity of the combination, the results on the M5 tumour are the most interesting. Mice treated with tallimustine plus L-PAM in either sequence with a 1 h interval survived much longer than with two doses of L-PAM or tallimustine.

In conclusion, the studies described in this paper indicate the possible clinical potential of combinations of DNA minor groove alkylators with conventional alkylating agents. The results in mice suggest that effective doses of each drug can be achieved without too severe toxicity, and with an additive or even synergistic antitumour effect.

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