In vitro and in vivo studies on the combination of Brequinar sodium (DUP-785; NSC 368390) with 5-fluorouracil; effects of uridine

G.J. Peters¹, I. Kraal & H.M. Pinedo¹²

¹Department of Oncology, Free University Hospital, PO Box 7057, 1007 MB Amsterdam; ²Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

Summary
Brequinar sodium (DUP-785; Brequinar) is a potent inhibitor of the pyrimidine de novo enzyme dihydroorotidate dehydrogenase (DHO-DH), leading to a depletion of pyrimidine nucleotides, which could be reversed by uridine. In in vitro studies we investigated the effect of different physiological concentrations of uridine on the growth-inhibition by Brequinar, the effect of the nucleoside transport inhibitor, dipyridamole, and the combination of Brequinar and 5-fluorouracil (5FU). Uridine at 1 μM slightly reversed the growth inhibition by Brequinar, while the effect of 5–500 μM was greater. However, at Brequinar concentrations >30 μM, uridine could not reverse the growth-inhibitory effects. Addition of dipyridamole could only partially prevent the reversing effects of uridine. The combination of Brequinar and 5FU was more than additive in the absence of uridine in the culture medium, but not in the presence of uridine. The combination of Brequinar and 5FU was tested in vivo in two murine colon tumour models, Colon 26 and Colon 38. Scheduling of both compounds appeared to be very important. In Colon 38 no potentiating effect of Brequinar could be observed. In contrast in Colon 26 a more than additive effect could be observed. Since uridine concentrations are considerably different in these tumours (higher in Colon 38), it was concluded from both the in vitro and in vivo experiments that uridine is an important determinant in combinations of Brequinar and 5FU.

Brequinar Sodium (Brequinar; DUP-785; NSC 368390) is a potent inhibitor of dihydroorotic acid dehydrogenase (DHO-DH), the fourth enzyme in the de novo pyrimidine nucleotide synthesis (Figure 1) which is located on the outer site of the inner membrane of the mitochondrion (Chen et al., 1986; Peters et al., 1987a). The Ki varies between 10 and 100 nM depending on the source of the enzyme. Brequinar is a 4-quinoline carboxylic acid with significant antitumour activity in experimental tumours (Dexter et al., 1985; Braakhuis et al., 1990) which was therefore selected for clinical Phase I and II investigations (Arteaga et al., 1989; Bork et al., 1989; Dodion et al., 1990; Noe et al., 1990; Schwartzmann et al., 1990). The maximum tolerated dose (MTD) proved to be 1,800 mg m⁻². A weekly treatment schedule showed no activity against a number of solid tumours (Dodion et al., 1990); the compound showed acceptable toxicity at the dose level 1,200 mg m⁻². Biochemical effects of Brequinar at the dose range from 600–2,250 mg m⁻² (Peters et al., 1990a) were related to the toxicity of the drug and consisted of the inhibition of DHO-DH and a depletion of pyrimidine nucleotides in lymphocytes of the patients; in plasma uridine levels decreased followed by a rebound. Biochemical effects observed in patients were comparable to those in mice and in vitro (Peters et al., 1987a; 1990b; Schwartzmann et al., 1988). Drug exposure resulted in accumulation of cells in the S phase (Schwartzmann et al., 1988). Growth-inhibitory effects of Brequinar could be prevented and reversed by addition of uridine or cytidine (Peters et al., 1987a; Schwartzmann et al., 1988), but not by thymidine or deoxycytidine. Depletion of pyrimidine deoxyribonucleotides was proportional to that of the ribo-nucleotides (Schwartzmann et al., 1988), while both could be reversed by uridine.

Inhibition of the pyrimidine de novo pathway can enhance the anti-tumour activity of 5FU; both aciclovir and N-phosphonomethyl-L-aspartate (PALA) have been investigated in preclinical in vitro and in vivo studies (Martin et al., 1983; Grem et al., 1988; Spiegelman et al., 1980). In initial trials the MTD of PALA was used while the dose of 5FU was lowered (Grem et al., 1988; Martin et al., 1983; Casper et al., 1983). However, a PALA dose of far below that recommended for Phase II trials, yet leading to a depletion of pyrimidine nucleotides, was sufficient to modulate 5FU activity (Martin et al., 1985). In a Phase II trial such a dose of PALA was selected and the dose of 5FU was escalated.

Correspondence: G.J. Peters, Department of Oncology, Free University Hospital, PO Box 7057, 1007 MB Amsterdam, The Netherlands. Received 8 May 1991; and in revised form 23 September 1991.

Figure 1 Schematic representation of pyrimidine metabolism, showing the effects of inhibition by Brequinar (DUP-785) which may affect the metabolism of 5FU; arrows indicate an increase of PRPP (phosphoribosylpyrophosphate) or a decrease of pyrimidine nucleotides and OA (orotic acid). In addition the possible role of the nucleoside transport inhibitor dipyridamol is shown. For comparison, PALA can inhibit the conversion of Carb-P (carbamoyl-phosphate) to Carb-asp (carbamoyl-aspartate) and pyrazofurin that of OA to OMP and UMP. UR, uridine.
leading to a high response rate (O'Dwyer et al., 1990). In addition to the encouraging results (30–40% response rate) with the combination leucovorin and 5FU (Arbuck et al., 1989) these data demonstrate that principles of biochemical modulation developed preclinically can be applied successfully in the clinic. However, conventional guidelines used for Phase II trials, should not be applied when the dose of the modifier is being selected (Leyland-Jones et al., 1986).

In vitro studies should take in vitro conditions into consideration. An example of such conditions is the relatively high in vitro concentration of uridine in tissues compared to e.g. plasma (Darnowski et al., 1986; Peters et al., 1987b); this might affect the antitumour activity of Brequinar. In vitro is referred to the higher sensitivity of cell lines cultured in dialysed serum compared to culturing in non-dialysed serum (Peters et al., 1987a), which contains a considerable amount of uridine. Since Brequinar can decrease pyrimidine nucleotides both in vitro and in vivo (Chen et al., 1986; Peters et al., 1990b; Schwartzmann et al., 1988; Anderson et al., 1989), the compounds theoretically can enhance the activity of 5FU. We evaluated this combination both in vitro and in vivo, while simulating in vivo conditions for in vitro experiments.

Materials and methods

Materials

Brequinar sodium (DUP 785, NSC 368390) was synthesised and obtained from the Medicinal Chemistry Section, DuPont Pharmaceuticals, Wilmington, Delaware, USA, and formulated as a 10 mg ml⁻¹ solution in saline. 5FU was from Hoffmann-La Roche, Mijdrecht, The Netherlands, and formulated as a 10 mg ml⁻¹ solution. Uridine and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma, Ohio. Other compounds were of analytical grade quality.

Cell lines and culture

A cell line (characterised as clone 10) from the murine colon tumour Colon 26 was obtained from Dr Klohs (Warner-Lambert, Ann Arbor, Michigan, USA) and was designated Colon 26-10. Mycoplasma-free cells were maintained at 37°C under an atmosphere of 5% CO₂ as subconfluent monolayers in 80 cm² culture flasks (Nunclon, Denmark) and subcultured once or twice weekly in Dulbecco's modification of Eagle's medium (Flow Laboratories, Irvine, Scotland) supplemented with 5% heat-inactivated foetal calf serum (FCS) (Gibco, New York, USA) and 1 mM L-glutamine.

Drug sensitivity testing was performed in triplicate using a modification of the Microculture Tetrazolium Assay (MTT assay) described elsewhere (Keepers et al., 1991). Briefly, cells were plated in 96-well flat bottom microtiterplates (Greiner Labrotechnik, Solingen, Germany) (100 μl of cell suspension per well) at optimal seeding densities (2,000–3,000 cells) to assure exponential growth during the 4-day assay. After 48 h, 100 μl of culture medium or culture medium containing drug was added to the wells. At the day of drug addition and at the end of the culture period the MTT assay was performed. Briefly, MTT was added (0.5 mg ml⁻¹ final concentration) to the wells, incubated for 2 h at 37°C, the medium was removed and the formazan crystals formed were dissolved with 150 μl dimethylsulphoxide containing 0.5% FCS. The absorbance was measured at 540 nm using a Titertek microplate reader (Titertek Multiskan MCC/340, Flow Laboratories, Irvine, Scotland).

Treatment of mice

Colon 26 and Colon 38 are murine colon adenocarcinomas maintained in 2–3 month old female Balb/c and C57Bl/6 mice, respectively. Their sources and growth characteristics have been described previously (Peters et al., 1987b, 1990b). Tumours were transplanted as 1–5 mm³ fragments subcutaneously in the flanks of the animals. Growth of the tumours was determined by caliper measurement (length × width × thickness × 0.5) once to twice a week. Treatment was started when tumour volume was between 50 and 150 mm³ (19 days after transplantation for Colon 38 and 10 days for Colon 26). The volume of the tumours was calculated relative to that of the first day of treatment (day 0). Before treatment mice were randomised in groups of each sex animals. The antitumour effect was evaluated by using the T/C (volume of treated tumours divided by that of control tumours) and the growth delay factor (Peters et al., 1990b): GDF = (TDₐ- TDₚ)/TDₚ, where TDₚ is the tumour doubling time of tumours from untreated mice and TDₚ is that from untreated mice.

Results

In vitro studies

From previous studies it was evident that endogenous nucleosides and bases present in cell culture medium and serum may prevent/rescue the growth-inhibitory effects of Brequinar and may influence the action of 5FU. So, several experiments on the effects of uridine and the interaction of Brequinar and 5FU were performed with dialysed serum. With reverse phase HPLC analysis no uridine (below detection limit of 0.1 μM) could be demonstrated in this serum, while in medium with 5% non-dialysed serum, the uridine concentration was about 1–2 μM. Figure 2 shows the effect of uridine addition to the cell cultures. Even at a low uridine concentration of 5 μM comparable to that in plasma, prevention of the growth-inhibitory effects of Brequinar was observed. This low concentration of exogenous uridine was clearly sufficient to support nucleic acid synthesis for the duration of the culture, even though uridine in the medium decreased. At higher uridine concentrations comparable to those in tissues complete reversal of growth-inhibition was observed until Brequinar concentrations of 20–30 μM. However, above a certain threshold (>50 μM) concentration of Brequinar no reversal could be observed.

For other inhibitors of pyrimidine de novo synthesis it has

![Figure 2](image-url)
been shown that inhibition of the uptake of uridine would enhance their cytotoxicity (Grem et al., 1988). This also holds for Brequinar (Figure 3). A non-toxic concentration of the nucleoside transport-inhibitor dipyridamole enhanced growth inhibition of Brequinar in the absence and presence of l or 10 μM uridine. However, dipyridamole failed to enhance the effect of Brequinar in the presence of 50 μM uridine.

Since uridine can influence the growth-inhibitory effect of both Brequinar and 5FU we investigated this combination in the absence and presence of uridine. 5FU was administered 2 h after Brequinar since we had previously demonstrated that at this time point pyrimidine nucleotides were depleted and DHO-DH was inhibited. Brequinar at 0.3 μM was equitoxic to the combination of 10 μM Brequinar in the presence of 50 μM added uridine (Figure 4). In the absence of uridine the growth-inhibitory effect of Brequinar and 5FU was more than would be expected in case of additive growth-inhibition (47% growth). However, in the presence of uridine only an additive effect was observed, since growth inhibition was comparable to the expected growth-inhibition.

In vivo studies

For in vivo studies several schedules of Brequinar and 5FU were tested. The most effective schedule for administration of Brequinar to tumour-bearing mice was the daily schedule (Braakhuis et al., 1990), while a schedule every 4 days showed some activity in Colon 38 and no activity in Colon 26. 5FU at its MTD (100 mg kg⁻¹ weekly for 4 weeks) was very active against Colon 38, there was only minor activity against Colon 26. For administration of 5FU every 4 days the MTD was 60 mg kg⁻¹. Therefore we compared the weekly and every 4 day schedules. Simultaneous administration of both drugs at their MTD in a weekly schedule resulted in an additive toxicity, as well as the combination at an interval of 1 h (data not shown).

An interval of 4 h, being the time point at which inhibition of DHO-DH by Brequinar is most pronounced (Peters et al., 1990b), was evaluated more in detail at several dosages (Table I). The weekly MTD dose of single agent 5FU (100 mg kg⁻¹) was too toxic in the combination with Brequinar and was lowered to 60 mg kg⁻¹, which resulted in combination with Brequinar in a comparable toxicity as single agent 5FU at 100 mg kg⁻¹. At a weekly schedule Brequinar (at 50 mg kg⁻¹) showed no activity in Colon 38 while the combination of Brequinar with 5FU was slightly more active but also somewhat more toxic than 5FU alone (Table I). At the q4d × 4 schedule Brequinar showed some activity against Colon 38, while 5FU (at 60 mg kg⁻¹) was more active. Although the combination was very active, toxicity was very severe (Table I). However, the same schedule was less toxic in Balb-c mice bearing Colon 26. Brequinar was inactive against this tumour, while 5FU showed some activity at the q4d × 4 schedule. The combination was very active.

Figure 4 Effect of several combinations of Brequinar (DUP) and 5FU on growth of Colon 26 cells in the absence and presence of 50 μM UR. 5FU was added 2 h after Brequinar as indicated by the arrow. Experiments were carried out in uridine depleted serum. When indicated, uridine was present during the whole experiment and added simultaneously with the first drug. Bars represent means ± SE of four separate experiments. The absence of a bar for 10 μM DUP indicates a complete growth inhibition (0% growth is < 0).

Discussion

Biochemical modulation has shown to be an effective way to improve the antitumour effect of 5FU. Enhancement of the activity of 5FU with Brequinar appeared to be feasible, but was complicated by external and endogenous conditions both in vitro and in vivo. Apparently the uridine concentrations determine the response to Brequinar and interfere with the potentiation of 5FU activity. In addition toxicity was difficult to predict and thus to control.

Theoretically Brequinar would be an ideal compound to modulate the effects of 5FU, as the drug is a very potent inhibitor of the pyrimidine de novo nucleotide synthesis (Chen et al., 1986; Peters et al., 1987a). A fundamental requirement for in vitro application of biochemical modulation is a clear-cut demonstration of the biochemical effects, which has been fulfilled in the case of Brequinar (Peters et al., 1987a, 1990b; Schwartzmann et al., 1988). However, physiological concentrations of uridine can already prevent growth-inhibition by Brequinar, but only at concentrations lower than 30 μM Brequinar. Even very high concentrations of uridine can not reverse the cytotoxic effects of Brequinar. Also dipyridamole, a nucleoside transport inhibitor, can reduce the effects of uridine only partly. In contrast, the cytotoxic effects of high concentrations of PALA and pyrazofurin, could be reversed by uridine (Grem et al., 1988). The lack of reversal of cytotoxicity of Brequinar at high.

Figure 3 Effect of 5 μM dipyridamol and additional uridine (UR) (1, 5 and 50 μM) on the growth-inhibition of 1 μM Brequinar (DUP). Experiments were carried out in medium with 5% non-dialysed serum, which contained 1.2 μM endogenous uridine. Brequinar, dipyridamole and uridine were added simultaneously to the cultures. Bars represent means ± SE of three separate experiments.
Table 1  Antitumour effect and toxicity of the combination of Brequinar with 5FU in mice bearing Colon 26 and Colon 38

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg kg(^{-1}))</th>
<th>Schedule</th>
<th>GDF</th>
<th>T/C (%)</th>
<th>Weight loss (%)</th>
<th>ILS/LS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon 26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breq</td>
<td>50</td>
<td>4q4d x 4</td>
<td>0.52(^a)</td>
<td>0.40 (21)(^a)</td>
<td>&lt;5</td>
<td>&gt;40</td>
</tr>
<tr>
<td>5FU(^i)</td>
<td>60</td>
<td>4q4d x 4</td>
<td>2.39(^b)</td>
<td>0.16 (14)(^b)</td>
<td>&lt;5</td>
<td>&gt;40</td>
</tr>
<tr>
<td>B-&gt;F(^i)</td>
<td>4q4d x 4</td>
<td>4.72(^b)</td>
<td>0.02 (14)(^b)</td>
<td>24 (12)(^d)</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Breq</td>
<td>50</td>
<td>4q4d x 4</td>
<td>0.62(^i)</td>
<td>0.52 (17)(^i)</td>
<td>&lt;5</td>
<td>&gt;40</td>
</tr>
<tr>
<td>5FU(^i)</td>
<td>60</td>
<td>4q4d x 4</td>
<td>2.39(^b)</td>
<td>0.17 (17)(^b)</td>
<td>&lt;5</td>
<td>&gt;40</td>
</tr>
<tr>
<td>B-&gt;F(^i)</td>
<td>4q4d x 4</td>
<td>3.27(^b)</td>
<td>0.08 (17)(^b)</td>
<td>7 (17)(^i)</td>
<td>&gt;40</td>
<td></td>
</tr>
</tbody>
</table>

Breq, Brequinar sodium; B->F, Brequinar followed after 4 h by 5FU. T/C, volume of treated tumours divided by that of control tumours; weight loss, maximum loss in percentages of weight at the first day of treatment; within parentheses the day at which these values were calculated. GDF, Growth Delay Factor. ILS, increase in life-span, i.e. median life-span of treated mice divided by that of non-treated mice x 100\(^\circ\) of median life-span of non-treated mice bearing Colon 26 after the first day of treatment was 12 days; that of mice bearing Colon 38 was more than 40 days, when tumour volume exceeded a size of 2,000 mm\(^3\) the experiment was discontinued. For Colon 26 the ILS is given, while for Colon 38 the life-span (LS) is given. Signs behind GDF, T/C and weight loss indicate the level of significance of the difference between tumours of treated animals compared to controls; \(^a\), <0.001; \(^i\), <0.01; \(^d\), <0.05. Signs in the first column after 5FU and B->F indicates the significance levels of the difference between Breq and 5FU and between Breq and 5FU and B->F, respectively.

concentrations and the different shape of the dose response curves, make it very likely that at high concentrations of the drug an additional mechanism of action may be responsible for irreversible cytotoxicity (at least not reversible by uridine). This additional target may be related to the first target (inhibition of the mitochondrial enzyme DHO-DH) leading to a dysfunctioning of the mitochondrial electron transport system. In tissues this target may be affected since 6 h after treatment with 50 mg Brequinar kg\(^{-1}\) the concentration of Brequinar still exceeded 50 \(\mu\)M in tumours and some other tissues (Shen et al., 1988), with accompanying plasma levels of >100 \(\mu\)M.

In principle this knowledge of in vitro interaction may be used for the design of in vivo modulation schedules. So, a relatively low dose of Brequinar which is biochemically active may be used for modulation of 5FU. However, scheduling and dosing of both drugs appeared to be very important, since at a weekly schedule at the MTD of 5FU, the toxicity was too severe. However, the MTD for 5FU for a q4d schedule could be combined with Brequinar. Several parameters may determine the activity of the combination, such as endogenous uridine concentrations. In Colon 38 the uridine concentrations are higher (50–100 \(\mu\)M) than in Colon 26 (about 10 \(\mu\)M) (Peters et al., 1990, 1987b). Plasma concentrations of uridine in mice are about 8.5 \(\mu\)M. The in vitro data have shown that high uridine concentrations will preclude a possible synergistic effect. So, in Colon 38 only an additive antitumour effect was observed, while toxicity was usually enhanced. The latter may also be related to an additional mechanism of action in Colon 38. This tumour is very necrotic and it has been observed for other DHO-DH inhibitors that cytotoxic effects may be enhanced under hypoxic conditions (Löffler, 1980). In contrast, Colon 26 lacks necrosis and a possible synergism is less likely to be precluded by high uridine concentrations, because of the low uridine in this tumour. So, in this tumour a potentiation of the activity of 5FU by modulating its mechanism may be possible. The advantage of Brequinar compared to other inhibitors of pyrimidine de novo synthesis may be the observation that reversal by uridine is limited. It is not clear from these studies how Brequinar would interact with 5FU, but it is very likely that the incorporation of 5FU into RNA would be enhanced, similar to the effect of PALA on 5FU metabolism (Greml et al., 1988). A decrease of pyrimidine deoxyribonucleotides including that of dUMP, would enhance the inhibitory effect of FdUMP on thymidylate synthase.

It may be concluded that the biochemical effects of Brequinar are more complex than initial biochemical studies indicated. An additional mechanism of action may exist at high concentrations (>50 \(\mu\)M) of Brequinar. At lower concentrations, which can already decrease pyrimidine nucleotides, Brequinar may be able to potentiate the effect of 5FU, depending on endogenous uridine concentrations. However, the timing and dosing of both compounds is very critical.

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