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CELLULAR HETEROGENEITY IN DNA DAMAGE AND GROWTH INHIBITION INDUCED BY ICI D1694, THYMIDYLATE SYNTHASE INHIBITOR, USING SINGLE CELL ASSAYS

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Abstract—Heterogeneity in the response of the HCT-8 (human ileocecal adenocarcinoma) tumor cell line to a new thymidylate synthase inhibitor, ICI D1694, was investigated in terms of induction of DNA single-strand breaks and cytotoxicity, applying the single cell alkaline gel (SCG) electrophoresis assay and the individual colony formation assay (iCFA), respectively. ICI D1694 induced maximal total DNA single-strand breaks 24 hr after a 2-hr drug exposure with incomplete repair by 72 hr. The level of DNA damage was concentration dependent and paralleled cellular growth inhibition *in vitro*. The proportion of cells with DNA damage and the extent of DNA single-strand breaks increased with drug concentration. At 1 μM ICI D1694 (IC_{95}), a significant level of DNA damage was detected in 58% of the cells; however, 25% of the cells had little or no damage. Using the iCFA system, it was observed that with 1 μM ICI D1694, only 2.6% of the seeded cells maintained a colony growth rate similar to that of the control colonies, and 22% of the cells were growing significantly more slowly. In conclusion, the SCG assay and the iCFA identified subpopulations of cells that were unaffected by ICI D1694. Although these cells represented only a small proportion of the total cell population, this phenomenon of heterogeneity in response to ICI D1694 might limit its therapeutic efficacy.

Key words: cellular heterogeneity; DNA damage; individual cells; colony formation assay; ICI D1694; *in vitro*

The extent and duration of DNA damage are considered important determinants of cellular response to radiation and chemotherapeutic agents with DNA as their primary target [1]. In contrast, antimetabolites, such as 5-fluorodeoxyuridine and the antifolate ICI D1694†, aimed at the enzyme TS can also potently induce DNA single- and double-strand breaks [2, 3]. The size of DNA fragments induced by 5-fluorodeoxyuridine in colorectal tumor cells has been demonstrated to measure 1–5 megabases [4]. This effect of TS inhibitors on DNA is felt to be due to uracil excision misrepair following depletion of thymidine nucleotide pools [3, 5, 6]. During the time when DNA damage occurs, cells swell but remain viable as indicated by trypan blue exclusion (Arredondo M, Yin M and Rustum Y, unpublished data). These findings support the hypothesis that the effect of antimetabolites, such as 5-fluorodeoxyuridine and ICI D1694, on DNA is induced specifically by these drugs, rather than general DNA fragmentation due to apoptosis or necrosis.

It has been recognized that cellular heterogeneity in drug response represents a major obstacle to curative chemotherapy [7]. Therefore, techniques that estimate cellular heterogeneity as determinants associated with drug response may provide the basis for identifying newer, more selective therapeutic strategies. Methods presently utilized to quantitate DNA damage rely on the use of radiolabeled DNA precursors and the availability of relatively large samples [8], which make these procedures less suitable for *in vivo* application. Furthermore, the results obtained from these assays represent an "average" response of the total cell population. The iCFA [9] and SCG assay for measurement of DNA single-strand breaks under alkaline conditions [10] are recently developed techniques capable of detecting drug treatment effects on individual cells. Cellular heterogeneity was evaluated in human ileocecal adenocarcinoma (HCT-8) cells treated with the quinazoline antifolate ICI D1694, a new and specific TS inhibitor [11–13], utilizing both the iCFA and the SCG assay.

MATERIALS AND METHODS

Drugs and chemicals. ICI D1694 was supplied by ICI Pharmaceuticals, U.K. The compound was

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† Abbreviations: ICI D1694, *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-yl-methyl)-*N*-methylamino]-2-thenoyl)-L-glutamic acid; iCFA, individual colony formation assay; SCG, single cell gel; SSB, single-strand breaks; TS, thymidylate synthase; and dHS, dialyzed horse serum.

dissolved in 0.1 M sodium bicarbonate (pH 8.3). Ethidium bromide was purchased from Molecular Probes (Eugene, OR); PBS, NaCl, Na_2EDTA, *N*-lauroylsarcosine (sodium salt), Triton X-100 and NaOH were obtained from the Sigma Chemical Co. (St. Louis, MO). Tris and agarose were from IBI (New Haven, CT); fully frosted slides and No. 1 cover slides were from Baxter (McGaw Park, IL). Proteinase K and tetrapropylammonium hydroxide were purchased from Bethesda Research Laboratories (Gaithersburg, MD) and the Fisher Scientific Co. (Pittsburgh, PA), respectively. [^{14}C]-Thymidine and [^{3}H]thymidine were obtained from Moravek Biochemicals, Inc. (Brea, CA) and the Amersham Corp. (Arlington Heights, IL), respectively.

Cells and culture conditions. Human ileocecal adenocarcinoma (HCT-8) cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained as a monolayer culture in RPMI-1640 medium supplemented with 10% dHS and 1 mM sodium pyruvate. The cell culture was serially passed twice weekly and kept in a humidified atmosphere containing 5% CO_2 at 37°. Cultures were checked monthly by a molecular probe for prokaryotic ribosomal RNA (Gen Probe Inc., San Diego, CA) and proven free of Mycoplasma contamination.

Drug treatment. Asynchronous HCT-8 cells (1×10^5) were seeded in Falcon T25 flasks. Twenty-four hours after seeding, cells in exponential growth exposed to ICI D1694 for 2 hr were washed twice and reincubated in drug-free medium. The cell cultures were then analyzed for DNA damage and growth inhibition as described below.

Collection of cells to assess DNA damage. Analysis of DNA damage was performed immediately after drug treatment, and thereafter at 24- and 72-hr intervals. These time points were selected from previous work in this laboratory characterizing the kinetics of DNA damage induced by ICI D1694. Maximal DNA damage was shown at 16–24 hr with considerable damage still present at 72 hr [3]. Cells were collected following a short trypsinization period, resuspended in medium, and chilled on ice.

SCG assay. The SCG assay was adopted from Singh *et al.* [14] with minor modifications. A suspension of approximately 50,000 cells in 80 μL of 0.5% low-melting point agarose was sandwiched between 1% normal melting point agarose for firm attachment and a third layer of 0.5% low-melting agarose on a fully frosted slide, allowing for solidification at 4°. The slides were placed in an alkaline lysing solution consisting of 1% sodium sarcosinate, 2.5 M NaCl, 1% Triton X-100, and 0.1 mg/mL proteinase K (pH 10) for 1 hr at 4°, and subsequent protein degradation was carried out for an additional 20 hr at 37°. DNA unwinding was facilitated with 1 mM EDTA and 300 mM NaOH (pH > 13) for 30 min, and then horizontal electrophoresis was performed for 20 min at 25 V and 300 mA. DNA fragments migrate through the agarose matrix from the cathode to the anode, while intact DNA remains *in situ*. The slides were neutralized for 10 min in Tris buffer (pH 7.5), and then DNA was stained with ethidium bromide for 10 min. The DNA fragments were viewed with a fluorescence microscope (Nikkon, Garden City, NY) at 400× magnification. Images obtained from a silicon intensified target camera (Hamamatsu Photonics, Japan) were transferred to a Quantimet 970 image analysis system (Leica, Deerfield, IL) via an Argus 10 image processor (Hamamatsu Photonics, Japan). Binary images were saved from 25 fields of view containing 1–3 cells each. The length of the migration (m), which represents the length of the total migration shadow minus the nuclear diameter of respective cells, was documented (Fig. 1). The length of the migration shadow is inversely proportional to the size of DNA fragments and thus is a quantitative estimate of the extent of DNA fragmentation [14, 15]. All experimental steps were carried out under dim light and repeated three times.

Total DNA SSB assay. DNA SSB were quantitated with the alkaline elution assay described by Kohn *et al.* [8]. The extent of DNA damage induced by ICI D1694, utilizing the alkaline elution assay, has been reported previously [3]. This assay was performed once in repetition of earlier work to serve as a comparative control with the SCG assay.

Irradiation. To obtain a reference curve for DNA single-strand breaks, exponentially growing cells were exposed to gamma-irradiation at a rate of 1.9 Gy/min with a 240 kV X-ray machine (General Electric) at ambient oxygen pressure and 4°. The dose–response curve of the migration length averaged from individual cells was linear in a range from 1 to 16 Gy, and the distribution of DNA migration was homogeneous as previously described [14, 15]. Therefore, we were able to assign a certain length of DNA migration to a corresponding dose level of irradiation.

Individual colony formation assay. A computerized image analysis system was used to quantitate the growth of individual colonies using the method described by Slocum *et al.* [9]. Briefly, 24 hr after drug removal, cells were trypsinized; approximately 5000 cells were suspended in a flat layer of 0.3% low-melting agarose suspension in RPMI and 10% dHS layered on a 1% agarose plane in 6-well plates (Falcon No. 3046). The cell suspension was covered with a second 0.3% agarose layer. The cell cultures were then incubated under standard conditions, 5% CO_2/95% air at 37°. Daily imaging was performed at 63× magnification. Individual colony areas were calculated after image processing with the Quantimet 970. The growth characteristics of colonies derived from individual cells following drug treatment were classified as unaffected (growth rate within the 95% confidence limits of untreated controls), growth slowdown, or growth arrest.

RESULTS

DNA damage in individual cells. Representative photomicrographs are shown in Fig. 2. The length of the observed cell/comet could be divided into three distinct categories representing different levels of DNA damage. Cells with migration patterns of less than 5 μm were judged to be undamaged or minimally damaged (panel A; upper cell, panel B). Low-level DNA damage was depicted by cells

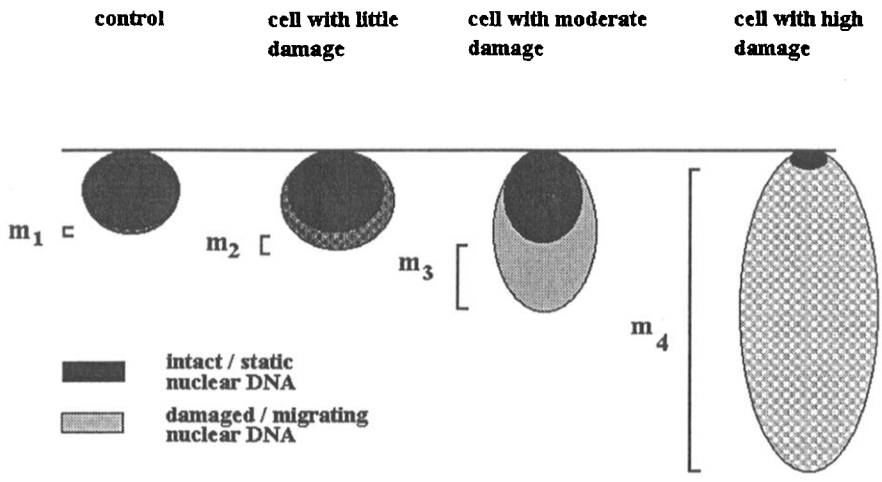

Fig. 1. Illustration of DNA migration patterns in individual cells following electrophoresis.

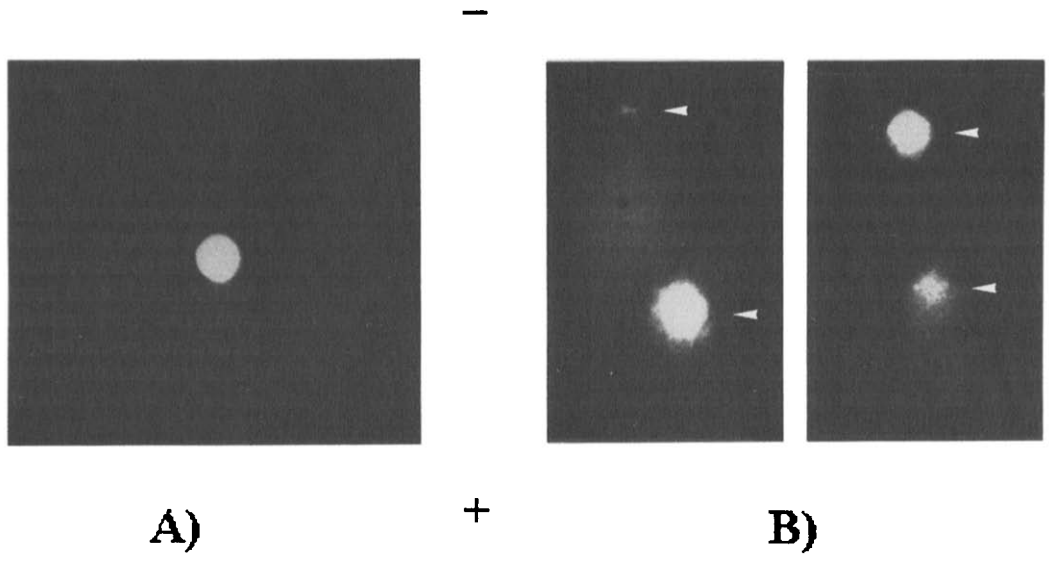

Fig. 2. Representative photomicrographs of HCT-8 cells following alkaline single cell gel electrophoresis. Cells in panel A were untreated; cells in panel B were assayed 24 hr after a 2-hr incubation with 3 μM ICI D1694. Note that in panel B clearly different DNA migration patterns are observed within the same field of view. Arrows indicate the position of the nucleus; (+) and (−) illustrate the direction of the current during electrophoresis.

demonstrating migration patterns between 5 and 10 μm (lower cell, left panel B). Moderate (panel B, lower cell) damage and high-level damage were judged to be characterized by migration patterns of 10–20 μm and greater than 20 μm, respectively. To clearly present differences between these categories with varying concentrations of ICI D1694 and different time points, bar graphs quantitating the number of cells in each category are presented rather than histograms of the migration patterns as previously reported [10, 14–19].

The amount of single-strand breaks detected in individual cells using the SCG assay, 24 hr after drug treatment, is shown in Fig. 3. The observed shifts in the distribution of these easily separable categories were related to the concentration of ICI D1694. At 1 μM ICI D1694 (IC_{95}), about 25% of the total cell population had no detectable DNA damage, whereas about 60% of the cells exhibited a moderate to high level of damage (Fig. 3). Increasing the concentration to 3 μM D1694 (IC_{99}) reduced the fraction of unaffected cells to 12% (see Fig. 5).

Total DNA damage. The level of DNA single-strand breaks induced by ICI D1694 was compared using alkaline elution and the SCG assays, and the results are summarized in Fig. 4. The data from the SCG assay represent the average length of the migration shadow calculated from a calibration curve

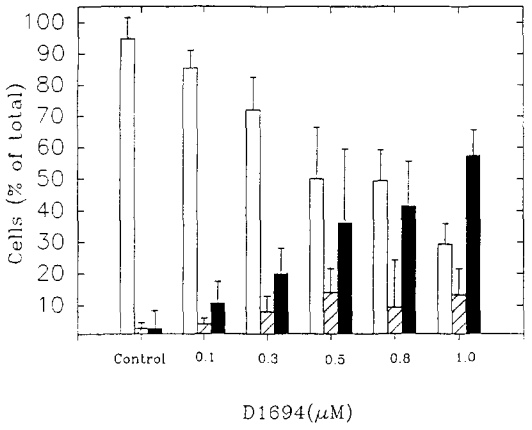

Fig. 3. Migration pattern of HCT-8 cells 24 hr after a 2-hr incubation with ICI D1694. Data (mean ± SD) from three separate experiments are summarized. Data were classified into three groups using the length of migration of DNA as a determining factor: (□) undamaged cells; (▨) little damage, and (■) moderate to high damage.

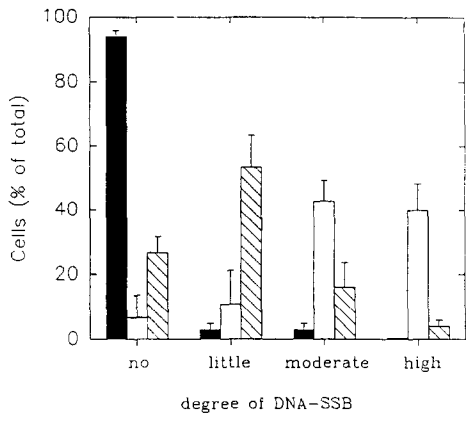

Fig. 5. Distribution of DNA single-strand breaks in HCT-8 cells 24 and 72 hr after ICI D1694 incubation. The experimental conditions were the same as shown in Fig. 4, except for the concentration, which was 3 μM. Error bars indicate the standard deviation of three experiments. Key: (■) control, (□) 24-hr postincubation, and (▧) 72-hr postincubation.

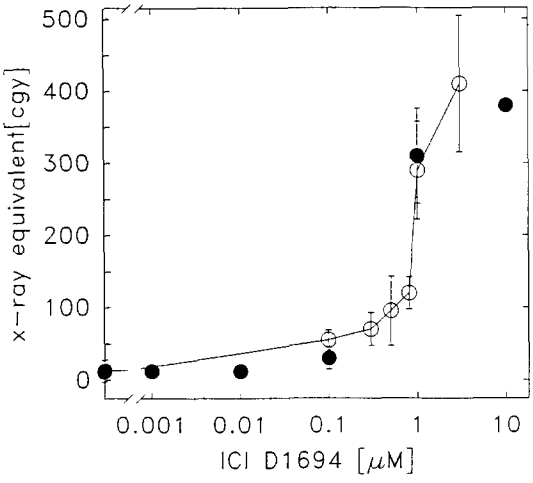

Fig. 4. Comparison of total DNA single-strand breaks evaluated with both the SCG assay and alkaline elution after a 2-hr exposure to ICI D1694. Results are expressed in X-ray equivalents. Error bars indicate the standard deviation of three experiments. The alkaline elution assay was performed once, as described in the text. Key: (○) SCG assay, and (●) alkaline elution.

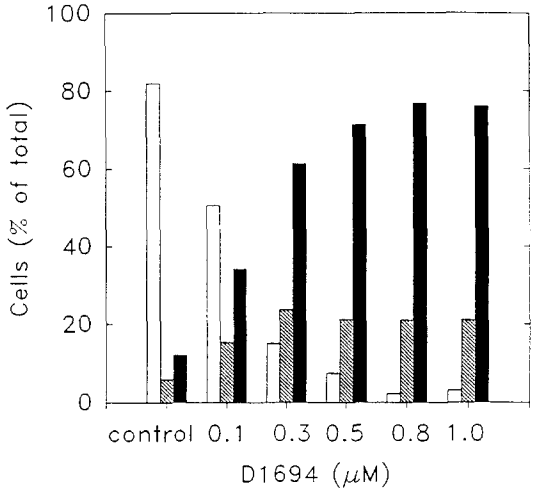

Fig. 6. Growth pattern of individual HCT-8 cells following a 2-hr incubation of ICI D1694 as assessed by the iCFA. Data from three separate experiments are summarized. Key: (□) unaffected growth, growth rate same as in untreated controls; (▧) growth slowdown, growth rate <95% of control cells; and (■) growth arrest, not growing at end of experiment.

in X-ray equivalents. As can be seen at 0.1 and 1 μM ICI D1694, the results obtained with both the SCG and alkaline elution assays were comparable.

DNA repair. Figure 5 demonstrates the kinetics of DNA damage induced by 3.0 μM ICI D1694 (IC_{99}). A significant level of DNA-SSB was achieved at 24 hr with only partial repair by 72 hr. At that time point, most cells exhibited a low to moderate extent of single-stranded DNA breaks. Twenty-five percent of the cell population continued to display no demonstrable DNA damage 72 hr after drug exposure.

Individual colony formation assay. The effects of ICI D1694 on the growth of individual cells were assessed using the iCFA. Drug effects can be classified into three distinct categories: (1) cells with a growth rate similar to control cells (unaffected growth); (2) cells with reduced growth rate (growth slowdown); and (3) growth arrest (Fig. 6). The data

demonstrate that the fraction of cells with unaffected growth rate decreased with increasing drug concentration. At 0.1 μM (IC_{50}), approximately 50% of colonies were growing at the growth rate of untreated controls (0.64 doublings/day; 95% confidence interval, 0.4–0.88 doublings/day). When the drug concentration was increased to an IC_{95}, 25% of the colonies continued growing, but only 2.6% did so with a growth rate similar to control.

Cytotoxicity and DNA damage. Comparing the results of Figs. 4 and 6, it becomes apparent that cytotoxicity was observed at a lower drug concentration than was required to produce significant DNA damage, e.g. IC_{50} for growth cessation by the iCFA was 0.13 μM and the EC_{50} for DNA damage was 0.9 μM.

DISCUSSION

The single cell gel assay and the individual clonogenic assay enable one to monitor the effect of ICI D1694 on individual HCT-8 tumor cells. Both techniques were able to identify distinctive subpopulations representing different responses to this drug. Using the iCFA, it was demonstrated that a small but significant number of cells continue to grow at rates similar to those of the controls. These cells have apparently escaped the drug's effect, and possibly represent populations with inherent resistance to the drug. Preliminary studies have indicated that 1 out of 4 colonies with high growth rates after drug exposure have shown consistent resistance to ICI D1694 when expanded as a subline of HCT-8 (data not shown).

The SCG assay is capable of distinguishing between a homogeneous and a heterogeneous pattern of DNA damage. Olive *et al.* [15] have shown that the distinction between these patterns of DNA migration is enhanced by the presence of alkaline lysing solution with detergent and proteinase K digestion. Irradiation and VP-16 have been shown to induce a homogeneous distribution of DNA migration patterns [10, 15–19, and our own data]. In contrast, a heterogeneous pattern of DNA damage, as seen with ICI D1694 in this report, has also been demonstrated with bleomycin [16].

We were able to confirm a relationship between growth inhibition and DNA single-strand breaks on individual cells caused by the TS inhibitor ICI D1694. A response to ICI D1694 could be observed with the iCFA at concentration levels where the SCG assay was unable to detect a significant amount of single-strand breaks. The limit of sensitivity of the SCG assay is in the region of 0.5 Gy equivalents [10], which corresponds to 70–140 breaks per $10-12 \times 10^{12}$ Da of DNA [8]. A further limitation of the technique, as described in this report, is the inability of the SCG assay to distinguish between single- and double-stranded DNA breaks. If the SCG assay is performed under neutral conditions, only double-strand breaks would be detected [15]. Furthermore, the SCG assay cannot distinguish whether DNA breaks are due to apoptosis or toxic fragmentation. Data from Yin *et al.* [3] and Dusenbury *et al.* [4] indicate that neither ICI D1694 nor FUdr induces cell death via the apoptotic mechanism in colorectal tumor cells.

Since the individual cell assays for DNA damage and colony growth were conducted on different aliquots of cells treated identically, we cannot conclude that the subpopulation showing no DNA damage is the same population that shows uninhibited growth, or that the cells with the highest damage are those that show growth arrest. However, this would seem to be a reasonable speculation. Cells showing little or no damage 24 hr after drug exposure may have escaped the drug's effect, or may represent cells with high DNA repair capacity. Similarly, at 72 hr, undamaged cells maybe those that have repaired damage, or those that have grown from an undamaged subpopulation [20, 21]. Inhibitors of specific repair enzymes might be used to examine this question.

It is possible that the lowest drug concentrations that cause growth inhibition cause DNA damage that is undetectable in the SCG. In principle, a single unrepaired break in DNA could be biologically significant [1]. The suspicion that DNA damage is responsible for growth inhibition by anticancer agents is supported by the fact that most drugs that are clinically useful in cancer therapy are DNA-active agents, whereas inhibitors of RNA or protein synthesis show only general toxicity [22]. Also, in a series of analogs of intercalating agents, those that were growth inhibitors caused DNA damage, and those that did not were not active agents [23]. Similar to the findings of the present report, the DNA damage was manifest only at drug concentrations that were higher than those that were required for growth inhibition.

Cellular response to the TS inhibitor ICI D1694 was heterogeneous, with some cells showing little or no damage, while others showed severe damage. This was true whether the damage was measured by individual colony growth or by individual cell nuclear damage. It is likely that more sensitive methods for detection of DNA damage will be required to examine the hypothesis that DNA damage is the cause of growth inhibition by ICI D1694 and other DNA-damaging agents.

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REFERENCES

1. Eastman A and Barry MA, The origins of DNA breaks: A consequence of DNA damage, DNA repair, or apoptosis. *Cancer Invest* **10**: 229–240, 1992.
2. Yin MB and Rustum YM, Comparative DNA strand breakage induced by FUra and FdUrd in human ileocecal adenocarcinoma (HCT-8) cells: Relevance to cell growth inhibition. *Cancer Commun* **3**: 45–51, 1991.
3. Yin MB, Guimares M, Zhang ZG, Arredondo M and Rustum YM, Time dependence of DNA lesions and growth inhibition by ICI D1694, a new quinazoline antifolate thymidylate synthase inhibitor. *Cancer Res* **52**: 5900–5905, 1992.
4. Dusenbury CE, Davis MA, Lawrence TS and Maybaum J, Induction of megabase DNA fragments by 5-

fluorodeoxyuridine in human colorectal tumor (HT29) cells. *Mol Pharmacol* **39**: 285–289, 1991.
5. Sedwick WD, Kutler M and Brawn OE, Antifolate-induced misincorporation of deoxyuridine monophosphate into DNA: Inhibition of high molecular weight DNA synthesis in human lymphoblastoid cells. *Proc Natl Acad Sci USA* **78**: 917–921, 1981.
6. Goulian M, Bleile BM, Dickey LM, Grafstrom RH, Ingraham HA, Neynaber SA, Peterson MS and Tseng BY, Mechanisms of thymineless death. *Adv Exp Med Biol* **195**: 89–95, 1986.
7. Goldie JH and Coldman AJ, The genetic origin of drug resistance in neoplasms: Implications for systemic therapy. *Cancer Res* **44**: 3643–3653, 1984.
8. Kohn KW, Ewig RAG, Erickson L and Zwelling LA, Measurement of strand breaks and cross-links by alkaline elution. In: *DNA Repair: A Laboratory Manual of Research Techniques* (Eds. Friedberg EC and Hanawalt PC), pp. 379–401. Marcel Dekker, New York, 1981.
9. Slocum HK, Malmberg M, Greco WR, Parsons JC and Rustum YM, The determination of growth rates of individual colonies in agarose using high-resolution automated image analysis. *Cytometry* **11**: 793–804, 1990.
10. Östling O and Johanson KJ, Microelectrophoretic study of radiation-induced DNA damage and repair in individual mammalian cells. *Biochem Biophys Res Commun* **123**: 291–298, 1984.
11. Marsham PR, Hughes LR, Jackman AL, Hayter AJ, Oldfield J, Waldleworth JM, Bishop JAM, O'Connor BM, Moran RG and Calvert AH, Quinazoline antifolate thymidylate synthase inhibitors: Heterocyclic benzoyl ring modification. *J Med Chem* **34**: 1594–1605, 1991.
12. Jackman AL, Gordon AT, Bibson W, Kimbell R, Brown M, Calvert AH, Judson IR and Hughes LR, ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth *in vitro* and *in vivo*: A new agent for clinical study. *Cancer Res* **51**: 5579–5586, 1991.
13. Bisset GMF, Pawelczak K, Jackman AL, Calvert AH and Hughes LR, Syntheses and thymidylate synthase inhibitory activity of the poly-γ-glutamyl conjugates of *N*-[5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-yl-methyl)-*N*-methylamino]-2-thenoyl]-L-glutamic acid (ICI D1694) and other quinazoline antifolates. *J Med Chem* **35**: 859–866, 1992.
14. Singh NP, McCoy MT, Tice RR and Schneider EL, A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* **175**: 184–191, 1988.
15. Olive PL, Wlodek D, Durand RE and Banáth JP, Factors influencing DNA migration from individual cells subjected to gel electrophoresis. *Exp Cell Res* **198**: 259–267, 1992.
16. Östling O and Johanson KJ, Bleomycin, in contrast to gamma irradiation, induces extreme variation of DNA strand breakage from cell to cell. *Int J Radiat Biol* **52**: 683–691, 1987.
17. Olive PL, Banáth JP and Durand RE, Detection of etoposide resistance by measuring DNA damage in individual Chinese hamster cells. *J Natl Cancer Inst* **82**: 779–783, 1990.
18. Olive PL, Banáth JP and Durand RE, Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. *Radiat Res* **122**: 86–94, 1990.
19. Singh NP, Tice RR, Stephens RE and Schneider EL, A microgel electrophoresis technique for the direct quantification of DNA damage in individual fibroblasts cultured on microscope slides. *Mutat Res* **252**: 289–296, 1991.
20. Malmberg M, Slocum HK and Rustum YM, Growth slow-down and growth arrest of human colon carcinoma cells HCT-8 *in vitro* after exposure to 5-fluoro-2′-deoxyuridine. *Cell Prolif* **26**: 291–303, 1993.
21. Zhang ZG, Malmberg M, Yin MB, Slocum HK and Rustum YM, Isolation and characterization of a human ileocecal carcinoma cell line (HCT-8) subclone resistant to fluorodeoxyuridine. *Biochem Pharmacol* **45**: 1157–1164, 1993.
22. Konopa J, G_2 block induced by DNA crosslinking agents and its possible consequences. *Biochem Pharmacol* **37**: 2303–2309, 1988.
23. Takeda K, Minowada J and Bloch A, The role of drug induced differentiation in the control of tumor growth. In: *Biological Characterization of Human Tumors* (9th International Symposia on the Biological Characterization of Human Tumor, Bologna, Italy, 1981) (Eds. Davis W, Maltoni C and Tanneberger S), pp. 275–281. Akademie Verlag, Berlin, 1983.