Dose reduction without loss of efficacy for 5-fluorouracil and cisplatin combined with folinic acid. *In vitro* study on human head and neck carcinoma cell lines

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Summary Folinic acid (FA) and cisplatin (CDDP) both potentiate the cytotoxicity of 5-fluorouracil (5-FU). The activity of various drug combinations including 5-FU, CDDP and FA was tested on two human cell lines derived from squamous cell carcinomas of the head and neck. Cytotoxicity was assessed by the semi-automated colorimetric MTT test. The drugs were tested in clinically achievable conditions (concentrations and duration of exposure). The dose response curves for 5-FU (0–100 ng ml⁻¹) associated with FA (10⁻¹ - 10⁻⁴ M) reflected a progressive increase in 5-FU cytotoxicity with increasing FA concentrations. When CDDP (0–5 μg ml⁻¹) was associated with 5-FU, CDDP-mediated enhancement of 5-FU cytotoxicity was apparent only when CDDP was given before 5-FU. The triple association CDDP, 5-FU and FA was also tested. In this case, for an identical final cytotoxicity, the presence of FA (10⁻¹ M) permitted reduction of the 5-FU concentration between 24.2 and 42% and reduction of the CDDP concentration between 13.8 and 72.7%. These observations may be beneficial for the design of more rational therapeutic trials associating CDDP, 5-FU and FA.

Material and method

Chemicals

5-FU was obtained from ROCHE Laboratories (Neuilly, France), in an injectable form dissolved in H₂O (final concentration 0.385 M). CDDP was obtained from R. Bellon Laboratories (Paris, France), in an injectable form dissolved in 0.9% NaCl (final concentration 1.66 10⁻² M). FA (d,l) was obtained from Sigma (La Verpillière, France), as a powder that was dissolved just before use in H₂O at a final concentration of 10⁻⁴ M. These stock solutions were stored at -20°C. The MTT test (Carmichael et al., 1987) was performed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DMSO, both from SIGMA.

DMEM medium, glutamine and foetal bovine serum (FBS) were purchased from GIBCO (Paisley, GB). Penicillin and streptomycin were from MERIEUX (Lyons, France).

Cell lines CAL 27 and CAL 33 were established in our institute (Gioanni et al., 1988) from squamous cell carcinomas of the head and neck; they were obtained from tumour fragments excised prior to any treatment. The doubling times were measured during exponential growth: 35 h for CAL 27, 43 h for CAL 33.

Experimental conditions

Cells were routinely cultured in a humidified incubator (Sanyo) at 37°C with an atmosphere containing 8% CO₂ in air. Initial cell densities were 2 500 cells per well (96-well plates). Four different types of experiments were performed (Table I): analysis of the specific effects of CDDP; analysis of the association 5-FU-FA (products given simultaneously); analysis of the association 5-FU-CDDP (effect of the order); analysis of the combination CDDP-5-FU-FA; in this last experiment, the preliminary results of the CDDP-5-FU association were known, so CDDP was given prior to the simultaneous association of 5-FU and FA. Experiments were repeated after an interval of 12 months.

Evaluation of cytotoxicity

The cytotoxic effects of the different drug combinations were assessed by the MTT semi-automated test (Carmichael et al.,

5-fluorouracil (5-FU) is increasingly used in the treatment of several types of malignancies, although not because of the efficacy of the drug itself. For example, even though 5-FU is widely used for the management of colorectal adenocarcinoma, it still has a disappointingly low efficacy when administered alone in this pathology (Moetel, 1978; Cohen et al., 1989). The renewed interest is linked to the results obtained with various drug combinations, and in particular 5-FU plus cisplatin (CDDP) for treatment of head and neck cancer (Kish et al., 1982; Amrein & Weitzman, 1985; Thyss et al., 1986), and 5-FU plus folinic acid (FA), which significantly improves the response rate in colorectal carcinoma (Machover et al., 1986; Rustum, 1989; O'Connell, 1989; Arbuck, 1989). The improved results obtained when 5-FU is administered in combination can be explained biochemically: reduced folates enhance the inhibition of thymidilate synthetase (TS) by 5-fluoro-2-deoxyuridine-5'-monophosphate (FdUMP), an active form of 5-FU (Houghton et al., 1982; Kerr, 1989). Although less work has been conducted on 5-FU potentiation by CDDP, a cisplatin-induced increase in intracellular levels of reduced folates may also be the central mechanism in CDDP-5-FU synergism (Scanlon et al., 1986). Tests of the triple association 5-FU-CDDP-FA were a logical consequence; not surprisingly, the first noncontrolled clinical trials have been reported recently. Objective responses have been observed in heavily pretreated patients with breast (Hart et al., 1989; Allegra et al., 1989), gastrointestinal (Leong et al., 1989; Fernandes et al., 1989), and head and neck carcinomas (Vokes et al., 1989). However, severe toxicity has also occurred, and has reportedly caused a number of deaths (Leong et al., 1989). These clinical trials differed considerably as concerns the drug sequences and dosages of 5-FU, CDDP and FA. Experimental data are required to develop rational guidelines for optimizing synchronisation of these three products and for analysis of the relative roles of CDDP and FA when combined with 5-FU. In an attempt to obtain such information, the present experimental study was developed using human cell cultures from head and neck carcinoma.

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1987) after 6–8 days of exposure in 96-well incubating plates. The MTT incubation time was 4 h. Results were expressed as the relative percentage of absorbance compared to controls without drugs. Absorbance was set at 540 nm and measured on a Titertek Twinreader. Each dose point was performed in sextuplicate. IC₅₀ was defined as the drug concentration causing a 50% reduction in growth compared to controls.

Results

Figure 1 shows the dose response curves of cell lines CAL 27 and CAL 33 to CDDP alone (concentration range 0–100 µg ml⁻¹). Both cell lines are sensitive to CDDP, but to different degrees; the IC₅₀ for CAL 27 and CAL 33 was respectively 2.5 µg ml⁻¹ and 15 µg ml⁻¹.

The dose response curves for 5-FU combined with FA are shown on Figure 2 for various FA concentrations (10⁻⁷ to 10⁻⁵ M). 5-FU activity increased progressively for both cell lines as the FA concentration rose. When the FA concentration changed from 0 to 10⁻⁵ M, the IC₅₀ (ng ml⁻¹) for CAL 27 and CAL 33 changed from 27.1 to 9.7 and from 44.4 to 27.1 respectively (Table II).

Figure 3 shows the dose response curves for cell line CAL 27 when 5-FU was combined with CDDP before, during and after exposure to 5-FU. CDDP-mediated enhancement of 5-FU cytotoxicity was apparent only when CDDP was given before or at the same time as 5-FU. It can be seen that the data compare well from the two separate experiments (Figure 3a vs Figure 3b). For each experiment condi

| Conditions       | Drug(s) used | Concentrations | Exposure | Cell line(s) |
|------------------|--------------|----------------|----------|--------------|
| CDDP alone       | CDDP         | 0–100 µg ml⁻¹   | 2 h      | CAL 27, CAL 33 |
| CDDP-FA          | CDDP, FA     | 0, 1, 2, 5 µg ml⁻¹ | 5 days   | CAL 27, CAL 33 |
| CDDP-FA          | CDDP, FA     | 0, 1, 2, 5 µg ml⁻¹ | 5 days   | CAL 27, CAL 33 |

Table 1 Experimental conditions
Table II  IC₅₀ values of 5-FU for head and neck carcinoma cell lines exposed to 5-FU and FA

| Cell lines | no FA | FA 10⁻³ M | FA 10⁻⁶ M | FA 10⁻⁹ M |
|------------|-------|-----------|-----------|-----------|
| CAL 27     | 27.1  | 24.3      | 13.6      | 9.7       |
| CAL 33     | 44.4  | 37        | 36.8      | 27.1      |

IC₅₀ values calculated from data on Figure 2 (sigmoid curve equations).

![Graphs](image)

Figure 3  Combined effects of CDDP and 5-FU on the inhibition of CAL 27 cell growth. a and b are separated experiments done at 12 months interval. Vertical bars = s.d.

Discussion

Most experimental results concerning use of 5-FU/FA associations have been obtained with colorectal carcinoma cell lines (Park et al., 1988), leukaemic cells (Kane et al., 1987; Bertrand & Jolivet, 1989; Keyomarsi & Moran 1986, 1988), and nasopharyngeal epidermoid cells (Kane et al., 1987). This in vitro study confirms and strengthens the findings of these earlier reports by demonstrating that 5-FU activity on human head and neck squamous cell carcinoma cell lines can be potentiated by both CDDP and FA. Careful attention was paid to use of clinically relevant drug concentrations. 5-FU exposure at a dose of 10–100 ng ml⁻¹ for 5 days compares well with the concentrations reported in patients treated by continuous, 5-day infusions (Fraile et al., 1980). Total concentration × time exposure (AUC) of CDDP ranged from 2 to 10 μg hr ml⁻¹; pharmacokinetic studies for patients treated by 2 h infusions indicate AUC values between 1.75 and 8.33 μg hr ml⁻¹ (Reece et al., 1989). Repeated oral administration of leucovorin during 5 days leads to plasma concen-
Figure 5  Combined effects of CDDP, 5-FU and FA on the inhibition of CAL 27 cell growth. a and b are separated experiments done at 12 months interval.

Figure 6  Iso-effect curves at IC₅₀ for the combination CDDP, 5-FU and FA on CAL 27. Data points were obtained by interpolation of cell growth inhibition at 50% on the respective curves shown on Figure 5a. The points were best fit by the equation \( y = ax + b \). For FA = 0, FA = 10⁻⁷ M and FA = 10⁻⁶ M; \( r² \) was respectively 0.9652 \( (P<0.001) \), 0.9720 \( (P<0.001) \), 0.9503 \( (P<0.01) \). The three regression lines were significantly different from one other: nonparametric ANOVA (Friedman test): \( \chi² = 6.00 \) (2df), \( P<0.05 \).

tractions of around 3 \( \mu \)M (Schilsky et al., 1989); in the present study, cells were exposed for 3–5 days to FA concentrations of 10⁻² to 10⁻⁻ M. Keeping in mind the differences between in vitro and in vivo situations, our experimental conditions (drug concentrations and the durations of cell exposure) appear pharmacologically achievable, and permit more satisfactory extrapolation of the present findings to the actual clinical situations.

The experiments were performed in a non-folate depleted medium. Theoretically, use of a folate-depleted medium would have allowed more precise evaluation of the specific role of FA on the activity of 5-FU. However, as stressed by others (Park et al., 1988), such tests could have resulted in overestimation of the effects of FA in comparison to physiological situations in which patients are not folate-deficient. On the other hand, the FA preparation contains an equimolar ratio of the natural (-) and unnatural (+) stereoisomers, and it is generally accepted that only the natural isomer is active. Parenteral administration of FA results in a very different blood behaviour of d/l FA, with a slower elimination phase of the unnatural isomer (Machover et al., 1986). This raises the possibility that the -d FA might interfere with the isomer's enhancement of 5-FU cytotoxicity. This eventuality was not tested in the present study, because earlier reports (Bertrand & Jolivet, 1989) demonstrated that the isomer did not impair 5-FU cytotoxicity enhancement by the l-isomer in similar tissue culture experiments.

Ever since the pioneer work of Dionet and Verrelle (1984), the association of CDDP and 5-FU has proven active in the
treatment of advanced head and neck cancers (Kish et al., 1982; Amrein & Weitzman, 1985; Thyss et al., 1986). However, up until now, there has been a lack of experimental investigations on the sequence-dependence of CDDP and 5-FU in this association. The present data suggest that CDDP administration before 5-FU potentiates the activity of the antimetabolite; simultaneous exposure to the two drugs results in simple additivity of their effects whereas the sequence 5-FU followed by CDDP gives lesser effects than could be expected. Considering that CDDP may promote increased intracellular retention of reduced folates (Scanlon et al., 1986), pre-exposure to CDDP leading to increased 5-FU activity appears pharmaceutically coherent (Houghton et al., 1982). These findings confirm the report by Scanlon et al. (1986) concerning human ovarian carcinoma line A 2780 in culture; for these authors, CDDP followed by 5-FU was more cytotropic than the opposite sequence or either drug used alone. However, these observations are in apparent contradiction with the recent work by Pratesi et al. (1988), who analysed the sequence-dependence of the antitumour effect of a 5-FU-CDDP combination on chemically-induced colon tumours in a murine model. For these authors, 5-FU followed 24 h later by CDDP was the most active sequence. The relevance of their experimental model can be questioned, though, because of 5-FU alone had only marginal activity compared to CDDP, which was more effective.

Consistent clinical data having demonstrated 5-FU poten-
tiation by CDDP (Kish et al., 1982; Amrein & Weitzman, 1985; Thyss et al., 1986; Scanlon et al., 1986) and FA (Machover et al., 1986; Rustum, 1989; O'Connell, 1989; Arbuck, 1989; Houghton et al., 1982), it was tempting to test a combination of all three drugs. Not surprisingly, limited noncontrolled trials of this drug combination have appeared recently (Hart et al., 1989; Allegra et al., 1989; Fernandes et al., 1989; Vokes et al., 1989). To date, little attention has been paid to the sequencing of the three drugs, a fundamental parameter influencing the effectiveness of this multiagent association. Our experimental data obtained with cell cultures of head and neck squamous cell carcinoma may shed some light on this important question. For a constant final cytotoxicity, administration of CDDP followed 6 h later by 5-FU plus FA (10^{-6} M) permitted reduction of the 5-FU dose between 24.2% and 42% or reduction of the CDDP dose between 13.8% and 72.7%. FA thus offers a means of significantly reducing exposure to potentially toxic drugs such as 5-FU and CDDP without loss of efficacy. Although definite benefits in terms of an improved therapeutic index remain to be proven clinically, these observations, obtained with clinically achievable drug concentrations, may allow the design of more rational therapeutic trials combining CDDP, 5-FU, and FA.

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Table III IC_{50} values of 5-FU and CDDP for head and neck carcinoma cell line CAL 27 exposed to CDDP, 5-FU and FA

| CDDP concentration | 5-FU (ng ml^{-1}) | FA 10^{-6} M | FA 10^{-4} M | Reduction* in 5-FU concentration % |
|---------------------|------------------|--------------|--------------|-----------------------------------|
| 1 µg ml^{-1}       | 25               | 18.5         | 14.5         | 42                                |
| 1.65 µg ml^{-1}    | 20.7**           | 17.7**       | 13.8**       | 33.3                              |
| 2.5 µg ml^{-1}     | 16.5             | 16           | 12.5         | 24.2                              |
| 5-FU concentration | CDDP (µg ml^{-1}) | FA 10^{-6} M | FA 10^{-4} M | Reduction* in CDDP concentration % |
| 10 ng ml^{-1}      | 2.9              | 3.2          | 2.5          | 13.8                              |
| 15 ng ml^{-1}      | 2.5              | 2.5          | 1           | 60                                |
| 17.5 ng ml^{-1}    | 2.2              | 1.65         | 0.6**        | 72.7                              |

*Reduction in drug exposure (5-FU or CDDP) allowed by FA, calculated as follows:

\[ \text{IC}_{50} (\text{FA} 10^{-6} M) = \frac{\text{IC}_{50} (\text{no FA})}{1 - \frac{\text{IC}_{50} (\text{FA} 10^{-4} M)}{\text{IC}_{50} (\text{no FA})}} \times 100 \]
DRUG COMBINATIONS INCLUDING 5-FU, CDDP AND FA

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