Modulation of the Cytotoxic Effect of 5-Fluorouracil by N-Methylformamide on a Human Colon Carcinoma Cell Line

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ABSTRACT

The cytotoxic effect of the combination of N-methylformamide (NMF) with 5-fluorouracil (5-FU) on cell survival of the human colon cancer line HT29 was assessed. The differentiating activity of NMF was evidenced by morphological maturation and conversion of cell culture characteristics to those consistent with a more benign phenotype. In combination experiments, the noncytotoxic concentration of 1% NMF was chosen and doses of 5-FU ranging from 5 to 25 μg/ml were employed. Two main schedules were tested either on exponentially or stationarily growing cells: (a) 1% NMF for 72 h followed by 12-h exposure to 5-FU; (b) 5-FU for 12 h followed by 72-h exposure to 1% NMF.

The results obtained demonstrated that the 5-FU→NMF sequence determined a powerful reduction in the surviving fraction of HT29 cells, while the reverse sequence did not increase the killing effect of 5-FU given alone. Immunocytochemical and scanning electron microscopy studies seemed to confirm that the association in which the differentiating agent followed the 5-FU treatment strongly impaired cellular integrity and function and that cytoskeletal elements, particularly microfilaments, and surface structures could play an essential role in the mechanisms of cytotoxicity. Furthermore, the results of this work indicate that drug sequence is a critical factor for the optimal combination of 5-FU and NMF.

INTRODUCTION

Colorectal cancer still represents one of the most important challenges in the field of cancer chemotherapy. The results obtained thus far with the combination of potentially active drugs have been very disappointing. The remission rate achieved in the largest series of patients has not been over 35–40%; few of these responses are complete and the median duration of remissions does not exceed 6 months (1–4). Up to date, 5-FU1 has been considered the first-choice chemotherapeutic agent in the clinical treatment of advanced gastrointestinal cancer. However, clinical reports indicated that the likelihood of response of locally advanced or metastatic colorectal cancer treated with 5-FU alone was not over 20% (4). Two different pharmacological tricks have been employed to enhance the activity of 5-FU: long term high dose infusion (5) or modulation with noncytotoxic drugs such as folic acid (5, 6), allopurinol (7), N-(phosphonacetyl)-l-aspartic acid (8), and thymidine (9). In all these regimes the response rate was higher than that of conventional 5-FU treatment. It therefore seems reasonable to increase these efforts aimed at detecting new experimental approaches for the pharmacological management of colon cancer.

Recent studies have focused on the ability of some polar solvents to induce cell differentiation, thus suggesting their potential use in cancer therapy at essentially noncytotoxic concentrations (10–12). However, it has been proposed (13) that a sequential use of cytotoxic and differentiating agents could improve the anticancer therapy by inhibiting tumor cell proliferation and phenotypic diversification. In vitro and in vivo studies clearly demonstrated that changes in biological characteristics are associated with a “better differentiated” phenotype, including alteration in cell morphology, increase in doubling time and decrease in clonogenic ability and tumorigenicity (14–16). The polar solvent NMF, the main metabolite of DMF, was shown to be active against certain experimental and human tumors (14, 17, 18). In addition, NMF as well as DMF treatments were reported to augment tumor cell sensitivity to several cytotoxic agents and X-ray treatment (19–22). We thus planned a morphological and functional in vitro and in vivo study on the effect of the association of NMF with 5-FU, in different cell lines.

In this paper we report the results concerning the effect of different combination regimens of the two agents on a human colon carcinoma cell line (HT29). Strikingly, the association which may account for a better pharmacological effect seems to be represented by the 5-FU administration followed by NMF treatment.

MATERIALS AND METHODS

Cell Culture. HT29 cells, originally isolated from a human adenocarcinoma of the colon, were kindly supplied by Dr. B. Giovanella (The Stehlin Foundation for Cancer Research, Houston, TX). Cells were grown in RPMI 1640 medium, supplemented with 10% fetal calf serum, L-glutamine, and antibiotics at 37°C in a humidified 5% CO2:95% air atmosphere. Cells were routinely subcultured using a 5-min exposure to trypsin-EDTA solution.

Population doubling times were determined from electronic cell counts of duplicate 60-mm dishes receiving inocula of 8 × 10⁴ cells. Cells from two dishes were harvested separately each day and counted. Results were plotted on semilogarithmic paper, and doubling times were calculated from the curves.

Population forming efficiency was determined from the plating of cells in quintuplicate and counting colonies containing 50 or more cells, after an incubation period of 2 weeks. Data on saturation density were calculated from the growth curves. The numbers of cells present in confluent dishes, which determined the plateau regions of the curves, accounted for the maximum cell number.

Chemicals. N-Methylformamide (Sigma M2769) was diluted in normal growth medium. 5-Fluorouracil (Roche S.p.A., Milano), as supplied for clinical use, was diluted in saline and in culture medium to obtain the desired final concentration. Stock solution of 5-FU was made immediately before use.

NMF Treatment and Induction of Differentiation. HT29 cells were exposed to graded concentrations of NMF (0.8–4%) for different times (6–24 h) in order to determine the cytotoxic effect of the compound in terms of impairment of cell colony efficiency. From the survival curve data, the noncytotoxic dose of 1% NMF (corresponding to 170 mM) was chosen to assess induction of differentiation of HT29 cells. 8 × 10⁴ cells were seeded into complete RPMI medium containing NMF and analyzed for growth characteristics in the inducing medium, as described before.
For histological studies on HT29 colon cancer growing in nude mice, cells were cultured in NMF containing medium for six passages. Both induced and control cells (non-NMF treated cells) were then injected into 6-week-old nude mice at a concentration of 1 x 10⁶ cells/mouse, in order to examine the morphological evidence of NMF-induced differentiation.

Cell Survival Assay. HT29 cells were exposed to graded doses of 5-FU (5 to 25 μg/ml) for progressively increasing times. In combination experiments cells were pre- or posttreated with 1% NMF for 72 h and then exposed to increasing doses of 5-FU for 12 h. For simultaneous treatment, 5-FU was added to the cells during the last 12 h of NMF incubation. For each combination, besides untreated control plates, controls with NMF alone for 72 h were performed and processed by changing the medium at the same time as the treated samples; duplicate plates were counted before and after NMF treatment in order to evaluate the effect of the agent on cell number.

After exposure, the drug containing medium was removed, the cells were washed with balanced salt solution, harvested as monodispersed suspension, and counted. Known aliquots of cell suspension were seeded into 60-mm Petri dishes into NMF-free medium so that colonies would appear after 14 days of incubation. Colonies were stained with 2% methylene blue in 95% ethanol. The surviving fraction was calculated by dividing the absolute survival of the treated sample by the absolute survival of the control sample at the same time. In combination experiments the NMF-treated cells were assumed as control sample. All experiments were repeated at least twice with five replicate samples for each point. Morphological and functional studies were performed in parallel.

Morphological Studies. For the study of the cytoskeletal components and of the cell surface features, observations were performed on HT29 cells treated as follows: 1% NMF for 72 h; 10 μg/ml or 20 μg/ml 5-FU for 12 h; 1% NMF for 72 h and then 10 μg/ml or 20 μg/ml 5-FU for 12 h; 10 μg/ml or 20 μg/ml 5-FU for 12 h and then 1% NMF for 72 h. Control samples were examined at the same times (12, 72, and 84 h).

Fluorescence Microscopy. For tubulin immunocytochemistry, a monoclonal antibody directed against α-tubulin (Amersham Internat. plc) and a sheep anti-mouse IgG fluorescein-linked whole antibody (Amersham Internat. plc) were used. The staining of actin was performed with fluorescein-phalloidin (NBD-phallacidin, Molecular probes). For keratin immunocytochemistry a polyclonal antibody directed against keratin (Ortho Diagnostic) and a sheep anti-rabbit IgG-fluorescein-linked whole antibody (Amersham Internat. plc) were used.

For the detection of tubulin and actin filaments, cells grown on coverslips were fixed in 3.7% formaldehyde in phosphate buffer (pH 7.4), for 5 min at room temperature. For the detection of keratin filaments, the cells were fixed with methanol at room temperature for 5 min and with acetone at −20°C for 5 s. For tubulin labeling, incubations with monoclonal antitubulin antibody at 37°C for 30 min were performed. After washing, cells were incubated with anti-mouse IgG, fluorescein linked, as described above. For keratin labeling, incubations with the polyclonal anti-keratin antibody at 37°C for 30 min were performed. After washing, cells were incubated with anti-rabbit IgG, fluorescein linked, at 37°C for 30 min. For actin detection, cells were stained with fluorescein-phalloidin at 37°C for 30 min.

Electron Microscopy. For SEM, control and treated HT29 cells grown on coverslips were fixed in 1.5% glutaraldehyde in cacodylate buffer (pH 7.3) at room temperature for 40 min. After fixation, cells were dehydrated through graded ethanol, critical point dried in CO₂ and gold coated by sputtering. The samples were examined with a Philips 515 scanning electron microscope.

For the embedding procedure, cells grown on 25-cm² flasks were fixed in 1.5% glutaraldehyde in 0.05 M cacodylate buffer, postfixed in 1% OsO₄, dehydrated in graded ethanol, and embedded in Agar 100 (Agar Aids); semithin sections, obtained with an ultramicrotome LKB (Ultratome Nova), were stained with toluidine blue (pH 8.0).

RESULTS

Biological and Morphological Effect of NMF. Fig. 1 shows the surviving fraction of HT29 cells as a function of different exposure times to graded concentrations of NMF ranging from 0.8 to 4%. A lethal effect was observed when the NMF concentration increased from 2 to 4%, while the lower doses (0.8 and 1%) were devoid of cytotoxicity. Since the percentage of surviving fraction was not modified at the dose of 1% NMF by increasing the exposure time up to 72 h (surviving fraction, 95.0 ± 7%), this concentration was chosen for combination studies.

The effect of NMF on growth behavior of colon cancer cells is reported in Table 1. Cells growing in the NMF-containing medium showed plating efficiency and saturation density values lower than control cells. Moreover, the saturation density was reached at different times of growth (17th day for NMF-treated cells versus 8th day for untreated cells). A marked increase in doubling time was also observed for cells cultured in the presence of 1% NMF, as compared with control cells.

Fig. 2a shows the morphological appearance of human colon carcinoma cell line HT29, as revealed by scanning electron microscopy. The cells are aggregated to form roundish clusters made up by a variable number of cell units. General morphological features seem to point to HT29 as being well-differentiated, polarized cells. In fact, peripheral cells exhibit a polygonal shape with the outer side flattened and firmly attached to the substrate while the opposite side appears to rise up. In the clusters, the inner cells are irregularly distributed and are characterized by a variable shape. Cytoplasmic bridges, likely connecting adjacent cells, can be observed. At higher magnification the cell surface morphology can be discerned (Fig. 2b). The microvilli are numerous and erect and cover the whole cell surface which appears to be very similar among the different cells.

NMF treatment does not seem to strongly modify the general appearance of the cell clusters, the only change being a more irregular cellular profile (Fig. 2c). In contrast, the cell surface structures appear to be remarkably modified (Fig. 2d). The structure of the microvilli is highly variable and most of them appear to be flabby and curved.

SEM observations of HT29 cells treated with 10 μg/ml 5-FU do not show any significant morphological change when...
Fig. 2. Control (a and b) and NMF treated (c and d) HT29 cells observed by SEM. At low magnification the cells appear to be aggregated to form roundish clusters. The general appearance of the cell cluster does not seem to be modified by NMF treatment, the only change being a more irregular cellular profile (c) as compared to the control (a). At higher magnification, a difference in the structure of the microvilli can be discerned. While in the control cells (b) the microvilli are erect and randomly distributed, in NMF-treated cells (d) they are flabby and curved and unevenly distributed. Cells observed after 5FU treatment at 20 µg/ml show noticeable changes in cell profile (e) and surface ultrastructure (f), large areas of the cell surface appearing deprived of microvilli. Bars, 10 µm (a, c, e, f) and 1 µm (b, d).

compared to control cells, whereas treatment with 20 µg/ml 5-FU for the same time, produces noticeable modifications in both cell shape and surface ultrastructure (Figs. 2, e and f). Under this condition the cells present a very irregular profile and large areas of the cell surface appear to be deprived of microvilli.

Evidence of a maturational effect of NMF was verified by implanting in nude mice HT29 cells growing in vitro in solvent-containing medium for six passages. In Fig. 3 histopathological features of tumors deriving from untreated and NMF-treated cells are compared. In the tumor arising from NMF-treated cells it is clearly evident that great changes both in cell morphology and structural organization have occurred. In fact, control cells (Fig. 3a) appear to be highly polymorphic in shape and size, with an irregular architecture. Conversely, NMF-treated cells (Fig. 3b) are much more uniform in shape and arranged in solid homogeneous nests regularly surrounded by stromal connective septa.

Cytotoxic Effect of Combined Treatment. Previous experiments from our laboratory (23) demonstrated that HT29 cells were relatively resistant to 5-FU and that the efficacy of the drug was time-dependent. In particular, an impairment in the cell colony forming ability was observed when cells were exposed to 5-FU (5–50 µg/ml) for prolonged times (6–12 h). A dose range of 5–25 µg/ml 5-FU and 12 h exposure were chosen for combination experiments.

Fig. 4 shows the survival curves of HT29 cells exposed to 5-FU and NMF during the exponential and the stationary phase of growth (Fig. 4, left and right, respectively). As is evident, the 5-FU→NMF sequence enhanced the cytotoxicity induced by 5-FU alone, primarily modifying the shoulder region of the 5-FU survival curve.

This effect, evident both on log and plateau phase, was also reflected by changes in the $D_0$ and $n$ values of the survival curves. The $D_0$ and $n$ values for the 5-FU→NMF sequence were 2.1 µg/ml and 1.2 for the exponential phase and 1.59 µg/ml and 1.0 for the plateau phase.

When the cells were exposed to 5-FU alone the $D_0$ and $n$ values were 6.0 µg/ml and 7.7 for the cells in log phase and 5.0 µg/ml and 4.0 for the cells in stationary phase.

On the contrary, when the cells were preincubated with NMF and then exposed to 5-FU, a decrease in lethal activity was
CYTOTOXIC EFFECTS OF SFU-NMF ASSOCIATIONS

Fig. 3. Light microscopical observation of semithin sections of HT29 cells implanted in nude mice. Untreated cells (a) appear to be highly polymorphic and irregularly arranged whereas HT29 cells, grown in NMF containing medium (b), are much more uniform and arranged in regular nests (1800 x).

Fig. 4. Effect of NMF in combination with 5-FU on HT29 colon cancer cells according to graded doses of 5-FU. Exponentially or stationarily growing cells (left and right, respectively) were exposed to •, 5-FU alone for 12 h; •, 5-FU for 12 h followed by 1% NMF for 72 h; O, 1% NMF for 72 h followed by 5-FU for 12 h. Percent survival values of cells after 78-h NMF exposure were 95.0 ± 7.0% for log phase, and 91.3 ± 5.0% for plateau phase. Following incubation with the first agent, cells were rinsed with balanced salt solution and then exposed to the subsequent treatment. Each point is an average ± SE of three separate experiments. When not shown, the standard error is included in symbols.

observed with the respect to the single drug administration ($D_0 = 5.0 \mu g/ml, n = 12.0$ in log phase; $D_0 = 8.6 \mu g/ml, n = 4.0$ in plateau phase). A similar cytotoxic effect was also induced by the exposure of cells to graded doses of 5-FU in presence of 1% NMF (data not shown).

When 5-FU treatment at the lower dose follows the treatment with NMF, particular morphological changes can be detected. The density of the microvilli appears to be reduced and numerous swellings are present on the cell surface. In this case too, large zones are devoid of microvilli (Fig. 5a).

When the two treatments are performed in the inverted order, i.e., 10 \mu g/ml 5-FU followed by NMF, cell modifications are much more evident. Cell profiles are very irregular (Fig. 5b) and the microvilli are distributed in patches (Fig. 5c); sometimes large blebs protrude from the cell surface and the cell edges are detached from the substrate. Such morphological changes appear to be even more dramatic when treatment with NMF follows cell exposure to 20 \mu g/ml 5-FU (Fig. 5d). In this case the modifications are so drastic as to involve lethal damages.

The observations performed by fluorescence microscopy on HT29 cells treated according to the different regimens (NMF alone, 5-FU alone, NMF→5-FU and 5-FU→NMF) revealed a differential effect of these treatments on the main cytoskeletal components. In fact, the microfilament network showed remarkable modifications after the various administration schedules, whereas the arrangement of tubulin and keratin did not seem to be affected even under treatments which caused drastic morphological changes and a reduced cell viability. Fig. 6, a and b shows the microtubular organization in untreated and 10 \mu g/ml 5-FU→NMF treated cell clusters, respectively. No significant difference is detectable. Fig. 7, a and b, shows the keratin distribution in control and 10 \mu g/ml 5-FU→NMF treated HT29 cells, respectively. The amount and distribution of this cytoskeletal component seem to be extremely similar in treated and control cells. In contrast, the actin pattern appears to be modified in a characteristic way after the different treatments, confirming the surface morphology changes observed by SEM. In control cells (Fig. 8a) the actin shows a normal distribution with numerous stress fibers crossing the cell body. After NMF treatment (Fig. 8b) the microfilaments become aggregated in thick fluorescent bundles unevenly distributed inside the cell. This finding suggests that the polar solvent exerted a powerful effect on the actin polymerization. A quite different effect is then exerted by 10 \mu g/ml 5-FU. In fact, in this case the actin does not appear to be organized in long filaments but is broken down into numerous small clusters, as indicated by the observation of numerous fluorescent spots inside the cell body (Fig. 8, c and d). The effect of 5-FU on the actin arrangement seems to be also confirmed by the intense fluorescence observed at the cell cluster periphery. Such an effect is partially inhibited by prior treatment with NMF. In fact, the actin pattern of HT29 cells exposed to the polar solvent and then to 10 \mu g/ml 5-FU (Fig. 8e) appears to be remarkably

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Fig. 5. Effect of NMF—5-FU administration on the morphology of HT29 cells as revealed by SEM. The cells show a reduced number of microvilli and numerous swellings are present on the cell surface. The cell edges tend to detach from the substrate (a). The morphological changes induced by the 5-FU—NMF association are much more evident than those produced by the inverted sequence. In cells treated with 10 μg/ml 5-FU and then with NMF (b and c) the cell edges appear to be detached from the substrate (b) and the microvilli are distributed in patches (c). The exposure to 20 μg/ml 5-FU followed by NMF induces remarkable morphological damages (d). Bars, 10 μm.

Fig. 6. Microtubular organization in untreated (a) and 10 μg/ml 5-FU—NMF treated (b) cell clusters revealed by indirect immunofluorescence. No significant difference is detectable (1800 x).

Fig. 7. Keratin distribution in control (a) and 10 μm/ml 5-FU—NMF-treated (b) cells detected by indirect immunofluorescence. The amount and distribution of keratin appear to be very similar in both control and treated cells (2000 x).

different when compared to that of cells treated with 5-FU alone. The stress fibers are still visible even though they are not as well organized as in control or NMF treated cells. The strong "stabilizing effect" of NMF on microfilaments was further confirmed by the observation of cells treated first with 10 μg/ml 5-FU and then with NMF (Fig. 8, f and g). In these conditions, NMF appears to be capable of once again forming the thick bundles of microfilaments. Finally, prior treatment
Fig. 8. Actin patterns revealed by fluorescein-phalloidin in HT29 cells treated with the different regimens. In control cells (a) the actin microfilaments are characterized by a regular appearance, with numerous stress fibers. In NMF treated cells (b) the microfilaments appear to be aggregated forming thick fluorescent bundles. Conversely, after 5-FU administration the actin microfilaments appear to be disaggregated. Moreover, the stress fibers are no longer detectable in the two micrographs showing different focal planes of the same cell cluster (c and d). When 5-FU treatment follows NMF administration (e) such actin rearrangement does not occur, the microfilament organization being very similar to that observed in cells treated with NMF alone (b). When HT29 cells are treated with the inverted sequence (10 μg/ml 5-FU—>NMF) the actin microfilament appear to be reorganized to form stress fibers (f) or thick fluorescent bundles (g). Finally, the treatment with 20 μg/ml 5-FU induces alterations of microfilament arrangement so drastic as to make the subsequent NMF treatment (h) ineffective (2200 x).

with 5-FU at a concentration of 20 μg/ml produces much lethal damage, to the extent that the subsequent NMF treatment (Fig. 8h) becomes ineffective.

DISCUSSION

The major chance of employing differentiating agents for cancer management lies in the possibility of treating tumors without causing cell killing (10, 17). Results reported in the present paper clearly demonstrate that, as previously suggested (24), the maturational effect induced by 1% NMF on HT29 colon cells is not associated with cytotoxicity. However, the expression in vitro of a more differentiated phenotype is evidenced by an increase in doubling time, reduction in saturation density, and clonogenic ability. In addition, an altered morphology of in vivo growing cells is observed. In fact, when NMF-adapted HT29 cells are implanted in nude mice, some modifications occur in the architecture of the tumor, accountable for a more organized and differentiated phenotype. These results could partially explain the previously reported data obtained by Dexter (14) on DMF-treated human colon cancer cells xenografted in nude mice.

The inhibitory action of NMF on the growth of HT29 cells is consistent with that previously reported in literature for other polar compounds on different tumor lines (11, 12, 16). As postulated by other authors, such an effect might be attributed to the polar solvent-mediated glutathione depletion (25), to the impairment in the production of growth factors, as well as an altered response of cells to these factors (26).

The morphological features of cells treated with polar solvents have been widely investigated at a subcellular level by several in vitro studies. Some of these organic compounds exhibited the capability of inducing a differentiative effect on a number of cell lines. Leukemic (27–29), neuroblastoma (30), rhabdomyosarcoma (16), and melanoma (31, 32) cells are among the induced cells. Human sarcoma cells incubated with NMF showed a pseudoepithelial differentiation, with cells appearing to be cytormorphologically similar to normal osteoblasts (33). Moreover, a normalization of transformed cells in terms of morphology, anchorage, and membrane antigen composition was also detected (34). However, colorectal carcinoma cells appear to represent the most suitable targets for therapy with
differentiating agents (14, 35, 36). In fact, these cancer cells exhibit morphological features that can be specifically related to the different differentiated cell stages (37). For instance, increased expression of desmosomes was detected in human colon cancer cells after DMF treatment as a sign of a differentiating process involving cytoskeletal components (15). Moreover, results obtained with radical scavengers (dimethyl sulfide, t-butanol, aminopyrine, hydroquinone, etc.) support the hypothesis that hydroxyl radicals are involved in solvent action (38) and the oxidant injury may result in a substantial increase of the F actin content and in a side-to-side aggregation of F actin bundles (39). Therefore, the microfilament arrangement observed in HT29 cells treated with NMF could be related to a peroxidative effect, as previously reported in other cell types (40). This effect can account for the thick fluorescent bundles observed in NMF-treated cells as well as the surface morphology modifications detected by SEM, such as the changes in the rootlets of microvilli (41–43). Moreover, these findings seem to confirm the previously observed desmosome changes (15) because of the probable interaction of microfilaments with keratin bundles (43). Finally, the oxidative effect seems to be also supported by the NMF-induced depletion of cellular-reduced glutathione revealed in human colon carcinoma cells (25).

In the past few years, several lines of evidence have shown that the combination of relatively nontoxic maturational agents with cytotoxic antineoplastic drugs might represent a potentially important strategy for cancer treatment (12, 14, 19–22, 44–48). In addition, some authors (13, 48) have suggested the sequential use of cytotoxic and cytostatic differentiating or immunostimulating agents in a treatment strategy direct at suppressing the diversification of tumor cells that survive cytotoxic treatment. Data reported in this study concern the effect of the polar solvent NMF in combination with 5-FU on the survival of HT29 colon cancer cells. The basic finding of our work is the remarkable enhancement of the killing effect of 5-FU by NMF administration on these tumor cells.

The powerful inhibition on cell proliferation exerted by the sequence 5-FU→NMF, seems to be partially explained by morphological and ultrastructural findings. It may be possible that surface changes occurring in 5FU→NMF-treated HT29 cells could impair cell substrate relationships following a remarkable change in membrane-underlying cytoskeletal elements (actin fibers in particular). Several antineoplastic agents have been previously reported to affect the cytoskeletal apparatus; in particular microfilament integrity has been shown to be modifiable by various drugs. The observed effect of NMF on microfilaments, when occurring after the rearranging effect induced by 5-FU, could lead to an impairment of membrane-underlying cytoplasm integrity and function. These changes, in addition to the well known effect of 5-FU on nucleic acids, may account for the impairment of cell proliferation.

As shown in Fig. 4, the sequence 5-FU→NMF, induces an increase in the steepness of the 5-FU survival curve because of the disappearance of the shoulder region of the same curve. The exact mechanism of action of NMF at subcellular level is yet to be elucidated. Nonetheless, taking the width of the shoulder region as an index of the ability of the cells to accumulate the sublethal damage, the enhancement of 5-FU cytotoxicity following the subsequent application of the polar compound, might be related to the lower ability of treated cells to bypass the sublethal damage produced by 5-FU.

When the opposite sequence is applied to HT29 cells, a different cytotoxic activity is displayed. By analyzing at a morphological level the effect of the NMF→5-FU administration, it becomes evident that the structural changes induced by NMF on the actin fiber arrangement may prevent the damages produced by 5-FU. In fact, cell surface and cytoskeletal changes induced by the NMF→5-FU sequence appear to be reduced when compared to those detected in HT29 cells treated with 5-FU alone. These findings seem to confirm a close relationship between cytoskeletal integrity and cellular function, as supported by the survival curves. As for other microfilament stabilizing substances, i.e., phalloidin, the thickening of stress fibers probably forming bundles of F actin, could induce an increased “rigidity” of cell shape and physiology which, in turn, reduces cell sensitivity to 5-FU treatment. This is confirmed by the SEM observations which only partially differ from the observation of NMF given alone. However, in our conditions, any evident morphological effect of the NMF→5-FU sequence on microtubules and intermediate filaments, i.e., cytokeratins, may be ruled out, and early changes at subcellular level seem to allow for a complete recovery of cell viability, as demonstrated by plating efficiency. It is not clear whether the morphological changes observed are the consequence of probable changes induced by the treatments on the cell cycle or a direct effect on the actin microfilaments. However, the above-captioned considerations and the absence of modification in the expression of microtubules seem to support the latter hypothesis.

As regards the cytotoxic effect induced by the sequence NMF→5-FU several lines of evidence suggested that 5-FU activity may be increased by modulating the biochemical pathway of the drug (49–52). In particular, the importance of the amount and the activity of several enzymes normally present in colon mucosa cells was stressed. The levels of enzymes concerned both in de novo pyrimidine biosynthesis and in the salvage pathways (thymidine kinase, uridine kinase, uracil-phosphoribosyl transferase) were found to be higher in malignant human colorectal tissue than in their normal counterpart (51). Moreover, a positive correlation between such enzymes activity, malignancy, and growth rate was demonstrated in several colon tumor lines (49).

In light of these reports, our findings suggest that NMF, inducing a normalization of the malignant phenotype of HT29 cells as well as a slowdown in their growth rate, also reduce the activity level of enzymes involved in the 5-FU metabolic pathway.

In conclusion, the results of this study support the hypothesis according to which the combination of 5-FU and NMF may be effective in the enzyme pattern-targeted chemotherapy of colon tumors. However, as this combination may trigger the opposite effect depending upon the sequence employed, further investigations also extended to in vivo systems are recommended before general conclusions are drawn.

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