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# Clonogenic Inactivation of Colon Cancer-derived Cells Treated with 5-Fluorouracil and Indomethacin in Hybrid Spheroids

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The clonogenic hybrid spheroid assay has been used to determine the toxicity of 5-fluorouracil (5-FU), alone or in combination with indomethacin, in LoVo cells (a human colon adenocarcinoma line). The principal finding was that 5-FU toxicity, determined as loss of colony-forming ability, increased as a function of dose (concentration  $\times$  duration of exposure), and that indomethacin causes a generalized alleviation of 5-FU toxicity, but only if given concurrently with 5-FU. The implications of these findings in the control of cancer cells by 5-FU are discussed.

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In situ tumors show a decreased responsiveness to treatment with 5-fluorouracil (5-FU) (1), in comparison to the response of cells in conventional in vitro cultures (2). This discrepancy has led to in vitro data being thought unrepresentative of in situ tumor responses, demonstrating the need for better tumor models (3, 4). An improved tumor model exists in the form of spheroids in culture, where cells are in an in vivo-like three-dimensional contact (5, 6). This system shows a more in vivo-like response to inactivating agents, in that often a higher survival is obtained than in comparable monolayer cultures (7, 8). We have improved the spheroid system by developing hybrid spheroids, which can be considered mini-tumors and are suitable for testing the clonogenicity of tumor cells (9). Using floating hybrid spheroids, we showed that HeLa cells, which had been artificially rendered as contact-inhibited in proliferation by the incorporation of 5-bromodeoxyuridine (BUdR), were resistant to 5-FU. However, after attachment of the cells to a growth surface, this resistance was lost (10), thus demonstrating the importance of three-dimensional cell-to-cell contact as a factor determining chemosensitivity.

One question that is particularly amenable for study in our hybrid spheroid system is whether or not concurrent and sequential treatments with two chemotherapeu-

tic agents are of equal effectiveness. Fludarabine (an adenine nucleoside analogue and inhibitor of chromosomal break repair) and indomethacin (an inhibitor of prostaglandin synthesis) have been found to act synergistically as radiosensitizers in the cure of murine tumors (11). However, without tumor cell clonogenicity measurements it is not clear whether lack of tumor detection in cure assays merely signifies delayed appearance of tumors rather than their eradication. These considerations are of particular importance when two agents with different modes of action are employed. For instance, fluorinated pyrimidines require cell-cycle progression for maximal effectiveness (12), while indomethacin prevents cell-cycle progression and proliferation (13). We have therefore examined the role of timing in the use of 5-FU and indomethacin in our hybrid spheroid system, and measured clonogenic inactivation following treatment with single and/or combinations of agents. Since in our previous study with primary cultures of tumor cells we used 1.5-h treatment periods (10), and it could be argued that the disproportionate resistance to higher concentrations of 5-FU observed with these cells in hybrid spheroids might be due to a (hypothetical) arrest in a drug-unresponsive cell-cycle phase, we used longer treatment periods, i.e., 6.5 and 16 h (at lower concentrations) in this study.

## MATERIAL AND METHODS

### Cell lines, media and maintenance

HeLa S3 cells, maintained over a decade in this laboratory (restarted periodically from cryopreserved stock), were cultured in Eagle's Minimal Essential Medium (MEM) supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml), and with 10% fetal calf serum (FCS), all purchased from GIBCO, Grand Island, NY. LoVo cells, a human adenocarcinoma line (14), were obtained from the Memorial Sloan-Kettering Cancer Center, NY, retrieved as needed from our cryopreserved stock, and maintained in Ham's F-12 medium (GIBCO), also supplemented with antibiotics and 10% FCS.

### Preparation of hybrid spheroids

This procedure has been described in detail elsewhere (9, 10). Briefly,  $5-7 \times 10^6$  HeLa cells containing BUdR and rendered non-proliferating by irradiation (feeder cells), were co-incubated in MEM for 16 h with the LoVo cells to be tested. The ratio of test to feeder cells was 1:30. After incubation of the mixture, agglomerates of various sizes were formed; these agglomerates were passed through a series of filters, and those in the size range 88–105 µm were harvested with a 30 ml volume of Ham's medium (to which LoVo cells are adapted). After counting spheroids in a small aliquot (0.2 ml) of the suspension, the spheroid density was adjusted to about 30/ml in the final volume of Ham's medium. It should be noted that with spheroids in this size range it has been reported that no problems with either penetration or retention of 5-FU have been found (5, 7).

### Treatment of hybrid spheroids with 5-fluorouracil and indomethacin

Aliquots of 9.0 or 9.5 ml hybrid spheroid suspension were transferred into 10-cm bacteriological Petri dishes (Fisher brand) to which spheroids do not attach, to which 0.5 ml of  $20 \times$  solutions of different concentrations of 5-FU (prepared from USP injection solution, SoloPak Laboratories, Franklin Park, IL) and 0.5 ml of a  $20 \times$  solution (sterile filtered, 0.2 µm pore size) of indomethacin (Sigma Chemical Co., St. Louis, MO) to yield 0.4 mM (reported to arrest cultured cells in the  $G_1$  phase (13)) were added, as indicated. After incubation at 37°C for the required time, drug treatment was terminated by spinning and washing the hybrid spheroid suspensions in 40 ml phosphate buffered saline, followed by resuspension of the pellets in 10 ml Ham's medium, of which 5 ml aliquots were plated in duplicate T25 flasks (Corning).

### Assessment of clonogenicity and survival after drug treatment

This was determined as described previously (9, 10). The

main requirement was that some, but not all spheroids formed colonies. In order to gain the benefit of binomial counting statistics when determining the frequency of colony formers among hybrid spheroids, shortly after plating and attachment of spheroids to the growth surface of flasks, a dissecting microscope was used to locate and mark the position of individual spheroids by circling with indelible ink on the bottom of tightly stoppered flasks. Only well-separated spheroids were circled; after a 2-week incubation period at 37°C, the T25 flasks were stained with crystal violet, and the clonogenicity of the test cells in selected spheroids was determined from the fraction of spheroids which did *not* form colonies. This fraction was taken as the zero term of the Poisson distribution of colony formers among spheroids. The effect of a particular treatment on survival was determined from the decrease in clonogenicity.

Survival curve parameters were determined from survival data, using the SigmaPlot (Jandel Scientific, Corte Madera, CA) program, to fit the single-hit, multitarget model. When the effect of different agents at lower doses (leaving high survival levels) was to be compared, the areas under the curves (AUCs), plotted on a linear scale (with a cut-off after the highest common dose used) were calculated for corresponding survival curves using the SigmaScan Pro (Jandel Scientific) program. This parameter (AUC) is analogous (but not identical) to the mean inactivation dose of Fertil et al. (15), and is subsequently used to calculate the enhancement ratio (ER) attributable to the presence of indomethacin in addition to 5-FU. SigmaPlot (Jandel Scientific) was used for graphical representation.

## RESULTS

### Effect of 5-FU on LoVo cells treated concurrently with indomethacin

In order to determine the interaction of indomethacin with 5-FU cytotoxicity, hybrid spheroids containing LoVo cells suspended in Ham's medium were exposed for 6.5 h to graded concentrations of 5-FU, in the presence or absence of 0.4 mM indomethacin. It can be seen from Fig. 1 that while 5-FU at a concentration of 60 µg/ml (the highest common dose) reduces survival of LoVo cells to about 0.1, in the presence of indomethacin the same concentration of 5-FU reduces survival to only 0.4. This four-fold difference is highly significant ( $p < 0.01$ ). At the same time, indomethacin alone did not exert an effect on the clonogenic ability of hybrid spheroids (data not shown).

Another way to assess differences between the two survival curves (and noting that the classical dose modification measurements in which survival curves have a common extrapolation number  $n$  does not apply) is by relating the AUCs, as defined in 'Material and Methods'. This and another ratio obtained from corresponding

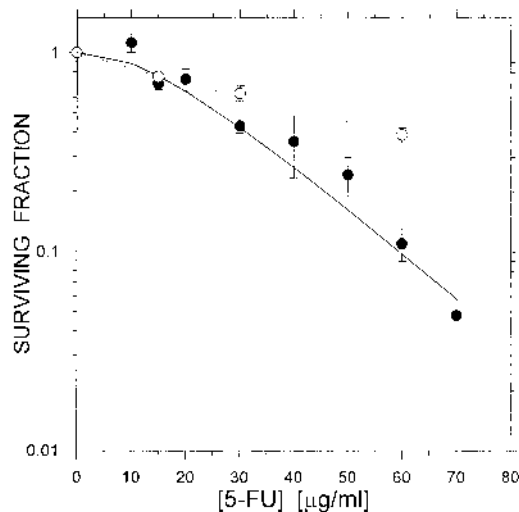


Fig. 1. Survival curves of LoVo cells treated with 5-FU for 6.5 h (filled circles,  $D_0 = 18.9 \pm 1.7$   $\mu\text{g/ml}$  5-FU, extrapolation number ( $n$ ) =  $2.4 \pm 0.5$ ), or for 6.5 h with a combination of 5-FU and indomethacin (empty circles,  $D_0 = 75.2 \pm 5.2$   $\mu\text{g/ml}$  5-FU,  $n = 0.8 \pm 0.04$ ).

paired survival curves are listed below. It should be noted that in all LoVo cell survival curves, no plateau in response to 5-FU was observed, rendering the use of the ratio of AUCs as a gauge of drug interaction acceptable.

With longer (16 h) 5-FU treatment of LoVo cells in hybrid spheroids, survival was decreased (Fig. 2). Taking into consideration the dose of 5-FU as a product of concentration  $\times$  time, the 16-h 5-FU treatment should have been  $16/6.5 = 2.46$  times more effective than the

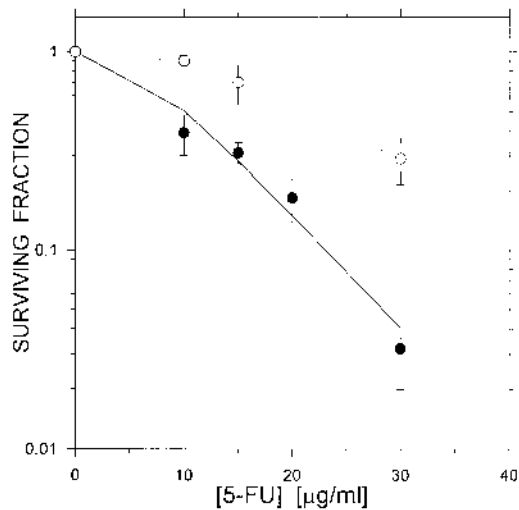


Fig. 2. LoVo cells treated for 16 h with 5-FU without indomethacin (filled circles,  $D_0 = 7.4 \pm 1.1$   $\mu\text{g/ml}$  5-FU,  $n = 2.3 \pm 0.9$ ), or with indomethacin (empty circles,  $D_0 = 12.0 \pm 0.6$   $\mu\text{g/ml}$ ,  $n = 3.9 \pm 0.3$ ).

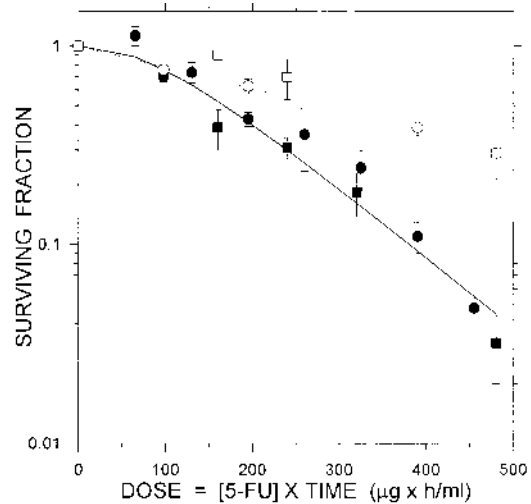


Fig. 3. Superimposed survival curves from Figs. 1 and 2, using a common measure for 5-FU exposure (concentration  $\times$  time in hours). Filled symbols, 5-FU alone ( $D_0 = 120.5 \pm 8.5$   $\mu\text{g} \times \text{h/ml}$  5-FU,  $n = 2.4 \pm 0.5$ ), empty symbols, indomethacin added to 5-FU ( $D_0 = 407 \pm 126$   $\mu\text{g} \times \text{h/ml}$  5-FU,  $n = 1.0 \pm 0.2$ ) (circles, 6.5 h treatment, squares, 16 h treatment).

6.5-h treatment. This value is borne out by replotting the data from Figs. 1 and 2 on a single graph (Fig. 3), using a unified scale of 5-FU concentration times length of exposure on the abscissa. It can be seen from Fig. 3 that 5-FU toxicity data are indeed superimposable when allowance for different exposure durations is made. Survival data with indomethacin are also congruent with a single survival curve, increasing the number of data-points and decreasing the statistical uncertainties involved (see description of Figs. 1–3). Using the data from Fig. 3, replotted on a linear scale, we calculated the AUCs for the two curves, with a cut-off after the highest common doses of 5-FU utilized, with and without indomethacin, and then calculated the enhancement ratio (ER) as the ratio of the two AUCs. Thus the ER for concurrent treatment with 5-FU and indomethacin is  $1.473 \pm 0.006$ .

#### Sequential treatment with 5-FU and indomethacin

When indomethacin was administered not simultaneously, but either (as close as possible within the limits of the experimental procedure) before or after 5-FU treatment (Fig. 4), the ER attributable to indomethacin was much smaller—a mere  $1.109 \pm 0.002$  (although still significantly higher than unity). A single ER was calculated from the two curves with indomethacin, as the individual values for the datapoints on the curve for pre- and post-treatment with indomethacin were not significantly different. Thus simultaneous treatment, rather than pre- or post-treatment, with indomethacin is most effective in alleviating 5-FU toxicity.

## DISCUSSION

One interpretation of the previously observed poor response of primary culture colon cancer cells in hybrid spheroids to 5-FU after a 1.5-h treatment period was that the resistant tumor cells were either in  $G_0$  or arrested in an unresponsive phase of the cell cycle (10). Based on this information, longer treatment times were used in this study, to allow differentiation between traversal and non-traversal through hypothetical unresponsive phases. However, there is no evidence in Figs. 1–3 of a plateau of diminished response with extended treatment time, as would be expected had cells been entering and been arrested in a 5-FU resistant phase of the cell cycle. Also relevant to this point is our observation on the equivalence of 5-FU exposures of different duration when the time was factored in (Fig. 3): no such equivalence would have been expected if the two treatment times had had to contend with non-traversal of a phase of fixed duration. It is therefore concluded that it is unlikely that 5-FU-treated cells (at least of the established immortalized type) are arrested in a cell-cycle unresponsive phase, or are retained in  $G_0$  under these experimental conditions.

Cell-cycle progression could also be invoked in yet another aspect of our investigation: indomethacin is reported to arrest cells in the  $G_1$  phase of the cell cycle (13), and the present finding of increased survival in the presence of indomethacin could be interpreted as showing that cells become less sensitive to 5-FU when they are held back in the  $G_1$  phase. This would be similar to the (sparing) action of aphidicolin (which blocks cells at the  $G_1/S$  interface) on the toxicity of fluorodeoxyuridine in HT29 human colon cancer cells (12). However, our data

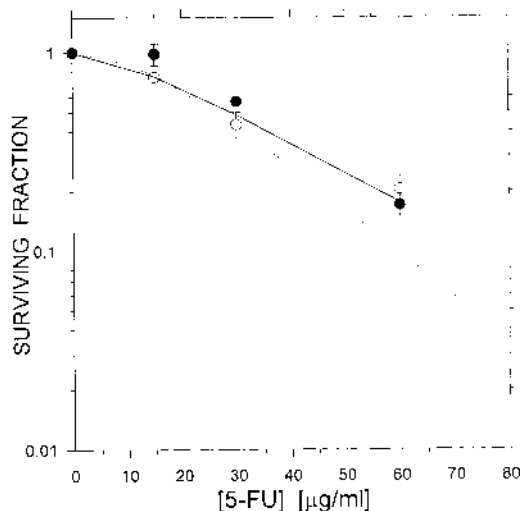


Fig. 4. Survival curves of LoVo cells treated with 5-FU for 6.5 h either after (filled circles), or before (empty circles) a 16-h treatment period with indomethacin. Common for both conditions  $D_0 = 27.2 \pm 2.4 \mu\text{g/ml}$  5-FU,  $n = 1.7 \pm 0.2$ . Dashed line refers to the control curve in Fig. 1.

show that all survival curves (with or without indomethacin) have a form without a resistant plateau, implying that no cell-cycle stage is totally resistant to 5-FU. On the other hand, cell-cycling itself may be deleterious to (subsequent) response to 5-FU, and by slowing cycling, indomethacin may actually spare cells from 5-FU toxicity. Without a technique for monitoring progression of viable cells through the cell cycle when in the presence of metabolically active feeder cells, this problem cannot be resolved at present. Whether cycling or some other process affected by indomethacin interferes with 5-FU toxicity remains to be determined, but an RNA altered by 5-FU is likely to be involved (16). How indomethacin may alleviate a dysfunctional RNA is also not known, but considering the inhibitory effect of indomethacin on prostaglandin synthesis (17), the participation of the latter seems most probable.

Whatever the mechanisms of 5-FU toxicity and the indomethacin rescuing effect might be, it is evident from Figs. 1–3 that concurrent treatment with indomethacin decreases the response of cells to 5-FU, irrespective of the length of treatment. On the other hand, when the two treatments were different in timing, i.e., when pre- or post-5-FU treatment with indomethacin was used, the total response was not very different from that with 5-FU alone (Fig. 4). Bearing in mind that hybrid spheroids allow treatment to be performed in an *in vivo*-like environment, as far as the three-dimensional cell-to-cell contact is concerned, the chief conclusion from the present study is that concurrent treatment with more than one chemical (such as when pain-killers involved with the prostaglandin pathways are used) should be approached with caution. Fortunately, their combined effect can be determined more accurately in our system than in conventional assays and trials.

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