ANTICANCER RESEARCH *24*: 2985-2990 (2004)

Does the Sequence of Gemcitabine and Vinorelbine Affect their Efficacy in Non-small Cell Lung Cancer *In Vitro*?

ANTONELLA DE LUCA[1], MICHELE GRASSI[1], MONICA R. MAIELLO[1], MASSIMO DI MAIO[2], MARIO MANCINO[1], ERMELINDA DE MAIO[2], CESARE GRIDELLI[3], FRANCESCO PERRONE[2] and NICOLA NORMANNO[1]

[1]*Department of Experimental Oncology and*
[2]*Clinical Trials Unit, National Cancer Institute, Fondazione Pascale, Naples;*
[3]*Division of Medical Oncology, "S.G. Moscati" Hospital, Avellino, Italy*

Abstract. *Background: We have recently completed a large phase III trial in advanced non-small cell lung cancer (NSCLC) in the elderly which showed that the combination of gemcitabine (GEM) plus vinorelbine (VNR), with GEM administered first, did not improve any outcome as compared with each single drug. Materials and Methods: The anti-tumor efficacy of different sequences of administration of GEM and VNR was investigated in a small panel of NSCLC cell lines, by using an in vitro cytotoxic assay and isobologram analysis. Results: Treatment of lung cancer cells with GEM followed by VNR resulted in moderate synergism in one cell line (A549), and in antagonism in two cell lines (H838 and H1355). However, treatment of NSCLC cells with VNR followed by GEM resulted in antagonism in all cell lines. Conclusion: The results of this study show that the GEM/VNR combination is not superior to both single agents against NSCLC cells, independently of the schedule of administration of the drugs.*

The modest results and the high toxicity of cisplatin-based therapy in patients with advanced non-small cell lung cancer (NSCLC) induced researchers to explore the efficacy of chemotherapeutic regimens containing different anticancer agents. Several new chemotherapeutic agents (paclitaxel, gemcitabine, docetaxel, vinorelbine and irinotecan) have shown good single-agent activity in NSCLC patients. More importantly, pre-clinical and clinical studies have suggested that the combination of gemcitabine (GEM) plus vinorelbine (VNR) might represent an alternative to platinum-based regimens for the treatment of NSCLC patients. In particular, by employing growth delay and isobologram analysis, it has been demonstrated that GEM in combination with VNR produces additive activity with little increased toxicity over a wide range of doses, in the mouse Lewis lung cancer model (1). Furthermore, this non-platinum regimen, when tested in several phase II clinical trials (2-7), showed good tolerability and sufficient clinical activity to deserve phase III testing. A small phase III trial has also shown that, in elderly patients with NSCLC, GEM plus VNR treatment is associated with significantly better survival than is VNR alone (8). However, we have recently completed a large phase III trial in which we compared the combination of GEM and VNR, with GEM administered first, *versus* the two drugs given singly to elderly patients with advanced NSCLC (9). The results of this study showed that the combination did not improve any outcome as compared with each single drug.

A recent phase II trial reported a 72.5% response rate when NSCLC patients were treated with a combination of VNR followed by GEM (10). Therefore, it has been suggested that the sequence VNR/GEM might be more efficient as compared with the GEM/VNR sequence. In order to verify this hypothesis, we investigated the activity of different sequences of the combination of GEM and VNR in a panel of NSCLC cell lines, by using an *in vitro* cytotoxic assays and isobologram analysis.

Materials and Methods

Cell culture. A549 human NSCLC cells were obtained from ATCC. H838 and H1355 human NSCLC cell lines were kindly provided by Dr. A. Budillon (INT-Fondazione Pascale, Naples, Italy). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 mM HEPES, pH 7.4, penicillin (100 U/ml) and streptomycin (100 μg/ml).

Drugs. GEM (Eli Lilly, Sesto Fiorentino, Florence, Italy), VNR (Pierre Fabre, Milan, Italy) and CDDP (Sigma Aldrich, Milan, Italy) were diluted in sterile saline solution and stored at -70˚C. Drug stocks were thawed and diluted in culture medium before each experiment.

Correspondence to: Dr. Nicola Normanno, Department of Experimental Oncology, National Cancer Institute, Fondazione Pascale, Via Mariano Semmola, 80131 Naples, Italy. Tel: 39-081-5903826, Fax: 39-081-7702938, e-mail: nicnorm@yahoo.com

Key Words: Lung cancer, gemcitabine, vinorelbine.

Cytotoxic assay. Cellular growth in the presence or absence of different drugs was evaluated using the previously described tetrazolium-based (MTT) colorimetric assay (11). Briefly, rapidly growing cells were harvested, counted and seeded at appropriate densities (A549 $0.5x10^3$; H838 $1x10^3$; H1355 $2x10^3$) in 96-well microtiter plates. After 24 hours, drugs were applied for 1 hour to quadruplicate culture wells. In combination experiments, the first agent was washed out prior to the introduction of the second drug. After 96 hours, MTT (Sigma Aldrich) was added to microculture wells. Following a 4-hour incubation at 37˚C, DMSO was added to the wells to solubilize the MTT-formazan product. Finally, absorbance at 540 nm was measured with a Biorad Model 550 microplate reader (Biorad, Milan, Italy).

Analysis of combination effect. The effects of combinations of different drugs were evaluated by using the method that was described by Chou and Talalay (12), and the Calcusyn software program (Biosoft, Cambridge, UK) for automated dose-effect analysis. For these experiments, fixed drug ratios were examined, with concentrations of drugs ranging from 0.1 N to 4 N, where N is a value close to the IC_{50} of an individual drug. The Chou and Talalay method is based on the calculation of a Combination Index (CI), with values of CI<0.1 indicating very strong synergism, <0.3 strong synergism, <0.7 synergism, <0.9 moderate to slight synergism, <1.1 nearly additive, <1.45 moderate to slight antagonism, <3.3 antagonism, <10 strong antagonism and >10 very strong antagonism (Calcusyn Software Instruction Brochure). The mutually nonexclusive hypothesis, which is more restrictive, was used to calculate the CI. Since the Chou and Talalay method has been criticized for not being accurate at extreme values (13), we evaluated the CI of the combinations at fractions of cells affected of 50% (*fa*50).

Results

Treatment of A549, H838 and H1355 human NSCLC cell lines with either GEM or VNR for 1 hour resulted in a dose-dependent growth inhibition (Figure 1 and data not shown). A549 cells showed a lower IC_{50} for GEM (8.5±0.2 μM) as compared with the H838 (25±0.7 μM) and H1355 (13±0.3 μM) cell lines. The IC_{50}s of VNR for the H838 and A549 cell lines were quite close (75±4 and 83±4 nM, respectively). In contrast, H1355 cells showed an IC_{50} for VNR that was significantly higher (240±15 nM) as compared with the other NSCLC cell lines.

In drug combinations experiments, cells were exposed for 1 hour to each drug. The first agent was removed and the cells were washed with medium before adding the second drug. By using this technique, treatment with a combination of GEM followed by VNR produced a more significant growth inhibition in A549 cells as compared with treatment with a single agent (Figure 1). Similar results were obtained in H1355 cells, although the efficacy of the combination was similar to VNR alone at high concentrations of the drugs (Figure 1). In H838 cells, the GEM/VNR combination showed an anti-tumor efficacy similar to single agent VNR (Figure 1). Combination analysis performed with the Chou and Talalay method demonstrated that the GEM/VNR combination resulted in a moderate synergism in A549 cells, as suggested by the CI value at *fa*50 of 0.75 (Table I and Figure 1). In contrast, this combination produced a moderate antagonism in H1355 cells (CI *fa*50 1.24) and a clear antagonism in H838 cells (CI *fa*50 1.71) (Table I and Figure 1).

We next determined the efficacy of the sequence VNR followed by GEM in the NSCLC cell lines. The anti-tumor effect of this sequence was similar to GEM alone in A549 cells, whereas it resulted in a lower cytotoxic effect as compared with treatment with any single agent in both H838 and H1355 cells (Figure 1). Combination analysis revealed that this sequence resulted in antagonism in A549 cells (CI *fa*50 2.74) and in strong antagonism in both the H838 and H1355 cell lines (CI *fa*50 6.11 and 4.47, respectively) (Figure 1 and Table I).

Discussion

Combination chemotherapy protocols are mainly designed empirically or on the basis of results derived from the retrospective analysis of clinical trials. In this respect, studies of appropriate animal models, or *in vitro* studies in cancer cell lines, might provide important information on the efficacy of drug combinations (14). However, these studies might sometime provide false-positive results. The GEM/VNR drug combination clearly offers an example of the pitfalls that might occur in pre-clinical studies. In fact, an additive activity of GEM in combination with VNR has been demonstrated in the mouse Lewis lung cancer model (1). However, a large clinical trial conducted by our group has shown that the GEM/VNR combination did not improve any outcome in elderly NSCLC patients as compared with each single drug (9). These results are in agreement with the findings of the present study, which clearly demonstrate that the GEM/VNR combination is not effective in NSCLC cell lines. In fact, this combination was synergistic in one NSCLC cell line and antagonistic in two different NSCLC cell lines. Interestingly, Budman *et al.* (15) found that treatment of MCF7 cells resistant to adriamicin with a combination of GEM and VNR resulted in antagonism, regardless of the sequence of administration of the two drugs. The contrasting results that have been obtained with the GEM/VNR combination in different *in vivo* or *in vitro* models (synergism, additive, antagonism) are clearly due to the high heterogeneity of tumor cell lines with respect to several phenotypic characteristics. This heterogeneity reflects the high variability that exists in and within human primary tumors. Therefore, studies conducted in a single cell line can give false-positive results with high frequency. However, the observation

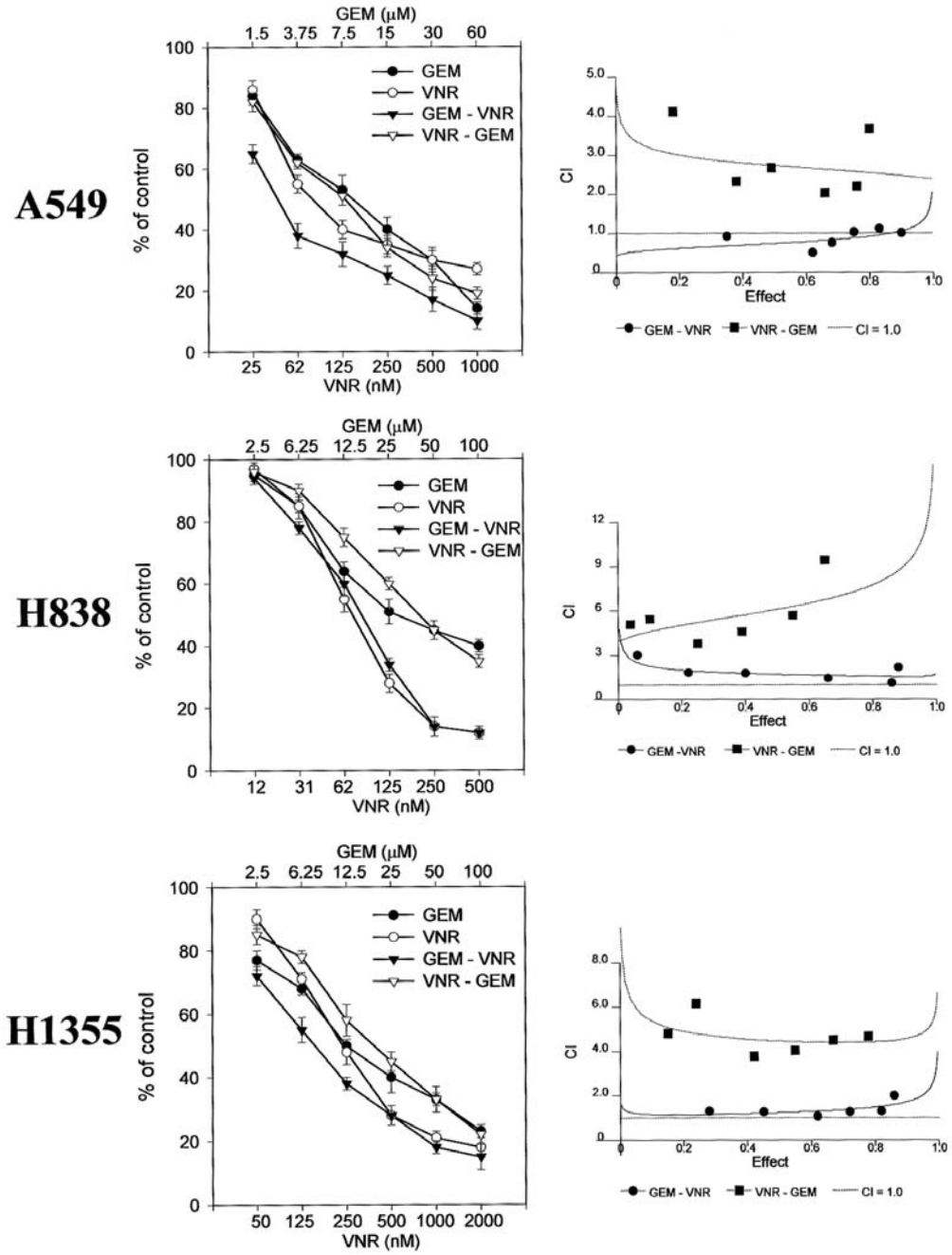

Figure 1. *Left panels: Dose-response curves of A549, H838 and H1355 cells treated with either GEM, VNR, or with the GEM/VNR and the VNR/GEM drug combinations. Right panels: Combination Index (CI) values of the GEM/VNR and VNR/GEM drug combinations, at different fractional effects, as evaluated by the Chou and Talalay method.*

that the GEM/VNR combination was synergistic in one cell line in this study, and additive in the mouse Lewis lung cancer model, suggests that it might be highly active in a subgroup of NSCLC patients. In this regard, additional studies are definitely required to confirm this observation and to establish criteria to select patients who might benefit from such treatment.

The efficacy of drug combinations has been frequently demonstrated to be dependent on the sequence of administration of the different drugs (14,16,17). In this

Table I. *Combination Index (CI) values at fa(50) for drug combinations.*

	CI values		
	A549	H838	H1355
GEM/VNR	0.75±0.09[a]	1.71±0.21	1.24±0.14
VNR/GEM	2.74±0.35	6.11±0.85	4.47±0.51

[a]Standard Deviation

context, patients in our phase III clinical trial were treated with GEM followed by VNR. However, a phase II study of VNR followed by GEM in NSCLC patients reported a very high response rate (10). In our *in vitro* system, treatment with VNR followed by GEM resulted in a reduced efficacy as compared with the inverse schedule in all tumor cell lines that we examined. It is difficult to make a hypothesis on this phenomenon.

GEM is a pyrimidine anti-metabolite and VNR pre-treatment might reduce its efficacy by inducing accumulation of cells in the G2/M-phase of the cell cycle (18). Although we pre-treated cells only for 1 hour with VNR before exposure to GEM, it is still possible that the interaction between these drugs is affected by cell cycle perturbations that might last for a long time following treatment with VNR. GEM is also a pro-drug that requires intracellular conversion by deoxycytidine kinase to its active forms (19). Therefore, we might hypothesize that any effect of VNR on the ability to activate GEM might result in a reduced efficacy of the latter drug. In this regard, both cortisol and dexamethasone have been demonstrated to reduce the efficacy of GEM by decreasing the activity of deoxycytidine kinase (20). However, the observation that the VNR/GEM combination is less active, when compared with both GEM and VNR as single agents, implies that different mechanisms might be involved in the antagonism of this combination.

In conclusion, the results of this study do not support the use of the GEM/VNR combination for the treatment of NSCLC patients, although they suggest that such a combination might be useful in selected patients. Our data also highlight the importance, as well as the potential pitfalls, of pre-clinical studies in the choice of novel drug combinations to be employed in the treatment of cancer patients.

Acknowledgements

This work was supported in part by a grant from the Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.), Italy, to N. Normanno.

References

1 Herbst RS, Lynch C, Vasconcelles M, Teicher BA, Strauss G, Elias A, Anderson I, Zacarola P, Dang NH, Leong T, Salgia R and Skarin AT: Gemcitabine and vinorelbine in patients with advanced lung cancer: preclinical studies and report of a phase I trial. Cancer Chemother Pharmacol *48*: 151-159, 2001.

2 Gridelli C, Frontini L, Perrone F, Gallo C, Gulisano M, Cigolari S, Castiglione F, Robbiati SF, Gasparini G, Ianniello GP, Farris A, Locatelli MC, Felletti R and Piazza E: Gemcitabine plus vinorelbine in advanced non-small cell lung cancer: a phase II study of three different doses. Br J Cancer *83*: 707-714, 2000.

3 Lilenbaum R, Cano R, Schwartz M, Siegel L, Lutzky J, Lewis M, Krill E, Barreras L and Davila E: Gemcitabine and vinorelbine in advanced nonsmall cell lung carcinoma: a phase II study. Cancer *88*: 557-562, 2000.

4 Krajnik G, Mohn-Staudner A, Thaler J, Greil R, Schmeikal S, Marhold F, Deutsch J, Preiss P, Malayeri R, Schafer-Prokop C, Wein W, Huber H and Pirker R: Vinorelbine-gemcitabine in advanced non-small-cell lung cancer (NSCLC): an AASLC phase II trial. Austrian Association for the Study of Lung Cancer. Ann Oncol *11*: 993-998, 2000.

5 Hirsh V, Langleben A, Ayoub J, Cormier Y, Pintos J and Iglesias JL: Flexible chemotherapy regimen with gemcitabine and vinorelbine for metastatic non-small cell lung carcinoma: a phase II multicenter trial. Cancer *92*: 830-835, 2001.

6 Herbst RS, Khuri FR, Lu C, Liu DD, Fossella FV, Glisson BS, Pisters KM, Shin DM, Papadimitrakopoulou VA, Kurie JM, Blumenschein GJ, Kies MS, Zinner R, Jung MS, Lu R, Lee JJ, Munden RF, Hong WK and Lee JS: The novel and effective non-platinum, nontaxane combination of gemcitabine and vinorelbine in advanced non-small cell lung carcinoma: potential for decreased toxicity and combination with biological therapy. Cancer *95*: 340-353, 2002.

7 Laack E, Mende T, Benk J, Chemaissani A, Scholtze J, Lorenz C, Niestroy A, Dalhoff KK, Muller T, Walter T, Durk H, Edler L and Hossfeld DK: Gemcitabine and vinorelbine as first-line chemotherapy for advanced non-small cell lung cancer: a phase II trial. Eur J Cancer *37*: 583-590, 2001.

8 Frasci G, Lorusso V, Panza N, Comella P, Nicolella G, Bianco A, De Cataldis G, Iannelli A, Bilancia D, Belli M, Massidda B, Piantedosi F, Comella G and De Lena M: Gemcitabine plus vinorelbine *versus* vinorelbine alone in elderly patients with advanced non-small-cell lung cancer. J Clin Oncol *18*: 2529-2536, 2000.

9 Gridelli C, Perrone F, Gallo C, Cigolari S, Rossi A, Piantedosi F, Barbera S, Ferrau F, Piazza E, Rosetti F, Clerici M, Bertetto O, Robbiati SF, Frontini L, Sacco C, Castiglione F, Favaretto A, Novello S, Migliorino MR, Gasparini G, Galetta D, Iaffaioli VR and Gebbia V: Chemotherapy for elderly patients with advanced non-small-cell lung cancer: the Multicenter Italian Lung Cancer in the Elderly Study (MILES) phase III randomized trial. J Natl Cancer Inst *95*: 341-343, 2003.

10 Chen YM, Perng RP, Yang KY, Liu TW, Tsai J Ming-Liu CM and Whang-Peng J: A multicenter phase II trial of vinorelbine plus gemcitabine in previously untreated inoperable (stage IIIB/IV) non-small cell lung cancer. Chest *117*: 1583-1589, 2000.

11 Heo DS, Park JG, Hata K, Day R, Herberman RB and Whiteside TL: Evaluation of tetrazolium-based semiautomatic colorimetric assay for measurement of human antitumor cytotoxicity. Cancer Res *50*: 3681-3690, 1990.
12 Chou TC and Talalay P: Quantitative analysis of dose effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul *22*: 27-55, 1984.
13 Greco WR, Bravo G and Parsons JC: The search for synergy: a critical review from a response surface perspective. Pharm Rev *47*: 331-385, 1995.
14 Zoli W, Ricotti L, Tesei A, Barzanti F and Amadori D: *In vitro* preclinical models for a rational design of chemotherapy combinations in human tumors. Crit Rev Oncol Hematol *37*: 69-82, 2001.
15 Budman DR, Calabro A and Kreis W: *In vitro* evaluation of synergism or antagonism with combinations of new cytotoxic agents. Anti-Cancer Drugs *9*: 697-702, 1998.
16 Bajetta E, Stani SC, De Candis D, Zaffaroni N, Zilembo N, Cortinovis D, Aglione S, Mariani L, Formisano B and Bidoli P: Preclinical and clinical evaluation of four gemcitabine plus carboplatin schedules as front-line treatment for stage IV non-small cell lung cancer. Ann Oncol *14*: 242-247, 2003.
17 Ricotti L, Tesi A, De Paola F, Ulivi P, Frassineti GL, Milandri C, Amadori D and Zoli W: *In vitro* schedule-dependent interaction between docetaxel and gemcitabine in human gastric cancer cell lines. Clin Cancer Res *9*: 900-905, 2003.
18 Fukuoka K, Arioka H, Iwamoto Y, Fukumoto H, Kurokawa H, Ishida T, Tomonari A, Suzuki T, Usuda J, Kanzawa F, Saijo N and Nishio K: Mechanism of the radiosensitization induced by vinorelbine in human non-small cell lung cancer cells. Lung Cancer *34*: 451-460, 2001.
19 Huang P, Chubb S, Hertel LW, Grindey GB and Plunkett W: Action of 2',2'-difluoro-2'-deoxycytidineon DNA synthesis. Cancer Res *51*: 6110-6117, 1991.
20 Bergman AM, Pinedo HM and Peters GJ: Steroids affect collateral sensitivity to gemcitabine of multidrug-resistant lung cancer cells. Eur J Pharmacol *416*: 19-24, 2001.

Received April 21, 2004
Accepted July 20, 2004