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Original Paper

Combination of Raltitrexed with other Cytotoxic Agents: Rationale and Preclinical Observations

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Agents for use in combination therapy should be effective as monotherapy in the tumour type of interest, have different mechanisms of action or pharmacology, and preferably non-overlapping toxicity profiles. Raltitrexed is effective as monotherapy in a number of tumour types, but it is hoped that combining it with other cytotoxic agents will lead to enhanced efficacy. Raltitrexed and 5-fluorouracil (5-FU) are specific and non-specific inhibitors, respectively, of thymidylate synthase, a critical enzyme in the *de novo* synthesis of DNA. Preclinical studies have indicated that raltitrexed and 5-FU have an incompletely overlapping spectrum of antitumour activity and may have additive or synergistic effects on colon carcinoma cells. These interactions are schedule-dependent (raltitrexed should precede 5-FU). Pre-treatment of colon carcinoma cells with raltitrexed has also been shown to increase intracellular levels of phosphoribosyl pyrophosphate resulting in increased incorporation of 5-FU nucleotides into RNA. Raltitrexed has a different mechanism of action from two other new agents active in colorectal cancer, irinotecan and oxaliplatin, and tumours are therefore not necessarily cross-resistant. Short pre-exposure of colon carcinoma cells to the irinotecan active metabolite, 7-ethyl-10-hydroxy-camptothecin (SN-38), prior to exposure to raltitrexed has consistently resulted in synergistic cell kill, whereas the reverse sequence is antagonistic. Preliminary results indicate that equitoxic doses of raltitrexed and cisplatin, or oxaliplatin, are antagonistic in two colon carcinoma cell lines. However, because there are major difficulties in translating preclinical drug combination results to the clinical setting, these results should be interpreted with caution. © **1999 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

COMBINATION THERAPY is aimed at maximising toxicity to cancer cells whilst minimising the toxicity to normal tissue. Clinically, combination therapy is directed at improving the response rate and outcome whilst maintaining acceptable toxicity. The decision to use particular agents in combination should be made rationally; clinical and preclinical studies, as well as an understanding of the biochemical and pharmacological mechanism of action of the agents and their toxicity profiles, should be taken into consideration.

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Rationale for combining agents

Primarily drugs for use in combination therapy should be effective as single agents in the tumour type of interest, have different mechanisms of action and, ideally, non-overlapping toxicity profiles. Another potential reason for combining agents could be that one agent causes biochemical, or pharmacological, modulation of the other agent. For example, leucovorin (LV) modulates 5-fluorouracil (5-FU) leading to improved response rates compared with 5-FU alone [1]. Preclinical studies are frequently used to search for combinations of agents that have incompletely overlapping spectra of antitumour activity, or that will cause synergistic cytotoxicity.

In vitro *preclinical models for investigating drug combinations*

To determine cytotoxicity *in vitro*, human tumour cell lines are grown as monolayers and drug-induced cytotoxicity is measured using different drug concentrations, exposure times and schedules of administration. For example, cells may undergo short (e.g. 1 or 4 h) or longer-term (24 h or 5 day) simultaneous or sequential exposure to the agents at differing concentrations. Dose-effect curves are generated as a plot of the fraction of unaffected cells versus the drug concentration. The end-point for determination of affected versus unaffected cells may be either a clonogenic or a surrogate one, such as an MTT assay.

The most generally accepted mathematical model used to calculate combined drug effects is the median-effect analysis of Chou and Talalay [2]. The median effect equation is defined as:

$$fa/fu = (D_x/D_m)^m$$

where fa and fu are the fraction of cells affected and unaffected, respectively, by a specific dose, D_x; D_m is the concentration for the median-effect dose (IC_{50}, the concentration required to produce 50% growth inhibition); and m is the coefficient depicting the shape of the dose-effect curve ($m = 1$, > 1 and < 1 indicate hyperbolic, sigmoidal and negative sigmoidal curves, respectively). The plot of the logarithmic form of the above equation yields a straight line where m is the slope and $\log(D_m)$ is the intercept. The interaction between two agents can then be quantified from the following equation.

$$\text{Combination index (CI)} = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$$

where $(D)_1$ and $(D)_2$ are the doses of the first and second drug that result in a specific degree of inhibition when used in combination, whereas $(D_x)_1$ and $(D_x)_2$ are the doses of the individual drugs which give the same degree of inhibition. Combination index (CI) values of < 1, 1 and > 1 indicate synergy, additivity and antagonism, respectively.

It is widely believed that an observed synergistic cell kill will, in clinical situations, result in an increased cytotoxicity for tumour cells and thereby lead to improved therapeutic benefit. However, in reality it is extremely difficult to translate the results of preclinical studies into the clinical situation, as the *in vivo* situation is far more complex. *In vitro* assays will only reveal mechanistic interactions, e.g. cell cycle arrest, enhanced metabolism to cytotoxic species or stabilisation of enzyme inhibition, whereas *in vivo* interactions may additionally modify the pharmacokinetics or metabolism of one or both drugs. The tumour cells used in *in vitro* studies behave very differently from tumours *in situ* as they are monoclonal and grown under specific conditions, in which they may be exposed to unnatural levels of nutrients that may modify drug activity. In addition, it is very difficult to represent the time versus concentration effects of drug pharmacokinetics. Furthermore, the use of one or two monoclonal cell lines will not be fully representative of the tumour type under investigation. Perhaps the most significant complication *in vivo* is the cytotoxic effect of the combination on normal tissues so that *in vitro* studies will give no guide to therapeutic index. Indeed, it may not be important whether the interaction is synergistic, additive or antagonistic provided that the effect on relevant normal tissues is different i.e. less synergistic or more antagonistic. Nevertheless, results of *in vitro* drug combination studies may provide some guidance, particularly if the mechanisms of interactions are understood.

In vivo *preclinical models for investigating drug combinations*

In vivo tumour models do provide the preclinical scientist with a better system for evaluating drugs in combination. However, problems do still exist, notably for the thymidylate synthase (TS) inhibitors. Rodents have unusually high levels of plasma thymidine (dThd), which circumvents TS inhibition through the dThd salvage pathway in proliferating tissues and tumours when TS inhibitors are given infrequently [3, 4]. This means that relatively high and frequent doses of TS inhibitors are required in rodents compared with those required in clinical settings. Furthermore, the clearance of drugs such as raltitrexed and irinotecan is more rapid in mice [5–7]. Taken together, these species differences present considerable difficulties for translation of results to a clinical setting.

Potential raltitrexed combination therapies

Raltitrexed has been shown to have: clinical activity as monotherapy in a number of tumour types, in particular colorectal cancer; a favourable toxicity profile; and can be used in a convenient three-weekly schedule [8, 9]. It is an antifolate that uses the reduced-folate carrier, a folate transporter, to enter the cell and is then metabolised by the enzyme folypolyglutamate synthetase (FPGS) to polyglutamate forms [10]. These polyglutamate forms are retained within the cells, accounting for relatively slow clearance of raltitrexed from tissues, which enables the infrequent three-weekly dosing regimen to be used [4]. A cascade of events is initiated following the polyglutamation of raltitrexed, involving inhibition of TS, a critical enzyme in the *de novo* synthesis of DNA. TS catalyses the methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (dTMP), which is phosphorylated to the triphosphate form (dTTP), an essential component for DNA replication and repair (see Figure 1). The rationale for combining raltitrexed with a number of other cytotoxic agents is discussed below.

RALTITREXED IN COMBINATION WITH CYTOTOXIC AGENTS

Raltitrexed + 5-FU

5-FU is metabolised to a number of different fluorinated intermediates, one of which 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP), binds to the pyrimidine binding site of TS [11]. In the presence of 5,10-methylenetetrahydrofolate, FdUMP forms a stable covalent complex with TS which leads to depletion of dTTP, thereby reducing the rate of DNA synthesis. There is also significant incorporation of the fluorinated base, 5-fluorouridine-5′-triphosphate (FUTP), into RNA and this is associated with some of the antitumour activity and toxicity of the drug [11]. Therefore, despite both raltitrexed and 5-FU being inhibitors of TS, they do so via different mechanisms (Figure 1), occupy different binding sites and have different specificities for the enzyme. Furthermore, particularly *in vivo*, 5-FU is a substrate for a very active pyrimidine catabolic pathway [11]. Thus, 5-FU and raltitrexed are two pharmacologically distinct drugs and consequently have an incompletely overlapping spectrum of antitumour activity against colorectal cancer cell lines

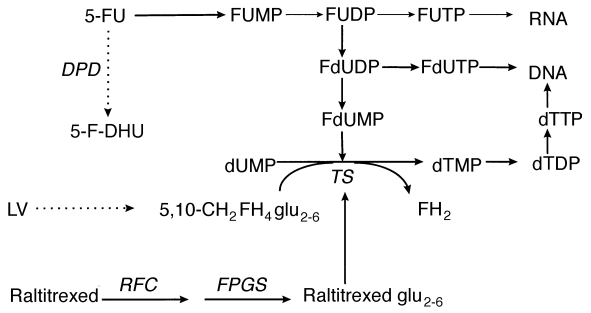

Figure 1. Mechanism of action of raltitrexed and 5-fluorouracil (5-FU). DPD, dihydropyridimine dehydrogenase; 5-F-DHU, dihydrofluorouracil; FUMP, 5-fluorouridine-5′-monophosphate; FUDP, 5-fluorouridine-5′-diphosphate; FUTP, 5-fluorouridine-5′-triphosphate; FdUDP, 5-fluoro-2′-deoxyuridine-5′-diphosphate; FdUTP, 5-fluoro-2′-deoxyuridine-5′-triphosphate; FdUMP, 5′-fluoro-2′-deoxyuridine-5′-monophosphate; dUMP, 2′-deoxyuridine-5′-monophosphate; TS, thymidylate synthase; dTDP, thymidine-5′-diphosphate; dTMP, thymidine-5′-monophosphate; dTTP, thymidine-5′-triphosphate; LV, leucovorin; 5,10-CH_2FH_4 $glu_{2–6}$, polyglutamated methylenetetrahydrofolate; FH_2, dihydrofolate; RFC, reduced folate carrier; FPGS, folypolyglutamate synthetase; raltitrexed $glu_{2–6}$, polyglutamated raltitrexed species.

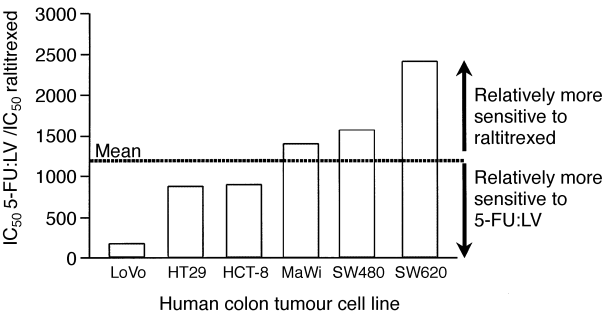

Figure 2. Relative sensitivity of human tumour cell lines to 5-fluorouracil/leucovorin and raltitrexed. Cells were grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum. Incubation was continuous with either 5-fluorouracil (5-FU) and 10 μM leucovorin (LV), or with raltitrexed. The surrogate end point for growth inhibition/cytotoxicity was an MTT assay after 5 days. Results are the mean of at least three separate experiments.

(Figure 2) [4]. Similarly, raltitrexed has been shown to be active in HT-29-1R and M2-1R cell lines (made resistant to 5-FU by a 1-h exposure) but HT29-24R and M2-24R cell lines (made resistant to 5-FU by a 24-h exposure) were highly cross-resistant [12]. Data emerging from a pharmacogenomic clinical study suggest that raltitrexed has activity in patients with advanced, metastatic colorectal tumours expressing high levels of dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase mRNA expression (data not shown). A separate study has demonstrated that high expression of DPD and thymidine phosphorylase mRNA, and TS, is associated with clinical resistance [13].

Many current standard regimens for advanced colorectal cancer use 5-FU modulated with LV [14–16]. Leucovorin increases the intracellular pool of the 5,10-methylene tetrahydrofolate cofactor, leading to stabilisation of the inactive FdUMP/TS/cofactor complex, and thereby increases 5-FU-mediated TS inhibition [17]. Raltitrexed and LV share the same transporter mechanism for entry into the cell and compete for polyglutamation. This results in decreased raltitrexed polyglutamation and, therefore, decreased cytotoxicity of raltitrexed [4, 10]. Combining raltitrexed simultaneously with 5-FU modulated with LV may, therefore, reduce clinical activity compared with raltitrexed/5-FU regimens without LV.

The partially overlapping biochemistry of raltitrexed and 5-FU suggests a number of potential sites for interactions. For example, Van der Wilt and colleagues [18] have demonstrated that FdUMP and the polyglutamates of raltitrexed can support a stable TS inhibitory complex and that this is at least additive. The depletion in dTTP that occurs following TS inhibition by either agent may significantly affect 5-FU action through release of feedback effects on deoxycytidylate deaminase, ribonucleotide reductase and thymidine kinase. *In vitro* drug combination studies have demonstrated that the combination of raltitrexed and 5-FU consistently causes additive or synergistic cell kill in colon carcinoma cells but that this is sequence-dependent. Pre-treatment of HCT-8 colon carcinoma cells with raltitrexed followed by 5-FU was additive/synergistic, particularly when 5-FU exposure time was for 4 h rather than 120 h, whereas the reverse order was antagonistic [19]. Expanded cell line panels have been used by ourselves and others to investigate whether the drug interactions are highly cell-line specific. Van der Wilt and colleagues demonstrated largely additive effects when raltitrexed and 5-FU were combined simultaneously and continuously [20]. Similar results were seen in our cell line panel (Table 1). However, if 5-FU preceded raltitrexed, antagonism was observed in four out of six cell lines. The reverse sequence was largely additive with some indication of synergy in the HT29 cell line. Overall, these results suggest that raltitrexed and 5-FU may have potential as a clinical combination, and that raltitrexed should precede 5-FU in the treatment schedule.

Table 1. *Combination index* values for the combination of raltitrexed and* 5*-FU in six human colon tumour cell lines*

	Cell line					
Regimen†	LoVo	MaWi	SW480	SW620	HCT-8	HT29
24-h continuous raltitrexed and 5-FU	1.0	1.0	1.2	1.1	1.2, 1.1	1.1 (0.04)
5-day continuous raltitrexed and 5-FU	1.1	1.0	1.2, 1.1	1.1	NT	1.0 (0.03)
4-h 5-FU followed by 24-h raltitrexed	1.1	1.0	1.7	1.3	1.7	1.4, 1.4
24-h raltitrexed followed by 4-h 5-FU	1.0	1.0	1.2	1.1	1.1, 1.0	0.87 (0.06)

NT, not tested. *Combination index values of < 1, 1 and > 1 indicate synergy, additivity and antagonism, respectively. Results are given as the mean (standard deviation, S.D.) where $n = 3$ or as individual results. †Raltitrexed and 5-FU were combined at equitoxic doses. Cells were grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% dialysed foetal calf serum. The surrogate end point for growth inhibition/cytotoxicity was an MTT assay after 5 days.

Table 2. Effect of combining raltitrexed and SN-38 in human colon tumour cell lines

Regimen	Aschele and colleagues 1998 [25] HCT-8 cell line	Kimbell and Jackman 1997 [24] Panel of 6 cell lines*
1-h SN-38 followed by 1-h raltitrexed (including a 24-h interval)	Synergistic	NT
4-h simultaneous exposure	Additive/synergistic	NT
4-h SN-38 followed by 4-h raltitrexed	Synergistic	Synergistic 6/6 lines
4-h raltitrexed followed by 4-h SN-38	Additive/synergistic	Additive 1/6 lines Antagonistic 5/6 lines
Simultaneous 24-h exposure	NT	Additive 1/3 lines Antagonistic 2/3 lines
24-h SN-38 followed by 24-h raltitrexed	Additive/antagonistic	NT
24-h raltitrexed followed by 24-h SN-38	Additive/antagonistic	NT
Continuous exposure for 5 days	NT	Antagonistic 5/5 lines
8-h SN-38 followed by simultaneous exposure for 5 days	NT	Antagonistic 5/5 lines
8-h raltitrexed followed by simultaneous exposure for 5 days	NT	Antagonistic 5/5 lines

NT, not tested. *Cell lines as listed in Table 1.

Interestingly, treatment of HCT-8 colon carcinoma cells with raltitrexed has also been shown to increase intracellular levels of phosphoribosyl pyrophosphate (PRPP), resulting in increased formation of 5-FU nucleotides and incorporation into RNA [19]. It is not clear why raltitrexed should cause such an increase in intracellular PRPP and it would be of interest to extend these studies to other colon carcinoma cell lines.

Raltitrexed and irinotecan

Irinotecan, a topoisomerase I inhibitor, is a semi-synthetic camptothecin derivative. It has demonstrated activity in advanced colorectal cancer [21, 22]. Topoisomerases are responsible for the topological control of DNA during its transcription and replication; therefore, inhibition of topoisomerase I results in DNA strand breaks and subsequently cell death [23]. Arrest occurs in the late S/G2 phase of the cell cycle in contrast with early S-phase for raltitrexed.

Irinotecan is a prodrug requiring activation via carboxylesterases to 7-ethyl-10-hydroxy-camptothecin (SN-38), so *in vitro* studies have utilised SN-38. As expected, raltitrexed and SN-38 are not cross-resistant and additionally SN-38 has shown activity in cell lines with acquired resistance to raltitrexed [24]. Schedule-dependent (short pre-exposure to SN-38) synergy has been observed when raltitrexed and SN-38 are combined in a range of colon tumour cell lines (Table 2), whereas the reverse sequence was generally antagonistic [24, 25]. Furthermore, longer exposures or co-exposure of cells to irinotecan and raltitrexed usually resulted in an antagonistic cell kill. It has been hypothesised that this observed antagonism is because raltitrexed reduces the rate of DNA synthesis, which in turn reduces the rate at which the DNA-topoisomerase I-SN-38 cleavable complex is converted into DNA strand breaks [25]. Due to the greater synergism seen when there was a 24 h interval between the SN-38 and raltitrexed [25] and the antagonism seen with simultaneous (including pre-exposure to SN-38) (Table 2) an interval of approximately 24 h is recommended.

Raltitrexed and platinum-based agents

Cisplatin is the longest established platinum-based cytotoxic agent, whereas oxaliplatin is a newer diaminocyclohexane platinum compound which has shown activity in pre-treated 5-FU-resistant colorectal cancer [26]. The use of cisplatin is limited by toxic effects, such as nephrotoxicity, myelosuppression, neurotoxicity, nausea and vomiting. However, oxaliplatin is associated with minimal myelosuppression and reduced nephrotoxicity in comparison with cisplatin [27].

Platinum-based agents largely act by forming intrastrand DNA-platinum adducts/crosslinks between two adjacent guanine:guanine or guanine:adenine base pairs, leading to DNA strand breaks and p53-dependent apoptosis. Excision or mismatch repair processes may repair these breaks provided deoxynucleotides are available. It has been hypothesised that depletion of dTTP following raltitrexed treatment may interfere with this repair process. However, *in vitro* experimental data using two human colon carcinoma cell lines have shown that if equitoxic doses of raltitrexed and cisplatin are employed, antagonism is observed irrespective of schedule (Table 3). Preliminary results of preclinical experiments combining raltitrexed and oxaliplatin in colon carcinoma cells indicate similar effects (data not shown). Generally, additive results with raltitrexed and cisplatin have been observed in ovarian tumour cells which may suggest some tissue-specific differences in response [28, 29]. These interactions need to be explored further in tumour types of different origins.

CONCLUSIONS

One or more of the factors that may be used to establish which cytotoxic agents can be combined comprise activity in tumour type of interest, different biochemistry or pharmacology, non-overlapping normal tissue toxicity and preclinical additivity or synergy. Raltitrexed has demonstrated efficacy in a number of tumour types, including colorectal cancer, and based on its different modes of action with other cytotoxic agents has the potential to be used in combination therapy. Raltitrexed is a more specific inhibitor of TS than 5-FU, which also interferes with RNA synthesis. Furthermore, it has a completely different mechanism of action from irinotecan,

Table 3. Effect of raltitrexed or cisplatin 4-h pre-treatment on combination index values in two human colon tumour cell lines

Cell line	Continuous exposure	4-h pre-treatment with raltitrexed	4-h pre-treatment with cisplatin
SW 620	1.8 (0.37)*	1.5	1.73 (0.29)*
MaWi	2.1 (0.15)*	1.3, 2.1	1.7, 1.6

Combination index values of < 1, 1 and > 1 indicate synergy, additivity and antagonism, respectively. Cells were exposed to equitoxic doses of raltitrexed and cisplatin. Values are given as mean (standard deviation, S.D.) where $n = 3$ or greater or as individual results. *$P < 0.05$ (compared with 1.0).

which is a topoisomerase I inhibitor, or platinum-based agents, which cause DNA adducts and strand breaks.

Drug combinations are widely investigated in a preclinical setting, generally using *in vitro* tumour cell lines. Additive or synergistic cytotoxicity has been observed in human colon carcinoma cell lines when raltitrexed was combined with 5-FU or SN-38. In combinations of raltitrexed and 5-FU, it was advantageous to sequence raltitrexed prior to 5-FU [12, 19, 30]. In the HCT-8 cell line, raltitrexed may be modulating the activity of 5-FU, as it has been shown to increase the intracellular levels of PRPP which leads to increased incorporation of 5-FU nucleotides into RNA. Whether this factor is responsible for the synergistic activity in this cell line, or if this observation extends to other cell lines is unknown. Perhaps more importantly, raltitrexed and 5-FU have different cellular pharmacology: they enter cells and are metabolised by distinct pathways, they occupy different binding sites of the enzyme and have different specificities for TS. There are, therefore, several potential sites for their interaction which may well be cell line or tissue specific. Thus, in the clinical situation, interactions between raltitrexed and 5-FU may be very complex, with potential for differential effects between tumours and normal proliferating tissue effects, and pharmacological interactions.

In studies with the irinotecan metabolite, SN-38, short pre-exposure (between 1 and 4 h) of cells to SN-38 followed by short exposure to raltitrexed resulted in synergy, whereas the reverse sequence, longer exposures or co-exposures were largely antagonistic. Reproducing these lengths of drug exposures in humans may be difficult. Studies with cisplatin or oxaliplatin suggest that this may be an antagonistic combination in colon tumours. Interestingly, ongoing and preliminary studies suggest some potentiation of raltitrexed cytotoxicity with very low concentrations of cisplatin. This result is very interesting and leads to questions concerning the practice of escalating both drugs to the maximum tolerated dose in clinical trials.

In conclusion, preclinical studies can be useful guides for determining non-cross-resistance, additive or synergistic combinations, sequencing preference and investigating mechanistic interactions. However, there are major difficulties in translating results of preclinical combination studies to the clinical situation and hence results should always be interpreted with caution and in a clinical context.

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