Schedule-dependent Synergism between Raltitrexed and Irinotecan in Human Colon Cancer Cells in Vitro

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ABSTRACT

The quinazoline folate analogue raltitrexed (ZD1694; Tomudex) and the camptothecin derivative irinotecan are two new agents showing clinical activity against colorectal cancer. To identify the optimal conditions to achieve synergistic cytotoxicity before the clinical development of their combination, we explored the interactions between ZD1694 and the active metabolite of irinotecan, 7-ethyl-10-hydroxy camptothecin (SN-38), in vitro. Cytotoxicity was evaluated with a clonogenic assay using the human colon cancer cell line HCT-8. Different schedules of administration and different dose ratios of the two agents were compared and evaluated for synergism, additivity, or antagonism with a quantitative method based on the median-effect principle of Chou and Talalay (T. C. Chou and P. Talalay, Adv. Enzyme Regul., 22: 27–55, 1984). Sequential short-term (1 and 4-h) exposures to SN-38 followed by ZD1694 resulted in synergistic cytotoxicity at broad dose-effect ranges. At a high level of cell kill, the synergism was greater when either equiactive doses of the two agents or higher relative doses of ZD1694 were used. A 24-h interval between exposure to SN-38 and ZD1694 significantly enhanced the magnitude of the synergy (P < 0.001). The opposite sequence or simultaneous exposures produced significantly less potentiation or nearly additive interactions (P = 0.0006 and P < 0.0001). The synergism was completely lost under conditions of more prolonged drug exposure (24-h continuous exposure). In conclusion, in this in vitro model of human colon cancer, ZD1694 and SN-38 produced synergistic cytotoxicity. Our findings support the clinical use of this combination and provide a rationale for a clinical trial using sequential short-term exposures to equiactive doses of SN-38 and ZD1694 administered sequentially with a 24-h interval.

INTRODUCTION

FUra and 5-fluorodeoxyuridine have been the only active drugs available to treat colorectal cancer for nearly 40 years. In the last decade, three new agents were developed that proved effective enough in this disease to reach Phase III clinical testing. The first is ZD1694, Tomudex, a quinazoline analogue of folic acid that directly and selectively inhibits TS (1); polypurinylamidation of ZD1694 allows its prolonged intracellular retention and enhances its affinity for TS (2). The second is CPT-11, a semisynthetic camptothecin derivative that inhibits the enzyme DNA topoisomerase I, thereby producing an accumulation of cleavable topopoioisomerase I-DNA complexes, which in turn results in DNA double-strand breaks (3). The third is oxaliplatin, a nonnephrotoxic cisplatin analogue that forms DNA adducts, producing inter- and intrastrand DNA cross-links (4).

Three very large Phase III studies have been completed that show that ZD1694 has overall equivalence with leucovorin-modulated bolus FUra (5, 6). CPT-11 has shown activity as a second-line chemotherapy in patients failing front-line FUra-based treatment (7, 8), and it is now being compared to best supportive care in a large Phase III randomized study in these patients. Oxaliplatin has produced very encouraging results in combination with chronomodulated FUra and leucovorin (9) and may be active in FUra-resistant patients (10), especially when combined with FUra (11).

Phase I and II combination studies of each of these novel agents with FUra are currently in progress (11–13). Registration of ZD1694, CPT-11, and oxaliplatin is still under way in many countries, and combinations of them are about to be launched. Using an in vitro model of human colon carcinoma, we have investigated the interactions between ZD1694 and the active metabolite of CPT-11, SN-38 (14). A quantitative method was used to assess the synergism or antagonism between these two agents and to compare different schedules of administration and different dose ratios in an attempt to provide the preclinical rationale for the optimal clinical development of this combination.
MATERIALS AND METHODS

Chemicals. ZD1694 was provided by Zeneca Pharmaceuticals (Alderley Parck, Macclesfield, United Kingdom). Stock solutions were prepared at 10 mM in 0.1 M sodium bicarbonate (pH 8.3) and stored in aliquots at -20°C. No appreciable loss of activity was detected over 12 months of storage. SN-38 was synthesized in the Preparative Synthesis Core Facility, Memorial Sloan-Kettering Cancer Center (New York, NY) and dissolved at 10 mM in DMSO, and aliquots were kept at -20°C. Further dilutions were made in sterile water immediately before use.

Cytotoxicity Assay. The human colon adenocarcinoma cell line HCT-8 was grown as a monolayer in 25-cm² sterile plastic flasks (Costar, Cambridge, MA) in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% horse serum and subcultured weekly. Under these conditions, the doubling time was 18 h, and the cloning efficiency was approximately 30%.

Drug-induced cell lethality, under different conditions of drug exposure (1 h, 4 h, 24 h, and 7 days), was measured with a monolayer clonal growth technique. Cultures were trypsinized for 3 min, and an essentially monolocular suspension was obtained by passing the trypsinized cells through a 25-gauge needle. Aliquots of 5-10 x 10⁵ cells in 5 ml of medium containing 10% horse serum were dispensed into sterile 60-mm Petri dishes and incubated at 37°C in 100% humidity with 5% CO₂. Eighteen hours later, when the cells were attached to the bottom of the Petri dish but had not yet divided, 0.1 ml of an appropriate dilution of drug in sterile water was added to each dish. Control dishes received the same volume of saline. After incubation as indicated, the medium was decanted, and the cells were washed twice with 5 ml of saline; 5 ml of fresh medium was then added. Clonal growth was determined after the cells were stained with orcein. Colonies containing more than 200 cells were scored at x10 magnification using a dissecting microscope. Under these conditions, more than 95% of the colonies contained more than 200 cells. Each experimental point was determined in triplicate with four replicate controls; all of the experiments were repeated at least three times.

Dose-Response Curves. For each drug, concentration-effect curves were generated as a plot of the fraction of unaffected (surviving) cells versus drug concentration. The ED₅₀ was defined as the drug concentration yielding a fraction of affected (nonsurviving) cells = 0.5, compared with untreated controls. The m coefficient gives information on the shape of the dose-effect plot, with values of 1, >1, and <1 indicating hyperbolic, sigmoidal, and negative sigmoidal curves, respectively. The conformity of the experimental data to the median-effect principle of the mass-action law was estimated in terms of the linear correlation coefficient (r value) of the median-effect plots. In this study, the r values for ZD1694, SN-38, and their combinations were all greater than 0.95.

Drug Combination Studies. To define the best schedule for the combination, either simultaneous or sequential short-term (1 and 4 h) or long-term (24 h, 7 days) exposures to the two agents were tested. The effect of allowing a 24-h incubation in drug-free medium in between the administration of the two drugs was also assessed.

To explore the relative contribution of each agent to the synergism, combinations with different ZD1694/SN-38 molar ratios were compared. For each schedule, three mixtures were tested using equiactive doses of the two agents, higher relative doses of ZD1694 (corresponding to doses of ZD1694 resulting in 70-90% cell kill and doses of SN-38 resulting in 20-30% cell kill), and higher relative doses of SN-38 (corresponding to doses of ZD1694 resulting in 20-30% cell kill and doses of SN-38 resulting in 70-80% cell kill), respectively.

The two drugs are equipotent when given for 4 h. Combinations at a 1:1 ZD1694:SN-38 molar ratio were thus used to test their interactions at equiactive doses. Mixtures at a 1:10 ZD1694:SN-38 molar ratio were prepared to test whether using higher relative doses of ZD1694 or SN-38, respectively, affects their interactions. Given the higher potency of SN-38 compared to ZD1694 for 1-h exposures, mixtures at a 1:1 ZD1694:SN-38 molar ratio were prepared to test equiactive doses. Accordingly, the molar ratios used in the mixtures prepared to assess the effect of using higher relative doses of ZD1694 or SN-38 were 100:1 and 1:1, respectively. For long-term exposures, mixtures were prepared at 1:2, 5:1, and 1:5 ZD1694:SN-38 molar ratios (equiactive doses and higher relative doses of ZD1694 and SN-38, respectively).

Assessment of Synergy. Drug interactions were quantitated using a computer program based on the median-effect equation (15, 16) to calculate combined drug effects. Each drug, or the mixture, was serially diluted 3-fold to 5-fold to generate a dose-effect relationship. The median-effect equation (17) is given by:

\[ \text{CI} = (D_1)/(D_1)_{50} + (D_2)/(D_2)_{50} \]  

\[ (D/D_1)^{\alpha} + (D/D_2)^{\beta} \]  

\[ (D/D_1)^{\beta} + (D/D_2)^{\alpha} \]  

Equation B may thus be solved, providing the iso-effective dose of the mixture, was serially diluted 3-fold to 5-fold to generate a dose-effect relationship. The median-effect equation (17) is given by:

\[ \text{CI} = (D_1)/(D_1)_{50} + (D_2)/(D_2)_{50} \]  

\[ (D/D_1)^{\alpha} + (D/D_2)^{\beta} \]  

\[ (D/D_1)^{\beta} + (D/D_2)^{\alpha} \]  

In equation A, D is the dose of ZD1694, SN-38, or one of their mixtures. D₅₀ is the median-effect dose (e.g., the dose required for 50% inhibition of cell growth, ED₅₀), fₐ is the fraction affected by dose D (e.g., fₐ = 0.7 for 70% inhibition of clonal growth), fₐ is the unaffected fraction (therefore, fₐ = 1 - fₐ), and m is a coefficient related to the shape of the dose-effect curve (m values of 1, >1, and <1 indicate hyperbolic, sigmoidal, and negatively sigmoidal curves, respectively).

Equation A may be rearranged as follows:

\[ D_i = D_{i0}[fa(1 - fa)]^{1/m} \]  

The parameters m and D₅₀ are easily determined by the median-effect plot x = log(D) versus y = log(fa(1 - fa)), which is based on the logarithmic form of equation A and yields a straight line where m is the slope and log(D₅₀) is the x intercept. In our studies, D₅₀ and D₅₀ were determined for each compound (e.g., ED₅₀ for fₐ = 0.7; ED₅₀ for fₐ = 0.9, and so forth).

The interactions between ZD1694 and SN-38 were quantitated by the CI derived from the following equation:

\[ CI = (D_1)/(D_1)_{50} + (D_2)/(D_2)_{50} \]  

CI values of <1, 1, and >1 indicate synergy, additivity, and antagonism, respectively (18). In our studies, (D₅₀) and (D₅₀) are the doses of ZD1694 and SN-38 that result in x% inhibition when used in combination: (D₅₀) and (D₅₀) in the denominators are the doses of ZD1694 and SN-38 alone, respectively, that...
give the same x% of inhibition. D0 can be calculated from equation B, where D is designated for x% inhibition. For CI = 1, equation C represents the classic isobologram equation. A computer program that generates fa-CI tables an fa-CI plots for any specified effect level was used for data analysis. For simplicity, a mutually exclusive assumption has been used that conforms with the classical isobologram equation.

The conformity of the experimental data to the median-effect principle of the mass-action law is automatically provided by the computer printout in terms of the linear correlation coefficient (r value) of the median-effect plots. In this study, the r values for ZD1694, SN-38, and their combinations were all greater than 0.95.

**Statistical Analysis.** The CI profiles (plot of CI at different levels of cell kill) obtained with different schedules of administration were compared using repeated-measure ANOVA with Statistica software (Version 4.1 for Macintosh; Statsoft, Tulsa, OK). All the presented P values are two-sided.

**RESULTS**

**Single-Agent Activity.** Fig. 1A shows the marked time dependency for the activity of ZD1694. The ED50 value after a 1-h exposure to the quinazoline (1.3 ± 0.2 μM) was 100 times higher than that obtained with a 24-h exposure (0.013 ± 0.001 μM); a 15-fold difference in ED50 values was observed between the 1 and 4-h exposures (0.08 ± 0.007 μM). Prolonging the time of exposure for more than 24 h did not substantially increase the activity of the quinazoline; ED50 values after a continuous 10-day exposure (0.004 ± 0.0003 μM) were only three times lower than those after 24-h exposures.

Time dependency was less pronounced for the activity of SN-38 (Fig. 1B), with ED50 values for 1- and 4-h exposures that were similar (0.19 ± 0.02 and 0.07 ± 0.01 μM, respectively) and were only 10–20 times higher than those for long-term exposures (0.01 ± 0.002 μM for 24 h and 0.008 ± 0.003 μM for continuous exposure).

Dose-response curves for ZD1694 were steeper than those for SN-38 (Fig. 1). This is underscored by slopes of the median effect plots (m values) that were approximately twice as high for the quinazoline as compared to SN-38 (m values: 2.26 ± 0.10 versus 0.91 ± 0.08, 2.25 ± 0.70 versus 1.54 ± 0.14, and 3.32 ± 0.20 versus 1.44 ± 0.18 for 4-h, 24-h, and continuous exposures to ZD1694 and SN-38, respectively). In particular, short-term exposures (1 and 4 h) to SN-38 produced a very shallow dose-response curve spanning over 2 logs that is rather unique for antineoplastic agents on this cell line (19).

**Combinations of Short-Term Exposures (Simultaneous ZD1694 + SN-38 versus Sequential ZD1694 → SN-38 versus Sequential SN-38 → ZD1694).** Because both ZD1694 and CPT-11 are administered to patients as short i.v. infusions, combinations of 4-h exposures to the two drugs were studied first.

To define the best schedule for combining the two agents, either simultaneous or sequential ZD1694→SN-38 or SN-38→ZD1694 4-h exposures were tested using combinations at different molar ratios of the two drugs. ZD1694 and SN-38 are equipotent when given for 4 h (ED50 = 0.08 ± 0.007 and 0.07 ± 0.01 μM, respectively). Combinations at a 1:1 molar ratio of ZD1694:SN-38 were thus used to test their interactions at equiactive doses. Mixtures with higher relative doses of ZD1694 (molar ratio of ZD1694:SN38 = 10:1, corresponding to doses of ZD1694 resulting in 70–90% cell kill and doses of SN-38 resulting in 20–30% cell kill) and mixtures with higher relative doses SN-38 (molar ratio of ZD1694:SN38 = 1:10, corresponding to doses of ZD1694 resulting in 20–30% cell kill and doses of SN-38 resulting in 70–80% cell kill) were also tested to explore whether the interactions between the two agents were affected by their dose ratio in the combination.

Fig. 2 illustrates the schedule-dependent synergism of ZD1694 and SN-38. Sequential 4-h exposures to SN-38 followed by ZD1694 (Fig. 2, G–J) resulted in synergistic cytotoxicity at broad dose-effect ranges and at all the dose ratios tested. The magnitude of potentiation was greater when either equiactive doses of the two agents (Fig. 2H, molar ratio of ZD1694: SN-38 = 1:1) or a higher relative dose of ZD1694 (Fig. 2G, molar ratio of ZD1694:SN-38 = 10:1) were used. This greater synergism was particularly prominent at high effect levels. An increase in the relative dose of SN-38 in the combination (Fig. 2I, molar ratio of ZD1694:SN-38 = 1:10) resulted in a lower
degree of synergism and a complete loss of potentiation at effect levels greater than 60%.

Sequential ZD1694→SN-38 4-h exposures (Fig. 2, D–F) and simultaneous 4-h exposures to the two agents (Fig. 2, A–C) produced only a slight-to-moderate synergism or nearly additive interactions, with profiles of CI values significantly higher (less synergism) compared to those for sequential SN-38→ZD1694. Similar to what happened with the sequence SN-38→ZD1694, combinations with higher relative doses of SN-38 (Fig. 2, C and F) resulted in a greater potentiation at low effect levels that was progressively lost at higher levels of cell kill.

Combinations of Long-Term Exposures. Prolongation of drug exposure to 24 h had a detrimental effect on the interactions: nearly additive or antagonistic effects were observed with the two sequences ZD1694→SN-38 and SN-38→ZD1694 (Fig. 3, B and C). Similar to what happened in short-term experiments, using equiactive doses of the two agents (molar ratio of ZD1694:SN-38 = 1:2) or a higher relative dose of ZD1694 (molar ratio of ZD1694:SN-38 = 5:1) produced better results compared to those for higher relative doses of SN-38 (molar ratio of ZD1694:SN-38 = 1:5).

Simultaneous 24-h exposures to the two agents produced clearly antagonistic interactions at all the three dose ratios tested (Fig. 3A). Similarly, less than additive cytotoxicity was obtained (Fig. 3). In conditions of continuous exposure to both drugs (data not shown).

Combinations of Short-Term Exposure with a 24-h Interval between the Two Agents (Sequential SN-38→ZD1694 versus Sequential SN-38→ZD1694). Two other approaches were pursued in the search for the optimal combination: (a) further shortening the time of exposure to the two agents; and (b) allowing a 24-h interval between them. Because protracted exposures to the two agents resulted in strong antagonism, 24-h exposures produced additivity or moderate antagonism, and 4-h exposures resulted in synergism, we evaluated whether further shortening the exposure time could maximize the potentiation. Sequential 1-h exposures to SN-38 followed by ZD1694 produced synergistic interactions (squares
Fig. 3 Sequence-dependent interactions between ZD1694 and SN-38 in HCT-8 cells in vitro (long-term exposures). Plots of the CIs for HCT-8 cells exposed to ZD1694 and SN-38 using different schedules of administration (simultaneous 24-h exposures, A; sequential ZD1694→SN-38 24-h exposures, B; sequential SN-38→ZD1694 24-h exposures, C) and different molar ratios of the two agents (ZD1694: SN-38, 5:1, 1:2, and 1:5 (●, ●, and ○, respectively). The line across the CI value of 1 indicates additivity; CIs above and below indicate antagonism and synergism, respectively. Symbols are the CIs of the actual data points. and dotted lines represent computer-derived CI at effect levels ranging from 10–100% inhibition of clonal growth.

Fig. 4 Influence of the drug interval on the effect of sequential ZD1694→SN-38 short-term exposures on HCT-8 cells in vitro. Plots of the CIs for HCT-8 cells treated with sequential 1-h exposures to SN-38 followed by ZD1694, with and without a 24-h interval between the two agents (△ and □, respectively). Combinations with different molar ratios were tested (100:1, 10:1, and 1:1 in A, B, and C, respectively). The line across the CI value of 1 indicates additivity; CIs above and below indicate antagonism and synergism, respectively. △ and □ are the CIs of the actual data points, and dotted lines represent computer-derived CI at effect levels ranging from 10–100% inhibition of clonal growth. The CI profiles obtained with and without the 24-h interval were compared by the use of repeated-measured ANOVA for comparison of entire curves. The reported P values are two-sided.

In Fig. 4, A–C; however, the magnitude of the potentiation was not greater than that with 4-h exposures.

Given the greater synergism obtained with the sequence SN-38→ZD1694 compared to the opposite sequence or to simultaneous exposures, the possibility of further enhancing potentiation by allowing 24 h of incubation in drug-free medium between the exposures to SN-38 and ZD1694 was explored. Sequential 1-h exposures with SN-38 preceding ZD1694 by a 24-h interval (triangles in Fig. 4, A–C) yielded significantly lower CI values (more synergism) as compared to sequential exposures with one drug backing the other (squares in Fig. 4, A–C). This enhancement was observed at broad effect ranges and at all the dose ratios tested (P = 0.0013, P < 0.0001, and P = 0.0007 with equiactive doses (Fig. 4B), higher relative doses of ZD1694 (Fig. 4A), and higher relative doses of SN-38.
In Vitro Synergism between ZD1694 and CPT-11

In drug combination studies, more than additive interactions were obtained in conditions of short-term exposure, independently of the schedule of administration. The lack of potentiation with prolonged drug exposure may be accounted for by the fully fatal consequences, without residual sublethal damage, that 24 h of treatment with each agent alone may have on sensitive cells. Conversely, the synergy can be explained by either the enhancement of the activity of one agent at a specific target site (TS, topoisomerase I) by the other drug (28) or a less specific potentiation between sublethal damages occurring at different target sites (28). We are presently studying the extent and duration of TS inhibition as well as double-strand DNA breaks, but the potentiation at specific target sites is less likely to occur because a certain degree of synergism is observed with both sequences of administration.

Synergism is clearly schedule dependent. Sequential SN-38→ZD1694 administrations with a 24-h interval between the two agents yielded a greater synergism than sequential back-to-back administrations that were, in turn, superior to the opposite sequence or concomitant administrations. This schedule dependency was also consistent between the three dose ratios tested.

Exposure to low doses of camptothecin has been shown to perturb the progression of HL60 cells through the S phase of the cell cycle (29). Pretreatment with low doses of CPT-11 might thus increase the proportion of cells that are sensitive to the S-phase-specific cytotoxicity of ZD1694. The synergism was in fact more prominent when the relative dose of CPT-11 in the combination was low. With the opposite sequence, ZD1694-induced TS inhibition may slow down the rate of DNA synthesis, reducing the conversion of cleavable complexes into DNA breaks and therefore decreasing the efficacy of the combination.

The ratio between the doses of the two drugs in the combination affects the synergism. The greatest absolute level of synergism was obtained when equiactive doses of the two agents were used. Increasing the relative dose of ZD1694 in the drug mixture did not improve the results, whereas using higher relative doses of SN-38 determined a complete loss of the synergism at high effect levels. This effect was consistent over the several conditions tested in this study. A remarkable similarity between the different schedules was observed in the CI profiles obtained at the three dose ratios tested, with equiactive doses and higher relative doses of ZD1694 consistently yielding CI values that decreased (more synergism) as the fractional cell kill increased, whereas combinations with higher relative doses of SN-38 produced higher CI values (less synergism) as the growth inhibition rose. This observation may have important practical implications, because interactions at high levels of cell kill are more relevant for clinical purposes.

The clinically relevant C×T values used in this study enhance the clinical interest of these findings. The administration of a 15-min ZD1694 infusion at a dose of 3 mg/m² has been shown to result in peak plasma concentrations in the micromolar range with a sustained plateau in the 0.01 μM range (30), and a 30-min infusion of CPT-11 at 100–300 mg/m² has been reported to produce plasma SN-38 concentrations ranging from 0.06–0.01 μM for approximately 24 h, with an area under the concentration curve value (230 ng × h/ml) that is in the same range as the in vitro C×T values of SN-38 obtained in this study (27).
The complex pharmacokinetics of CPT-11 in humans, coupled with the strict time dependence of the synergism observed in this study, suggests that care should be taken when attempting to transfer these results to the clinical setting. The long terminal half-life of SN-38 (27) may result in loss of the synergism in vivo, because we have found that long-term exposures (24 h, 7 days) to ZD1694 and SN-38 produce less-than-additive interactions, independent of the sequence of administration. The large interpatient variability in the CXT profiles of SN-38 after CPT-11 administration (31) and the potential pharmacokinetic interactions between ZD1694 and CPT-11 when they are combined may further increase the heterogeneity in the duration of drug exposure compared to the tissue culture setting. A careful monitoring of the pharmacokinetic behavior of the two agents in initial clinical trials of this combination is therefore recommended. Little help may in fact be expected through the use of animal models, because the CPT-11 terminal half-life is substantially longer in humans (12 h; Ref. 32) compared to either mice (1.1 h; Ref. 14) or rats (1.2–2.4 h; Ref. 33). In addition, the high plasma thymidine levels in mice, with the consequent salvage of ZD1694-induced TS inhibition through the thymidine kinase pathway, make murine models inadequate to test this combination.

The potential gastrointestinal toxicity of these drugs should also be considered. Although the two agents have a different spectrum of toxicity, moderate-to-severe diarrhea is in fact common to both. It is present in 15% of ZD1694-treated patients (34), and it is the dose-limiting toxicity in patients receiving CPT-11 (35). Nevertheless, the time of onset of this side effect is slightly different, and effective treatment with high-dose loperamide (12) may help to overcome this potential problem. The reduction in the dose of the two agents, achievable when they are combined, and the possibility of using a combination with a lower relative dose of CPT-11 raised by these results may also be useful at this purpose.

With these notes of caution, these in vitro studies warrant the clinical testing of the combination ZD1694 + CPT-11 and provide some basic information for the rational design of a clinical protocol using sequential short-term exposures to SN-38→ZD1694 with a 24-h interval between the two agents over other schedules of administration. A suggestion to maintain adequate effective doses of ZD1694 in the combination is also provided.

Additional studies should include in vitro experiments aimed at identifying the mechanisms responsible for this synergism along with a pharmacokinetic evaluation of the interactions between the two agents, attempts at minimizing the potential toxicity, and, possibly, the assessment of intratumoral levels of TS, topoisomerase I, and poly(ADP-ribose) synthetase expression in patients entering the initial clinical trials of this combination.

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