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Synergy between the non-classical thymidylate synthase inhibitor AG337 (Thymitaq®) and cisplatin in human colon and ovarian cancer cells

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AG337 is a recent non-classical thymidylate synthase inhibitor with promising activity and manageable toxicity in phase I clinical trials. In this study, we investigated the cytotoxic activity of AG337 alone and in combination with cisplatin in cultured human colon (HT29) and ovarian (2008) cancer cell lines and their derived counterparts selected for their resistance to 5-fluorouracil (5-FU) (HT29-5-FU) and cisplatin (2008C13). We observed that AG337 had potent cytotoxic effects in colon (IC_{50} = 0.17 μM) and ovarian cancer cells (IC_{50} = 0.65 μM). The cytotoxic activity of AG337 was higher than that of 5-FU in the two models. The activity of AG337 was not significatively affected in 5-FU-resistant HT29-5-FU colon cancer cells characterized by an amplification of the thymidylate synthase gene (IC_{50} = 0.27 μM, p = 0.15). Combinations of cisplatin and AG337 exert synergistic activity in both ovarian and colon cancer cells. Interestingly, this synergism was maintained in 5-FU- and cisplatin-resistant cells. Therefore, our data encourage further examination of combinations of AG337 with cisplatin in cancer chemotherapy.

Key words: Antimetabolites, combination chemotherapy, platinum, quinazoline antifolate.

Introduction

Thymidylate synthase, the terminal enzyme mediating *de novo* formation of thymidylate, is a crucial enzyme for DNA synthesis in tumor cells.[1] Inhibition of thymidylate synthase is the primary mechanism of 5-fluorouracil (5-FU) cytotoxicity and is achieved by the formation of a ternary complex between the enzyme, fluorodeoxyuridine monophosphate (FdUMP), and a 5,10-methylenetetrahydrofolate cofactor.[1] Moreover, the amplification of the thymidylate synthase gene is thought to be an important mechanism of resistance to 5-FU in tumor cells[2] and an important factor of therapeutic failure.[3] Recent knowledge of human thymidylate synthase protein structure and the development of molecular modeling allowed the design and synthesis of new compounds which bind thymidylate synthase with high specificity.[4,5] Classical folinic acid analogs that contain a glutamate residue require facilitated transport across the cell membrane and are generally converted to polyglutamate derivatives inside the cells. Non-classical inhibitors, lacking the terminal glutamate moiety, are more lipophilic, cross rapidly the cell membrane by passive diffusion and are not subject to polyglutamation.[6] Therefore, such compounds could inhibit thymidylate synthase in cells resistant to classical antifolates. Among these drugs, 3,4-dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridylthio)-quinazolone dihydrochloride (AG337) inhibits the active site of human thymidylate synthase, the growth of several human cancer cells and has demonstrated potent antitumor activity against human cancers with acceptable toxicity in phase I trials.[7,8]

Cisplatin remains one of the most efficient agents in cancer chemotherapy. Its cytotoxicity results from the formation of platinum–DNA adducts.[9] An interesting potentiation of cisplatin cytotoxicity by 5-FU is observed in several tumors systems.[10] The proposed mechanism of interaction between cisplatin and 5-FU implies a rise in the folate pool, although the role of thymidylate synthase in this interaction has not yet been clearly established. Therefore, the favorable cytotoxic effects of AG337 in cells resistant to classical antifolate, its mechanism of action and its

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non-overlapping toxicity with cisplatin in clinical trials deserved further analysis, and make cisplatin and AG337 a suitable pair of agents for future clinical combination chemotherapy.

The purpose of our study was to evaluate the cytotoxic interactions between cisplatin and AG337 in human colon and ovarian cancer cells. To parallel clinical situations, we selected the colon HT29 and the ovarian 2008 cancer cell lines, and their derived counterparts resistant, respectively, to 5-FU and to cisplatin. Since protracted or repeated infusions and continuous oral treatment with AG337 seem to induce superior antitumor effects in clinical trials, we decided to expose cells to 48 h exposures. We applied the combination index–isobologram method to analyze the experimental results and to determine synergism or antagonism between drugs. This method allows quantitative determination of synergism occurring at different drug concentrations. This study provides further insight into the modalities of cancer chemotherapy using cisplatin and the non-classical thymidylate synthase inhibitors AG337 in human cancers.

Materials and methods

Drugs

Cisplatin was purchased from Sigma (St Louis, MO). AG337 was provided by Agouron (La Jolla, CA).

Cells

The colon cancer cell line HT29 was obtained from Dr J Fogh (Sloan Kettering Institute for Cancer Research, NY). The HT29-5-FU cell line, obtained from Dr T Lesuffleur (INSERM U178, Villejuif, France), was selected from parental HT29 cells after progressive growth adaptation to 5-FU.[11] The A2008 ovarian cancer cell line and the platinum-resistant subline 2008 C13, selected *in vitro* by stepwise increases of cisplatin concentrations in the medium,[12] were obtained from Dr P Canal (Centre Claudius Regaud, Toulouse, France).

Culture conditions

Colon cancer cells were cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2 in Dulbecco modified Eagle's minimum essential medium (DMEM; Eurobio, Paris, France). Culture medium contained 10% (v/v) heat-inactivated fetal calf serum (FCS; Boehringer, Mannheim, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin and 8 mM glutamine. Ovarian cancer cells were maintained in RPMI 1640 medium containing 10% FCS and antibiotics. The cisplatin-resistant 2008C13 cells were challenged weekly with 50 μM cisplatin for 1 h. The HT29-5-FU cells were maintained in media containing 10 μM of 5-FU. The medium was renewed every 2 days and the cells were passaged once or twice a week by trypsin/EDTA to maintain the cultures in exponential growth.

Cytotoxicity studies

Two days before drug additions, $1.5–2 \times 10^5$ cells were seeded in 35 mm 6-well flasks (Corning, New York, NY) containing 2 ml of growth medium. The cells underwent at least one doubling before drug exposure. Growth rates were determined from day 0 to 4. Cell numbers were determined after trypsinization using a Coulter counter ZM (Coultronics, Luton, UK). Results are given as mean ± SD of three experiments performed in duplicate.

Functional interactions between drugs

The combined drug effects were evaluated by using the Chou and Talalay analysis based on the median-effect principle.[13] This method involves plotting of dose–effect curves for each drug and for multiply diluted, fixed-ratio combinations by using the equation: $f_a/f_u = (C/C_m)^m$, where C is drug concentration, IC_{50} is the concentration required for half-maximal effect (i.e. 50% inhibition of cell growth), f_a is the cell fraction affected by drug concentration C (e.g. 0.9 if the cell growth is inhibited by 90%), f_u is the unaffected fraction and m is the coefficient of sigmoidicity of the concentration–effect curve. Based on the slope of the concentration–effect curves, it can be determined whether the drugs have mutually non-exclusive effects (e.g. independent or interactive mode of action). The combination index (CI) is then determined by the equation:

$$CI = [(C)_1/(C_x)_1] + [(C)_2/(C_x)_2] + [\alpha(C)_1(C)_2/(C_x)_1(C_x)_2],$$

where $(C_x)_1$ is the concentration of drug 1 required to produce x percent effect alone and $(C)_1$ is the concentration of drug 1 required to produce the

same x percent effect in combination with $(C)_2$. If the mode of action of the drugs is mutually exclusive or non-exclusive, then α is 0 or 1, respectively. CI values were calculated by solving the equation for different values of f_a (i.e. different degrees of inhibition of cell growth). CI values below 1 indicate synergy, values equal to 1 indicate additive effects and values above 1 indicate antagonism. Data analysis was performed using the concentration–effect analysis for microcomputer software (Biosoft, Cambridge, UK) on an IBM-PC computer. Statistical analysis and graphs were performed using Instat and Prism software (GraphPad, San Diego, CA). The dose–effect relationships for the drugs tested alone or in paired combinations were subjected to the median-effect plot in order to determine their relative potency (IC_{50}), shape (m) and conformity (r) in each selected cell line. As defined previously, the IC_{50} and m values were used for calculating synergism or antagonism based on the CI equation. Results were given as mean ± SD of three experiments performed in duplicate. In each experiment, cells were exposed to the paired combinations for 48 h. Comparisons of means and standard deviations used Student's t-test (two-side p value).

Results

Single-drug cytotoxicity

The cytotoxic activity of AG337 has been compared to the cytotoxic effect of 5-FU and cisplatin in colon and ovarian cancer cell lines (Figure 1). In the HT29 colon cell line, AG337 shown a potent cytotoxic activity with an IC_{50} of 0.17 ± 0.1 μM as compared to 2.7 ± 1.2 μM for 5-FU ($p = 0.0004$) and 2.8 ± 0.5 μM for cisplatin ($p < 0.0001$). The HT29-5-FU cell line cultured in medium containing 5-FU displayed a stable 5-fold increased resistance to 5-FU. The resistance to 5-FU did not affect the cytotoxic activity of cisplatin in HT29-5-FU cells. Interestingly, the cytotoxic activity of AG337 was not significatively affected by the acquired resistance to 5-FU ($IC_{50} = 0.27 \pm 0.12$ μM, $p = 0.15$).

In the 2008 ovarian cancer cell line, the cytotoxic activity of AG337 ($IC_{50} = 0.65 \pm 0.1$ μM) was higher than that of cisplatin ($IC_{50} = 1.9 \pm 0.9$ μM, $p = 0.007$) and 5-FU ($IC_{50} = 11.75 \pm 1.9$ μM, $p < 0.0001$). The 2008C13-derived cell line, weekly challenged with cisplatin, was characterized by a stable 6- to 7-fold increased resistance to cisplatin

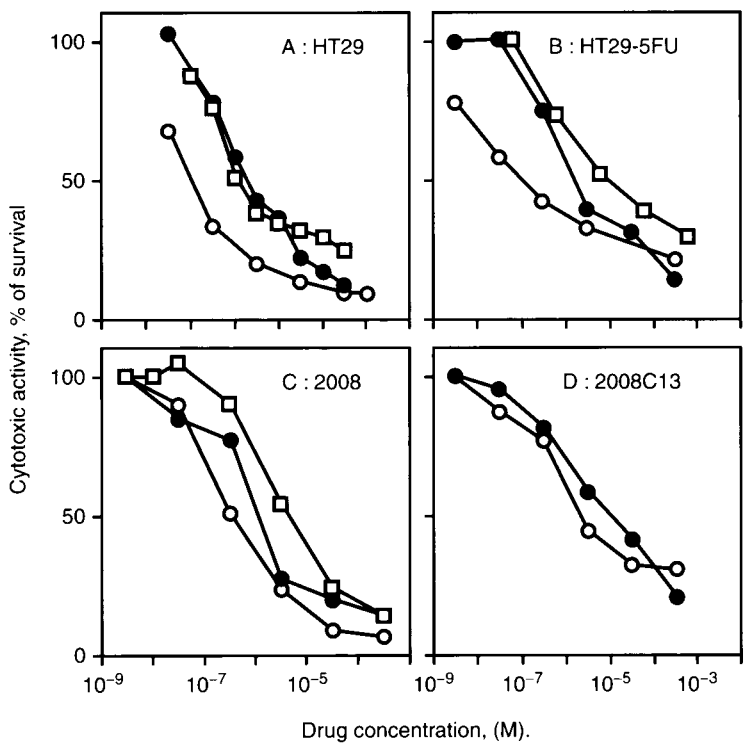

Figure 1. Cytotoxicity of AG337 and cisplatin in colon and ovarian cancers cell lines. The activity of AG337 (○), 5-FU (□) or cisplatin (●) was evaluated after 48 h exposure in the colon HT29 cell line (A), in the 5-FU-resistant HT29-5-FU cells cultured in a media containing 5-FU (B), in the ovarian 2008 cell line (C) and the 2008C13-derived cell line selected for resistance to cisplatin (D).

(IC_{50} = 13 ± 3 μM). Surprisingly, in the 2008C13 cells, resistance to cisplatin led to a significative decrease of AG337 cytotoxicity (IC_{50} = 7.5 ± 2.8 μM, p = 0.0001).

Drug combination studies

The antiproliferative effects of a simultaneous exposure to AG337 and cisplatin was evaluated in parental and 5-FU-resistant colon cancer cells. In the parental HT29 cells, cisplatin very effectively enhanced the cytotoxic activity of AG337 with major synergistic effects for concentrations lower than IC_{75} (Figure 2A). This contrasts with the antagonistic or additive effects previously observed with cisplatin and 5-FU in this cell line (data not shown). Interestingly, except for concentrations below IC_{25}, the synergistic activity between AG337 and cisplatin was maintained in the 5-FU-resistant cancer cells (Figure 2B). These data suggest that cisplatin may interfere positively with the mechanism(s) of resistance to 5-FU and partial resistance to AG337 in this colon cancer cell line.

Subsequently, we investigated the effects of AG337 in combination with cisplatin in the 2008C13 cisplatin-resistant ovarian cancer cells. In this model, it appears that AG337 reinforced the cytotoxic activity of cisplatin, with potent synergistic effects for concentrations above IC_{25} (Figure 2C). Therefore, our data suggest that exposure to AG337 is able to thwart mechanisms of resistance to cisplatin in 2008 cancer cells.

Discussion

Our data demonstrate that AG337 exerts a remarkable cytotoxic activity against human HT29 colon and 2008 ovarian cancer cells. Moreover, we observe that the cytotoxicity of AG337 was not significatively decreased in the HT29 5-FU-resistant cells. The mechanism of resistance to 5-FU in this cell line has been extensively studied and appears to be a consequence of an amplification of the thymidylate synthase gene, leading to an increased activity of thymidylate synthase.[12] In a recent report using the human HT1080 sarcoma cell line continually maintained in the presence of AG337, AG337-resistant subclones have been isolated.[14] The authors suggested that resistance to AG337 could also be mediated by an increase in thymidylate synthase activity and/or by an alteration of the thymidylate synthase. Surprisingly, despite different chemical

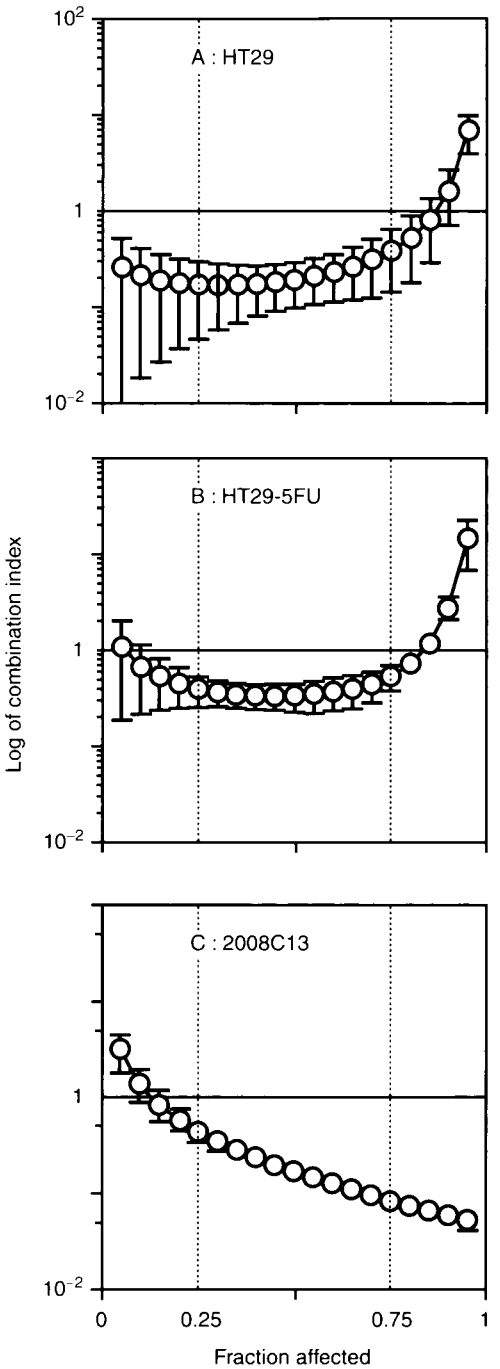

Figure 2. Cytotoxicity of AG337 in combination with cisplatin in human colon and ovarian cancer cells. The cytotoxic activity of combinations was studied using the quantitative method based on the median-effect principle (Chou and Talalay[13]). Drugs were given simultaneously at a 1:1 molar ratio during 48 h. A combination index below 1, 1 or greater than 1 indicates synergy, additivity or non-functional interaction, respectively. Cytotoxic effects of cisplatin-AG337 combinations (○) were evaluated in HT29 (A), in the 5FU-resistant HT29-5-FU (B) and in the cisplatin-resistant 2008C13 ovarian cancer cells (C).

structures and mechanisms of action, both AG337 and cisplatin displayed reduced activity in the 2008C13 cisplatin-resistant ovarian cancer cells. The described mechanisms of resistance to cisplatin in 2008C13 cells are modification of the plasma membrane, glutathione level or alteration of mitochondria.[15,15–16] It is unlikely that these changes could alter AG337 activity. However, previous reports have shown that carcinomas which are resistant to cisplatin are collaterally resistant to antimetabolites because of enhanced dTMP synthase activity.[17] Therefore a similar unexpected mechanism could account for the cytotoxicity profile of AG337 and cisplatin in 2008C13 cisplatin-resistant cells and will deserve further studies.

Subsequent experiments have shown that simultaneous 48 h exposure to AG337 and cisplatin had synergistic activity in colon and ovarian cancer cells. Moreover, in comparison with our previous studies, the interactions between AG337 and cisplatin seem to be more efficient in the HT29 colon cancer cells than that between 5-FU and cisplatin. At the time of this study, there were no data supporting the administration of these drugs in a time sequence. Therefore, it remains possible that the synergy could be further improved by modifications of the schedule.

Interestingly, the positive interaction between AG337 and cisplatin was maintained in cell resistant to 5-FU and cisplatin. This suggests that AG337 may interact positively with the mechanism of resistance to cisplatin and therefore supports further evaluation of the paired combinations of these two drugs in 5-FU- and cisplatin-resistant tumors.

Conclusion

Our data have shown that AG337 exerts a potent cytotoxic activity in colon and ovarian cancer cell lines. A synergistic activity with cisplatin was observed in colon and ovarian cancer cells. This synergism is maintained in 5-FU- and cisplatin-resistant cells *in vitro* and therefore gives a basis for further investigation of the AG337–cisplatin combinations in 5-FU- and cisplatin-resistant tumors.

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