Cancer Chemother Pharmacol (2001) 48: 141–144
DOI 10.1007/s002800000273

ORIGINAL ARTICLE

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Interactions of gemcitabine, carboplatin and paclitaxel in molecularly defined non-small-cell lung cancer cell lines

Received: 10 August 2000 / Accepted: 20 December 2000 / Published online: 6 June 2001

Abstract *Purpose*: To evaluate in vitro interactions of carboplatin, gemcitabine and paclitaxel in molecularly defined non-small-cell lung cancer lines. *Materials and methods*: Three NSCLC lines, A549 ($p16^-$,p53 wt, Rb wt), Calu-1 ($p16^-$, $p53^-$, Rb^+) and H596 (p16 wt, p53 mut, Rb^-) were utilized. Cells were exposed to carboplatin, gemcitabine and paclitaxel as individual drugs and in two- and three-drug combinations with various sequences of administration. Cytotoxicity was assessed with the MTT assay. Interactions between the drugs (additive, synergistic and antagonistic) were evaluated by median effect analysis. *Results*: Gemcitabine and carboplatin were synergistic in all three cell lines. In the A549 line, this synergy was most pronounced when gemcitabine preceded carboplatin. For three-drug combinations, paclitaxel was synergistic with gemcitabine and carboplatin regardless of sequence of administration. *Conclusions*: In vitro modeling of gemcitabine and carboplatin as well as gemcitabine/carboplatin and paclitaxel demonstrates synergistic interaction regardless of p16, p53, or Rb status.

Keywords Carboplatin · Gemcitabine · Paclitaxel · Lung cancer · In vitro

Introduction

Of 150,000 patients who develop non-small-cell lung cancer (NSCLC) in the United States in the next year, at least 40% will present with metastatic disease, and the vast majority of the remainder (>80%) will eventually develop metastases. The introduction of new chemotherapy agents such as gemcitabine, vinorelbine and paclitaxel has improved the outlook for patients with stage IV disease. Recent clinical studies evaluating combinations of platinum agents with "newer" agents (carboplatin/paclitaxel, cisplatin/gemcitabine, cisplatin/vinorelbine) have shown improved median survivals to 8–10 months with 1-year survival of 30–35%, and/or reduced toxicity relative to older combinations [3, 12, 15]. In a recent four-arm trial of chemotherapy regimens, the combination of cisplatin and gemcitabine demonstrated the best results in terms of time to progression [13].

Although these results represent a therapeutic advance, only 10–15% of patients will survive 2 years and there are few long-term survivors. Theoretically, three-drug combinations ("triplets") should be superior to current two-drug regimens. However, it has historically been difficult to demonstrate that the addition of a third drug to a standard two-drug combination improves survival in NSCLC. Therefore, it is important prior to embarking on such trials to determine whether there is a reasonable possibility of success based upon known mechanisms of activity as well as at least the demonstration of favorable preclinical interactions. Synergistic interactions between cisplatin and gemcitabine were anticipated based upon the known mechanisms of activity and have been demonstrated in vitro [2, 14]. Carboplatin, though similar in many respects to cisplatin, has some differences in mechanism and activity and has not been previously evaluated with gemcitabine [8]. Furthermore, the interaction of paclitaxel with this combination in vitro is not known.

Paclitaxel's mechanism of action includes G_2/M phase arrest through prevention of microtubule disassembly as well as phosphorylation and consequent inactivation of the antiapoptotic gene product, bcl-2 [11]. As a consequence of bcl-2 phosphorylation, increased cell death due to the DNA-damaging effects of the gemcitabine/carboplatin combination might be expected. However, prior treatment with paclitaxel might

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result in cell cycle arrest at the G_2/M checkpoint and a consequent reduction of incorporation of gemcitabine and/or carboplatin into DNA, resulting in decreased activity. Thus, this in vitro study was undertaken to explore whether there is evidence of synergy between carboplatin and gemcitabine and whether paclitaxel is synergistic with this combination, and to determine whether sequence of administration is important for these three drugs. In addition, given the known influence of the p53 and Rb gene products upon susceptibility to cell cycle arrest and apoptosis from DNA-damaging and antimicrotubule agents, molecularly defined cell lines were utilized in this study to determine if there was any relationship between activity and potential synergy and the presence or absence of functional p53 and/or Rb genes.

Materials and methods

Cytotoxicity analysis

A modified MTT assay was employed to determine drug cytotoxicity [5]. The cell lines utilized were A549, Calu-1, and H596. The known molecular characteristics of these cell lines are indicated in Table 1. All cell lines were grown in RPMI-1640 supplemented with 5% FCS at 37°C in an atmosphere containing 5% CO_2. Subconfluent cultures were trypsinized and seeded at 5000 cells/well in 96-well plates. Cells were allowed to adhere for 18 h, then exposed to drug dissolved in medium for 24 h, washed and exposed to the second drug. After 24 h the drug-containing medium was removed, the cells washed and drug-free medium added. When control wells were 70–90% confluent, MTT was added, the plates were incubated for 2 h and then the medium was removed, 175 µl DMSO was added, the plates agitated for 15 min and the optical density read at 540 nm. Optical density readings for experiments were compared with previously obtained standard curves (four-parameter analysis) to determine the cell numbers per well (fraction unaffected, Fu). There were five wells per drug concentration. The fraction of cells affected (Fa) was derived from the expression 1–Fu. The 50% inhibitory concentrations for each cell line (IC_{50}) was determined from an initial group of experiments. These values were then utilized to determine the ratio of carboplatin and gemcitabine in subsequent experiments. Single-drug controls were also done with each experiment. Carboplatin and paclitaxel were obtained from Bristol-Myers Squibb (Princeton, N.J.). Gemcitabine was obtained from Lilly Oncology (Indianapolis, Ind.). All experiments were done in duplicate and repeated at least three times. Mean values were then obtained. Thus, each data point represents at least six separate determinations.

Data analysis

The results from all experiments were pooled and mean values obtained for each data point. Median effect analysis using the combination index (CI) was employed to determine whether the drugs interacted synergistically, additively or antagonistically [4].

This method of determining synergy derives from the mass action law and is independent of mechanism. The median effect equation is given by: $Fa/Fu = (D/Dm)m$ where Fa is the fraction affected, Fu is the fraction unaffected, D is the dose, Dm is the median effect dose and m is the coefficient signifying the shape of the dose response curve ($m = 1$ hyperbolic, $m > 1$ sigmoidal and $m < 1$ negatively sigmoidal). The shape of the curve is easily determined from a plot of dose and effect, and rearrangement allows calculation of Dm, also known as the 50% inhibitory concentration, IC_{50}. Median effect analysis determines whether two (or more) agents interact in an additive, superadditive (synergistic) or antagonistic manner.

The CI is defined by the following equation:

$$CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2}$$

in which (D)1 is the dose necessary for a particular effect in the combination, (Dx)1 is the dose of the same drug which will produce the identical level of effect by itself, (D)2 is the dose of a second drug which will produce a particular effect in the combination and (Dx)2 is the dose of the second drug which will produce the same level of effect by itself. For true combinations, a CI of >1 would imply antagonism and a CI of <1 would indicate synergy. It should be noted that that the CI may vary across drug concentrations, so that a particular combination may be antagonistic at one set of concentrations, additive at a second and synergistic at a third. In this eventuality, the clinically significant effect is usually at the higher drug concentrations. Simplifying this analysis is a computer program (Calcusyn, Biosoft) which provides a CI plot and allows easy computation of Dm (IC_{50}). The strength of this analysis is that it allows determination of synergy irrespective of exact mechanism and therefore if synergy is found for a cell line with one set of molecular characteristics, e.g. deleted p53, and not in another then the interaction was due to the molecular difference.

The equation cited above is for mutually exclusive drugs, i.e. those with the same mode of action. A third term, (D)1(D)2/(Dx)1(Dx)2, is added if the assumption is that the drugs are mutually nonexclusive, i.e. have completely different modes of action. The mutually nonexclusive assumption is therefore more rigorous in its definition of synergy and was employed in these studies.

Table 1 Molecular characteristics of cell lines (*wt* wild type, *mut* mutated)

Cell line	p16	p53	Rb
A549	Null	Wt	Wt
Calu1	Null	Null	Wt
H596	Wt	Mut	Null

Results

The results of single-drug experiments are displayed in Table 2. In Fig. 1 the results of two- and three-drug combination experiments are graphically presented. CI values <1 indicate synergistic interaction. Interactions at Fa levels >0.5 are generally the most relevant. In the A549 cell line, all sequences of gemcitabine and carboplatin demonstrated some degree of synergy. The

Table 2 Carboplatin, gemcitabine and paclitaxel single-drug effects (r = correlation coefficient)

Cell line	Drug	IC_{50} (±95%)	r
A549	Carboplatin	27 (20–37) µ*M*	0.98
	Gemcitabine	11 (8–17) n*M*	0.96
	Paclitaxel	1.8 (1.2–2.6) n*M*	0.97
Calu-1	Carboplatin	36 (26–50) µ*M*	0.97
	Gemcitabine	18 (12–26) n*M*	0.96
	Paclitaxel	6 (4–10) n*M*	0.95
H596	Carboplatin	2.9 (1.6–5.2) µ*M*	0.92
	Gemcitabine	10 (8–13) n*M*	0.99
	Paclitaxel	1.4 (1.1–1.8) n*M*	0.99

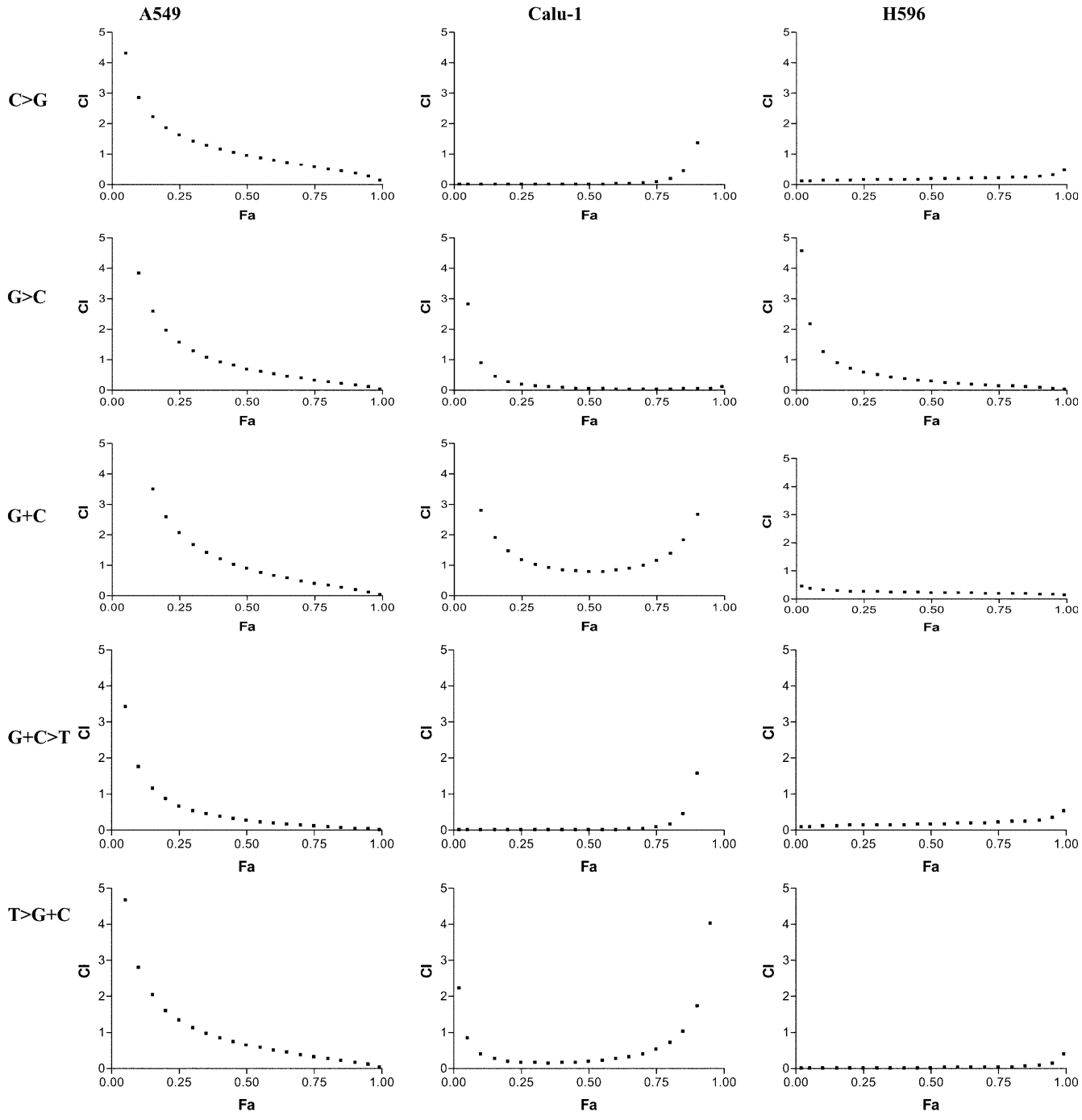

Fig. 1 CI plots: CI < 1 synergism, CI = 1 additivity, CI > 1 antagonism (*C* carboplatin, *G* gemcitabine, *T* paclitaxel; *Fa* fraction affected)

most profound degree of synergy was demonstrated for the sequence of gemcitabine preceding carboplatin and the least for when the two drugs were combined simultaneously. In all cell lines, either gemcitabine and carboplatin preceding the administration of paclitaxel or the reverse sequence was synergistic. Of note, the simultaneous administration of gemcitabine and carboplatin in the Calu-1 line was, at best, additive. In contrast this combination was clearly synergistic in both the A549 and H596 lines. Given the molecular profiles of the different cell lines (Table 1), a possible explanation is that the simultaneous combination requires either an intact p53 pathway or an intact p16 pathway to exert cytotoxicity. However, this explanation would fail to account for the clear synergism when the two drugs were administered sequentially. More likely, concurrent administration in this particular cell line failed to allow optimal interaction of the two drugs to produce DNA damage. Simultaneous administration of all three drugs was not evaluated.

Discussion

Synergistic interactions between cisplatin and antimetabolites such as cytarabine and gemcitabine have been attributed to numerous mechanisms including inhibitory effects of cisplatin on ribonucleotide reductase leading to depletion of dCTP pools, DNA distortions and other mechanisms [1, 7]. This series of experiments demonstrates interactions between carboplatin and gemcitabine similar to those seen for cisplatin and gemcitabine and extends those findings by demonstrating that synergy occurs despite (and to some extent may even be enhanced by) the presence of mutations commonly found in lung cancer. Synergistic interaction of carboplatin and gemcitabine with paclitaxel was also demonstrated and also appeared to be unaffected by the presence of p16, p53 or Rb abnormalities. Although cell lines with defined molecular abnormalities were employed in this study, these were lines derived from different tumors and therefore almost certainly differed in other genes. Future studies employing cell lines which differ in only a single gene are planned.

With the plethora of new agents currently available, preclinical studies may be helpful in designing clinical trials by indicating promising combinations as well as potential antagonistic relationships between drugs. This study provides a strong rationale for the three-drug combination of carboplatin, gemcitabine and paclitaxel. Phase I and II studies employing these three drugs concurrently have recently been reported [9, 10]. A strategy of planned sequential administration of the three drugs, in which the combination of carboplatin and gemcitabine is followed by paclitaxel, has also been reported and was partially based upon this in vitro evaluation [6].

References

1. Abbruzzese JL, Frost P (1982) Studies on the mechanism of the synergistic interaction between 2′-deoxy-5-azacytidine and CDDP. Cancer Chemother Pharmacol 30:31–36
2. Bergman AM, Ruiz van Haperen VWT, Veerman G, Kuiper CM, Peters GJ (1996) Synergistic interaction between cisplatin and gemcitabine in vitro. Clin Cancer Res 2:521–530
3. Bonomi P, Kim K, Chang A, et al (1996) Phase III trial comparing etoposide-cisplatin versus taxol with cisplatin-GCSF versus taxol-cisplatin in advanced non-small cell lung cancer. An Eastern Cooperative Group trial. Proc Am Soc Clin Oncol 15:382
4. Chou TC, Talalay P (1983) Quantitative analysis of dose effect relationship: the combined effects of multiple drugs on enzyme inhibitors. In: Wever G (ed) Advances in enzyme regulation, Pergamon Press, New York, pp 27–55
5. Cole SPC (1986) Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. Cancer Chemother Pharmacol 17:259–263
6. Edelman MJ, Gandara DR, Lau D, Lauder J (2000) Sequential chemotherapy in non-small cell lung cancer: carboplatin and gemcitabine followed by paclitaxel. Proc Am Soc Clin Oncol 19:525a
7. Fram RJ, Robichaud N, Bishov SD, Wilson JM (1987) Interactions of cis-diamminedichloroplatinum(II) with 1-β-D-arabinofuranosylcytosine in LoVo colon carcinoma cells. Cancer Res 47:3360–3365
8. Go RS, Adjei AA (1999) Review of comparative pharmacology and clinical activity of cisplatin and carboplatin. J Clin Oncol 17:409–422
9. Hainsworth JD, Burris III HA, Erland JB, et al (1999) Phase I/II trial of paclitaxel by 1-hour infusion carboplatin, and gemcitabine in the treatment of patients with advanced non-small cell lung carcinoma. Cancer 85:1269–1276
10. Kelly K, Mikhaeel-Kamel N, Pan Z, et al (2000) A phase I/II trial of paclitaxel, carboplatin and gemcitabine in untreated patients with advanced non-small cell lung cancer. Clin Cancer Res 6:3474–3479
11. Rowinsky EK (1997) The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. Annu Rev Med 48:353–374
12. Sandler A, Nemunatis J, Denham C, et al (2000) Phase III trial of gemcitabine plus cisplatin versus cisplatin alone in patients with locally advanced and metastatic non-small cell lung cancer. J Clin Oncol 18:122–130
13. Schiller JH, Harrington D, Sandler A, et al (2000) A randomized phase III trial of four chemotherapy regimens in advanced non-small cell lung cancer. Proc Am Soc Clin Oncol 19:1a
14. Storniolo AM, Allerheiligen SRB, Pearce HL (1997) Preclinical, pharmacologic and phase I studies of gemcitabine. Semin Oncol 24 [2 Suppl 7]:S7-2–S7-7
15. Wozniak AJ, Crowley JJ, Balcerzak S, et al (1992) Randomized trial comparing cisplatin with cisplatin plus vinorelbine in the treatment of advanced non-small cell lung cancer: a cooperative group study. J Clin Oncol 10:1066–1073