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Studies on the mechanism of gastrointestinal absorption of melphalan and chlorambucil*

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Summary. The uptake of melphalan into tumour cells has been shown previously to involve active transport, while that of chlorambucil is by passive diffusion. In view of these findings, the mechanism of their gastrointestinal absorption was investigated using the in situ rat intestinal model. Segment lengths in all cases were (mean ±SD) 47.2±4.7 cm. Drug absorption was monitored from control intestinal segments and from segments pretreated with the metabolic inhibitors 2,4 dinitrophenol (DNP) or carbonylcyanide-*M*-chlorophenyl-hydrazone (CCCP). Aliquots of gut-perfusing solution were removed at 5-min intervals over 30 min and assayed for drug using a high-performance liquid chromatography (HPLC) method selective for the alkylating agents. Absorption of melphalan by control animals was (mean ±SD) 1.22%±0.25% cm^{-1} gut length, as against 0.59%±0.13% cm^{-1} in DNP- and 0.45%±0.07% cm^{-1} in CCCP-treated animals. Absorption of chlorambucil was 1.47%±0.17% cm^{-1} (control), 1.49%±0.06 cm^{-1} (DNP), and 1.58%±0.23% cm^{-1} (CCCP).

It was clear, therefore, that pretreatment of intestinal segments with metabolic inhibitors led to a reduced absorption of melphalan ($P<0.01$) but did not influence that of chlorambucil. The experimental data suggest that melphalan uptake from the intestine involves an energy-dependent system whereas chlorambucil is passively absorbed.

Introduction

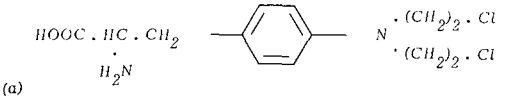
Melphalan and chlorambucil are structurally related cytotoxic drugs (Fig. 1) currently used in the therapeutic management of a number of malignancies. Melphalan was first synthesised by Bergal and Stock [4] in 1954, with the expectation that it would be incorporated into proteins due to the presence of the amino acid group (L-phenylalanine), thus enhancing antitumour specificity. It is used effectively to treat multiple myeloma, and carcinoma of the breast and ovary [11]. Chlorambucil, although similar in structure to melphalan, does not contain an amino acid group. Clinically it forms part of the chemotherapy for chronic lymphocytic leukaemia, Hodgkin's and non-Hodgkin's lymphoma, and carcinoma of the breast and ovary [8].

A major problem encountered with these and other antineoplastic agents is tumour resistance. Research into the mechanisms of resistance has shown that melphalan is transported into cells by an energy-dependent system [13]. It is thought that the transport mechanism is involved in the uptake of several amino acids and may be blocked by certain metabolic inhibitors and amino acids, such as leucine and glutamine [13, 18]. In addition, drug uptake into cells appears to proceed against a concentration gradient. The uptake of chlorambucil into cells is unaffected by metabolic inhibitors and does not take place against a concentration gradient, thus suggesting that it enters cells by passive diffusion [14].

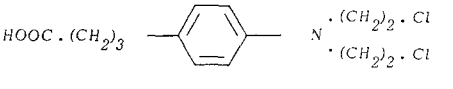
The aim of the present study was to extend the investigation of drug uptake to intestinal absorption using the metabolic inhibitors dinitrophenol (DNP) and carbonyl cyanide-*M*-chlorophenyl hydrazone (CCCP). Clearly, if active processes are involved in the intestinal absorption of a potent drug, bioavailability may be adversely influenced by the gastrointestinal contents at the time of administration. Such erratic absorption may lead to a poor therapeutic response in the patient.

Materials and methods

In situ intestinal model. The method adopted for the study was an in situ rat intestinal model based on those described by Doluisio et al [7] and Swintosky and Pogonowska-Wala [16]. Albino Wistar rats weighing 218–307 g were fasted for 16–24 h before the experiment with water allowed ad libitum. The rats were anaesthetised by IP injec-



(a)



(b)

Fig. 1. Structural formulae of melphalan (a) and chlorambucil (b)

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tion of pentobarbitone sodium (60 mg/kg). Via a midline incision two L-shaped glass cannulae were inserted through small slits at the duodenal and ileal ends of the small intestine. The cannulae were secured with linen sutures and were connected to 20 ml syringes via three-way stopcocks. This arrangement allowed fluid to be withdrawn from and replaced into the intestinal segments as required. Animal body temperature was maintained at 37 °C by an infrared lamp.

The segments in each case were first flushed with 20 ml of saline at 37 °C to clear any debris. The saline remaining in the isolated segment was expelled by means of air from a syringe and replaced with either 12 ml metabolic inhibitor (DNP or CCCP) or saline (control). These solutions were allowed to remain in the intestinal segment for 2 min, followed by flushing with 20 ml saline and finally with air. Buffered solutions (12 ml) of the drugs were then added to the segments and drug absorption monitored. Experimentation was carried out in a randomised fashion and involved study of melphalan and chlorambucil absorption from control and inhibitor-pretreated segments.

Sample collection and storage. Samples of drug solutions (0.5 ml) were removed from the intestinal segment at 5-min intervals up to 30 min, the volume of perfusate being maintained by replacement with 0.5 ml original drug solution on each occasion. The latter solution was incubated at 37 °C from the beginning of each experiment to ensure that the rate of spontaneous hydrolysis was the same as that in the luminal perfusate. Goldenberg et al. [12] showed that the cellular uptake of parent compound and hydrolysed derivative took place by independent mechanisms, so that it was necessary to use intact drug to study the absorption mechanism. After collection, all samples were kept on ice (2 °C), as hydrolysis does not occur at this temperature over short periods of time. For overnight storage prior to analysis, samples were frozen at −20 °C.

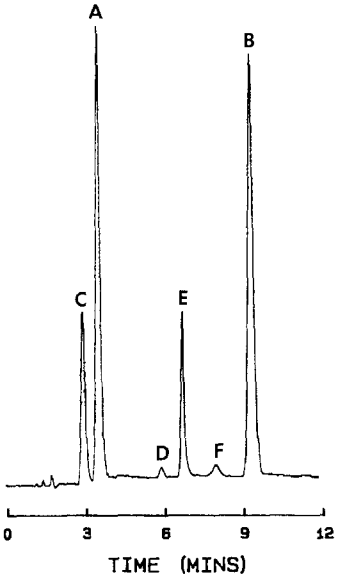

Fig. 2. Chromatogram of chlorambucil *(A)* and melphalan *(B)*. The products of hydrolysis are monohydroxychlorambucil *(C)*, dihydroxychlorambucil *(D)*, monohydroxymelphalan *(E)* and dihydroxymelphalan *(F)*

Perfusing media. The perfusing buffer for both drugs was an iso-osmotic (263 mosmol/kg) phosphate buffer (sodium phosphate monobasic/sodium phosphate dibasic) at pH 5.2. This solution was chosen because an acidic environment increases melphalan stability. Because both alkylating agents are sparingly soluble in aqueous solution and are subject to rapid spontaneous hydrolysis, stock solutions (0.1 mg/ml) were prepared in methanol. The solution used to perfuse the intestinal segments was a mixture of the methanolic drug solution (2.5%) and phosphate buffer (97.5%). The perfusion concentration for each alkylating agent was 2.5 μg/ml. The total dose administered to each animal was 30 μg, which approximates to the equivalent adult human daily dose (mg/kg) for intermittent low-dose therapy.

A concentrated solution of the nonspecific metabolic inhibitor DNP (50 μ*M*) was prepared in alkaline iso-osmotic phosphate buffer (pH 9). The test perfusing solution consisted of DNP stock solution (5%) and normal saline (95%). A perfusing solution of CCCP (0.25 m*M*) was prepared in alkaline iso-osmotic phosphate buffer (pH 9) immediately before perfusion. The concentrations of metabolic inhibitors used were based on preliminary experimentation on their systemic toxicity.

Drug analyses. Melphalan and chlorambucil were determined by HPLC [1, 2]. Separation of the agents was performed on a Spherisorb ODS (5 μm particle size) column (250 × 4.6 mm ID), which was maintained at 40 °C using a block heater. The mobile phase was a mixture of 80% methanol, 20% water, and contained 0.0135% (w/v) sodium dodecyl sulphate as ion-pairing agent. It was adjusted to pH 3.10 with concentrated sulphuric acid. The flow rate was 1.3 ml/min.

The potential presence of proteins in the perfusate necessitated their precipitation with 20 μl concentrated perchloric acid. After centrifugation, the supernatant was flushed through a sample clean-up cartridge (C_{18} Sep-Pak), and the polar components were eluted by washing the cartridge with 10 ml 15% (v/v) methanol in water. Both drugs were eluted with 2 ml cold (−20 °C) methanol, 200 μl of which was injected onto the column. Drug retention times were in the order of 4.2 min (chlorambucil) and 9.5 min (melphalan). The system accomplished resolution of parent compounds from the products of hydrolysis (Fig. 2). Since only the parent compounds are considered to be pharmacologically active, the data reported relate solely to them.

Results

The drug absorbed in each case was calculated as a percentage from the initial dose (30 μg) and the residual (t = 30 min) amounts of drug. These values were obtained by regression analysis of the log drug concentration versus time curves. The coefficients of correlation for the regression analysis of melphalan and chlorambucil absorption from the in situ segments were (means ± SD) 0.909 ± 0.053 and 0.982 ± 0.013, respectively. Loss of intact drug from the intestinal segment is brought about by processes of absorption and spontaneous hydrolysis. To account for this latter effect, in vitro hydrolysis rate constants were determined for each alkylating agent in perfusate at 37 °C, and subtracted from the observed in situ rate constant for drug

Table 1. Summary (mean ± SD) of absorption data for melphalan in control and inhibitor-pretreated animals

Group	Number of animals	Intestinal segment length (cm)	Residual amount of drug (μg)	Percentage of dose absorbed	Percentage of dose absorbed per cm segment length
Control	5	42.4 ± 2.6	14.8 ± 2.4	50.8 ± 8.0	1.22 ± 0.25
DNP	5	50.0 ± 9.5[a]	21.0 ± 3.4*	30.0 ± 11.4**	0.59 ± 0.13**
CCCP	5	43.6 ± 5.8[a]	24.0 ± 1.2**	19.9 ± 3.9**	0.45 ± 0.07**

Statistical difference from control animals:
[a] Not significant
* $P<0.05$; ** $P<0.01$

Table 2. Summary (mean ± SD) of absorption data for chlorambucil in control and inhibitor-pretreated animals

Group	Number of animals	Intestinal segment length (cm)	Residual amount of drug (μg)	Percentage of dose absorbed	Percentage of dose absorbed per cm segment length
Control	5	48.8 ± 4.8	8.7 ± 1.7	71.1 ± 5.6	1.47 ± 0.17
DNP	5	51.8 ± 1.3	6.8 ± 1.2	77.3 ± 3.9	1.49 ± 0.06
CCCP	5	46.6 ± 4.0	8.1 ± 2.0	73.1 ± 6.8	1.58 ± 0.23

No statisticaly significant difference was observed between control and inhibitor-pretreated animals for any parameter

disappearance (absorption and hydrolysis). This enabled the process of drug absorption to be quantified separately, and data reported relate solely to the absorption of parent compound.

After each experiment was complete the intestinal segment was dissected out and measured. To account for variations in segment length the results are also reported as percentages of drug absorbed per centimetre of gut length. Summaries of results for the two alkylating agent are given in Tables 1 and 2. Statistical analysis was performed using the Mann-Whitney U-test (two-tailed) for nonparametric data.

In experiments involving melphalan the amount of drug absorbed differed significantly ($P<0.01$) between control and inhibitor-pretreated animals (Fig. 3). This reduced absorption indicated that the metabolic inhibitors did indeed impair the absorption of melphalan. No significant difference ($P>0.05$) was observed between absorption data for DNP- and for CCCP-pretreated animals. The absorption of chlorambucil did not differ significantly between control and test segments ($P>0.05$; Fig. 4). The intestine segment length did not differ significantly between control and test animals for either melphalan or chlorambucil ($P>0.05$).

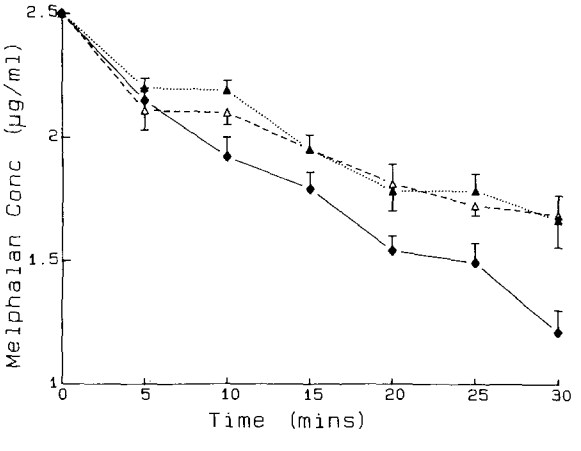

Fig. 3. Gastrointestinal concentration (mean ± SE) versus time profiles for melphalan in control (—◆—), DNP-treated (—▲—) and CCCP-treated (—△—) rats

Discussion

The inhibition of melphalan absorption by DNP suggests that an active process is involved in its absorption from the gastrointestinal tract. The present experimental findings are supported by physicochemical considerations as well as by clinical pharmacokinetic data. Since melphalan

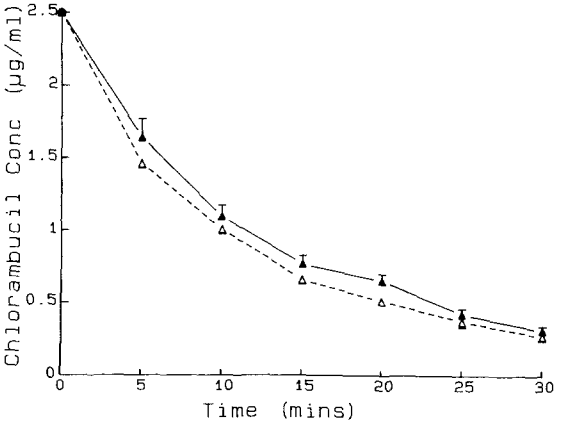

Fig. 4. Gastrointestinal concentration (mean ± SE) versus time profiles for chlorambucil in control (—▲—) and CCCP-treated (—△—) rats. Data for DNP-treated animals have been omitted for clarity. No significant differences was observed between CCCP and DNP and CCCP and control data (concentration profiles have not been corrected for hydrolysis or volume depletion).

contains an amino acid group (L-phenylalanine) it possesses both acidic and basic properties. Values for pKa are available in the literature [10] for the L-phenylalanine moiety only, and are 1.83 and 9.13; this will result in almost total ionisation of the molecule throughout the intestinal pH range, so that according to the pH partition theory, diffusion across cellular membranes would be limited. Nonetheless, clinical pharmacokinetic studies show the drug to be rapidly absorbed [3, 5, 6]. In addition, it has been shown that bioavailability can be 100% under fasting conditions [6]. Cellular experimentation has shown that melphalan uptake into tumor cells is dependent on an energy-requiring system [13], which has been linked with two possible amino acid transport systems [13, 18]. However, our results differ from those recorded in cellular studies, which have shown DNP to have little effect on the cellular uptake of melphalan. This difference may be caused by the existence within cancer cells of other energy sources unaffected by DNP, and may reflect metabolic differences between LPC-1 plasmacytoma and gastrointestinal epithelial cells.

The pKa value for chlorambucil is 5.8 [8], which would result in the drug existing largely in the unionised form at duodenal pH. Absolute bioavailability data concerning chlorambucil are lacking because of the absence of an intravenous preparation, but rapid absorption rates with peak plasma levels occurring 15–30 min after oral administration to fasting subjects have been reported [9, 15]. Although the above data do not allow determination of the mechanism of absorption the present experimental findings suggest strongly that chlorambucil absorption is by passive diffusion rather than by active transport. Chlorambucil uptake into tumour cells has been shown to occur by passive diffusion [14]; intestinal absorption appears to proceed by the same process.

Two recent pharmacokinetic studies in patients have shown that chlorambucil bioavailability is unaffected by concurrent ingestion of food [9], whereas food markedly reduced melphalan bioavailability [6]. In addition, it has also been shown that melphalan uptake into tumour cells is reduced in the presence of leucine and glutamine [17].

In view of the present evidence supporting an active absorption mechanism for melphalan, it is possible that amino acids present in food may compete with melphalan for intestinal transport sites, thus reducing the efficiency of drug absorption.

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