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EFFECTS OF ANTIMETABOLITES ON ADENOVIRUS REPLICATION IN SENSITIVE AND RESISTANT HUMAN MELANOMA CELL LINES

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Abstract—Methotrexate (MTX), 6-thioguanine (6-TG) and cytosine arabinoside (ara-C) inhibited the replication of adenovirus (viral capacity) more in drug-sensitive than in resistant human melanoma cell lines. By comparison, inhibition of cellular DNA and RNA synthesis after short treatment periods (<48 hr) was not a good predictor of cellular sensitivity. MTX, an inhibitor of *de novo* nucleotide synthesis, was most effective when added to cells just before infection with virus and inhibited viral capacity at doses 10–1000-fold lower than those required to affect cell survival. The MTX-sensitive cell lines, members of a DNA repair deficient group sensitive also to killing by methylating agents (the Mer^- phenotype), were not deficient in dihydrofolate reductase but exhibited DNA fragmentation after treatment with MTX for 48 hr. 6-TG and ara-C, inhibitors of purine and pyrimidine salvage, were most inhibitory to viral capacity when added >36 hr before virus infection and were less effective than MTX (doses 5–7-fold and 4–24-fold higher than for cell survival respectively). No correlation was found between MTX sensitivity and sensitivity to 6-TG or ara-C. These results indicate that (i) inhibition of viral capacity is a more comprehensive test of antimetabolite cytotoxicity than inhibition of cellular DNA or RNA synthesis; (ii) the viral capacity assay correctly predicts cellular sensitivity to MTX, 6-TG and ara-C and therefore has potential for application to primary cultures of human tumours; and (iii) MTX-sensitive cell lines and adenovirus replication rely heavily on *de novo* nucleotide synthesis, which in Mer^- cells appears to be linked to a DNA repair defect as yet undefined.

Methylating agents such as DTIC† have been used extensively as chemotherapeutic agents in the treatment of melanoma [1, 2]. Such agents react with DNA to form a variety of products including 0^6-MeG, a lesion associated with mutagenesis and toxicity in mammalian cells [3, 4]. The methylation sensitive Mer^- phenotype lacks 0^6-MeG methyltransferase, a protein which removes methyl groups from DNA [5]. Mer^- cell lines are also sensitive to killing by the antimetabolites hydroxyurea and MTX compared with Mer^+ cell lines and remain so after becoming resistant to methylating agents [6]. This co-sensitivity may reflect a structural genetic linkage or a common functional defect such as sensitivity to deoxynucleotide (dNTP) depletion. Hydroxyurea inhibits ribonucleotide reductase [7] and MTX inhibits DHFR and other enzymes [8], with consequent depletion of pyrimidine and purine dNTPs essential for DNA synthesis. There is therefore a need for determining the effects of antimetabolites on DNA synthesis in these phenotypes, preferrably by a nonisotopic method to avoid pool size artifacts.

Viral capacity (the ability of agent-treated cells to replicate untreated virus), was found to reflect cell survival following ultraviolet irradiation of sensitive and resistant cell types [9]. This assay is a test of the ability of the treated cell to replicate DNA, irrespective of the cytotoxic mechanism of action of the agent studied, and with adenovirus allows the measurement of chemosensitivity in both proliferating and non-proliferating cells [10–12]. The assay has correctly identified human tumour cell lines sensitive to hydroxyurea, deoxyadenosine [10], Adriamycin® or etoposide [13]. In the present study, viral capacity was compared with clonogenic cell survival for detecting the chemosensitivity of human tumour cell lines to the antimetabolites MTX, 6-thioguanine (6-TG) and cytosine arabinoside (ara-C), for eventual application to primary cultures and because clonogenic survival assays are difficult to perform even on cell lines, especially with MTX [6, 14, 15]. The assay was then used in conjunction with measurement of cellular nucleic acid synthesis, DHFR activity and DNA strand breaks to investigate the relationship between sensitivity to methylating agents and antimetabolites in Mer^- cells.

MATERIALS AND METHODS

Cell culture. The origins and properties of the human melanoma cell lines MM96L, MM253C1, and MM418 have been described previously [13, 16, 17]. MM466 and MM470 are human melanoma cell lines established in this laboratory from lymph node metastases. Cells were cultured in RPMI medium 1640 (Commonwealth Serum Laboratories, Melbourne, Australia) containing 10% (v/v) fetal calf serum, penicillin (100 IU/mL), streptomycin (100 μg/mL) and 3 mM HEPES. Routine tests for

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† Abbreviations used: DTIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; 0^6-MeG, 0^6-methylguanine; MTX, methotrexate; dNTP, deoxynucleoside triphosphate; ara-C, cytosine arabinoside; 6-TG, 6-thioguanine; Hu, hydroxyurea; DHFR, dihydrofolate reductase.

Mycoplasma using an agar growth assay were negative.

Cell survival. Drug treatments for clonogenic cell survival assays were carried out using duplicate cultures seeded 24 hr previously at 1000 cells per 6-cm dish. In some experiments the fetal calf serum was first dialysed for 24 hr at 4° against RPMI 1640. The cultures were maintained until colonies in the control plates had more than 50 cells (7–14 days), then fixed with methanol and stained with Giemsa for enumeration. To obtain the single cell response, the surviving fraction was corrected for the contribution of multicell clusters present at the time of treatment [18]. Five doses of each drug were used, and the D_{37} (dose require to reduce survival to 37%) was determined from a plot of log percent survival vs dose. In the nonclonogenic assay [6], cells (3×10^3 cells per 16-mm well) were treated with drug for 6 days, then labelled for 4 hr with [^{3}H]thymidine, lysed in H_2O and harvested onto glass fibre discs for liquid scintillation counting of covalently incorporated label.

DNA and RNA synthesis. Cells seeded in duplicate 24 hr previously (5×10^3 per 6-mm microtitre well) were treated with the drug and at various times labelled for 4 hr with a mixture of [^{14}C-*methyl*]thymidine (1 Ci/mol, 0.02 μCi/mL; Radiochemical Centre, Amersham, U.K.) and [5-^{3}H]uridine (10 μCi/mL, 20 Ci/mmol; Radiochemical Centre). The cells were dislodged with trypsin (200 μg/mL in phosphate buffered saline), lysed with water and washed onto glass fibre discs for liquid scintillation counting of covalently incorporated label. Five doses were used for each drug, and the D_{37} was determined from plots of log percent control vs dose.

Viral capacity assay. The ability of cells to replicate adenovirus was assayed by immunochemical detection of viral antigens 2–3 days after infection, using cells grown and treated in the microtitre plates as previously described [10]. Virus titres in untreated cells were 10^4–10^8 infectious doses/mL, depending on the cell line.

DHFR assay. Dihydrofolate was prepared from folic acid by the method of Blakley [19] and used immediately. NADPH was purchased from Sigma Chemical Co. (St Louis, MO). DHFR activity was measured as the decrease in absorbance at 340 nm with time [20].

DNA strand breaks. Cells were labelled for 24 hr with [2-^{14}C]thymidine or [*methyl*-^{3}H]thymidine, the latter culture treated with drug and a mixture of the two labelled cell populations lysed and eluted from Millipore BS-2 filters with tetrapropylammonium hydroxide (pH 12.3) as described previously [21].

RESULTS

Cell survival

MTX toxicity *in vitro* was recently reported to be greatly increased by the use of dialysed fetal calf serum, presumably through removal of thymidine and other nucleosides [14]. The previous study of Mer$^-$ cell lines [6] was therefore reassessed with dialysed serum using four cell lines suitable for clonogenic survival assay. The results (Fig. 1A) confirmed that Mer$^-$ cell lines were sensitive to MTX, and that the drug was significantly more toxic to both phenotypes in dialysed serum (Table 1).

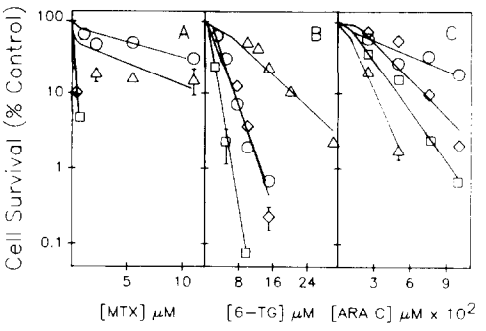

Fig. 1. Comparison of the sensitivity of Mer$^-$ (□, MM253cl; ◇, HeLa) and Mer$^+$ (△, MM96L; ○, MM418) to killing by MTX (A), 6-TG (B) and ara-C (C).

Many human cell lines form diffuse colonies and clone poorly at very low cell densities. Cell survival was therefore also measured by [^{3}H]thymidine incorporation after 6 days of drug treatment, a method which has given results similar to clonal assays with a variety of agents [22]. To more closely simulate physiological conditions, the serum was not dialysed for this assay or for the following studies. The results (Table 1) were qualitatively similar to clonogenic survival in a comparison of four cell lines, although the slowly proliferating MM418 line (doubling time 48 hr) gave a much higher D_{37} in the labelling assay presumably because less proliferation had occurred in the 6-day culture period.

Similar studies were undertaken with two antimetabolites not previously tested in these cell lines. The Mer$^+$ cell line MM96L exhibited the greatest resistance to 6-TG in the clonogenic assay, but another Mer$^+$ cell line, MM418, was not significantly more resistant than the Mer$^-$ line HeLa (Fig. 1B). All four cell lines were considerably more resistant to 6-TG in undialysed fetal calf serum (Table 2), presumably due to competition with normal purines. The labelling assay (Table 2) gave quantitatively similar results, and identified two lines more resistant than MM96L (MM466 and MM470).

No relationship between Mer phenotype and clonogenic cell survival was found for ara-C, the third antimetabolite studied (Fig. 1C). The MM96L cell line, although the most resistant to 6-TG, was the most sensitive to ara-C compared with other cell lines. In the labelling assay, MM470 was found to be highly resistant to ara-C (Table 3).

DHFR assay

To investigate the biochemical basis of the MTX sensitivity observed in Mer$^-$ cell lines, assays for DHFR activity were undertaken for a representative panel of cell lines (Table 2). No consistent relationship emerged, with HeLa, a Mer$^-$, MTX-sensitive cell line showing the highest activity of the target enzyme. The lowest activity was found in MM418, a

Table 1. Inhibition of viral capacity, cell survival and nucleic acid synthesis by MTX

	D_{37} (μM)*				
			DNA synthesis†		RNA synthesis†
Cell line	Viral capacity	Cell survival‡	144 hr	24 hr	(24 hr)
Mer⁺					
MM96L	0.014	8.5 (0.67)§	0.45	>20	>20
MM127	0.050		2.90	>20	>20
MM170	0.034		0.31	>20	>20
MM229	0.024				
MM418	0.019	>10 (0.085)§	>20	>20	>20
MM466	0.160		>20		
MM470	>2		0.20	>20	>20
Mer⁻					
MM253cl	0.007	0.080 (0.010)§	0.08	>20	>20
HeLa	0.008	0.095 (0.027)§	0.09	>20	>20

* Dose required to reduce the response to 37% of untreated cells.
† Cells were treated with drug for the time stated and then isotopically labelled with thymidine (DNA synthesis) or uridine (RNA synthesis).
‡ Determined by colony formation.
§ Determined in dialysed fetal calf serum.

Table 2. Inhibition of viral capacity, cell survival and nucleic acid synthesis by 6-TG

	D_{37} (μM)				
			DNA synthesis		RNA synthesis
Cell line	Viral capacity	Cell survival	144 hr	48 hr	(48 hr)
Mer⁺					
MM96L	50	10	7.9	38	45
MM127	54	(1.2)*	3.5		
MM170			2.2	10	5
MM418	18	3.5	1.5	42	80
MM466	>100	(0.38)*	18	75	75
MM470	27		30	180	>200
Mer⁻					
MM253cl	15	1.9 (0.22)*	0.70	22	30
HeLa	17	2.8 (0.35)*	1.0	3.7	4.5

* Determined in dialysed fetal calf serum.

slowly-proliferating Mer⁺ cell line derived from a primary melanoma of the skin.

Induction of DNA strand breaks by MTX

To determine whether DNA strand breaks reported to be induced in mammalian cells by MTX [23, 24] were being formed and correlated with cellular sensitivity, a Mer⁺ and a Mer⁻ cell line were treated with MTX for 48 hr after prelabeling the DNA with [^{3}H]thymidine. Analysed by alkaline elution, the Mer⁻ HeLa cells showed a more rapid elution of DNA from treated cells compared with MM96L, indicative of a higher level of strand breaks (Fig. 2).

Time course for inhibition of viral capacity by drugs

Adenovirus DNA synthesis commences 8–12 hr after infection and intact particles are formed after 24 hr [11, 12]. Experiments were therefore conducted to determine the optimum time to commence drug treatment before infecting the cells with virus (Fig. 3). Inhibition of virus replication by MTX sharply diminished if added any later than 6 hr before virus infection, indicating that a cell exposure of

Table 3. Inhibition of viral capacity, cell survival and nucleic acid synthesis by ara-C

	D_{37} (μM)				
			DNA synthesis		RNA synthesis
Cell line	Viral capacity	Cell survival	144 hr	48 hr	(48 hr)
Mer$^+$					
MM96L	0.10	0.018	0.067	0.038	>5
MM127	0.30		0.069		
MM170		0.150	0.045	>5	
MM418	1.25	0.053	0.25	2.8	>5
MM466	1.30		0.16	7.5	>5
MM470	>2.50		>0.5	7.0	>5
Mer$^-$					
MM253cl	0.20	0.024	0.17	6.0	>5
HeLa	0.24	0.055	0.26	0.5	>5

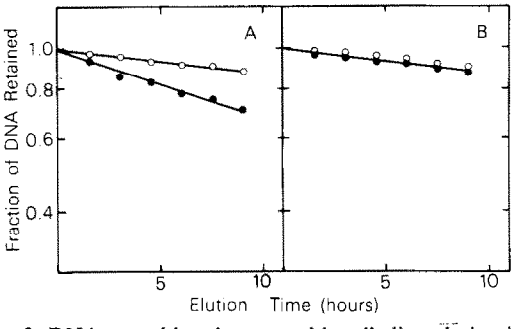

Fig. 2. DNA strand breaks assayed by alkaline elution in HeLa (A) and MM96L (B) cells, 48 hr after treatment with 10 μM MTX. (○) ^{14}C-Labelled control; (●) treated cells.

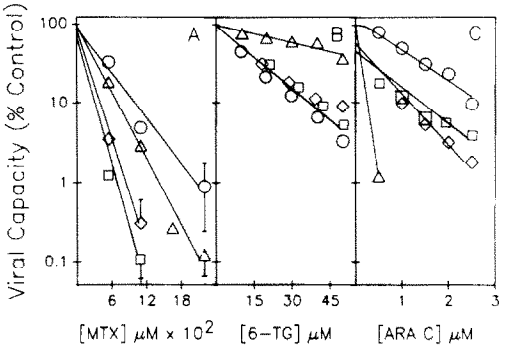

Fig. 4. Effect of continuous drug treatment on the viral capacity of human cell lines. (A) MTX added to cells at the time of virus infection. (B) 6-TG added 48 hr before virus infection. (C) Ara-C added at the time of virus infection. (◇) HeLa; (△) MM96L; (□) MM253cl; (○) MM418.

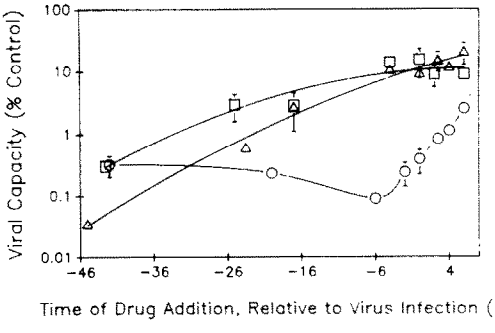

Fig. 3. Replication of adenovirus in HeLa where treatment of cells with MTX (○), 6-TG (△) or ara-C (□) was commenced at various times before or after infection.

14 hr before the onset of viral DNA synthesis is required to maximally inhibit virus replication.

6-TG and ara-C showed similar time responses, with maximum inhibition of viral capacity occurring at the longest times of exposure before infection. These antimetabolites were both equally inhibitory if added immediately before, or after infection, indicating some direct effect on viral DNA synthesis.

The time chosen for the addition of each drug in the following viral capacity assays was that at which the drug effect was close to maximal. Drug exposures of longer than 46 hr before infection were not feasible because the control cultures became too dense.

Viral capacity assays

The pattern of cellular MTX resistance associated with Mer phenotypes was also observed when assayed by viral capacity (Fig. 4A), although the difference in the D_{37} for viral capacity between sensitive and resistant lines was less than the difference determined by clonogenic cell survival. In a larger number of lines assayed by [^{3}H]thymidine labelling after long term treatment, there was poor correlation within the range exhibited by the resistant Mer$^+$ group. One cell line, MM470, was exceptionally resistant to MTX in the viral capacity assay (Table 1).

The enhanced cellular resistance to 6-TG of the melanoma line MM96L was paralleled in the viral capacity assay (Fig. 4B), with three sensitive cell lines exhibiting a much greater inhibition of viral replication. Two Mer$^+$ lines (MM418, MM470) were almost as sensitive to this agent as the Mer$^-$ lines

Table 4. DHFR activity in Mer$^+$ and Mer$^-$ cell lines

Cell line	Mer phenotype	DHFR activity (A_{340}/min per 10^8 cells)
MM96L	+	0.084 ± 0.027*
MM170	+	0.059 ± 0.007
MM418	+	0.008 ± 0.003
MM127	+	0.071 ± 0.026
HeLa	−	0.198 ± 0.032
MM253cl	−	0.023 ± 0.001

* Mean ± SE of 2–3 determinations.

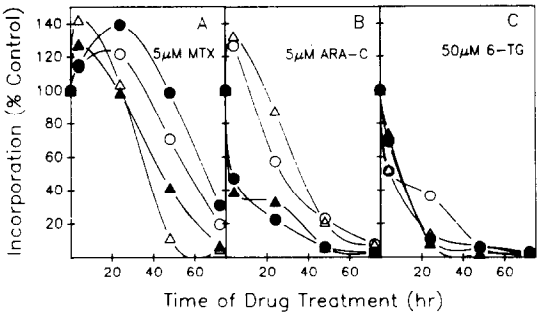

Fig. 5. Temporal response of nucleic acid synthesis in HeLa (△, ▲) and MM96L (○, ●) cells by 5 μM MTX (panel A), 5 μM Ara-C (panel B) or 50 μM 6-TG (panel C). ●, ▲: [^{14}C]Thymidine incorporation (DNA synthesis). ○, △: [^{3}H]Uridine incorporation (RNA synthesis).

tested (Table 3), indicating that 6-TG sensitivity is not associated with the Mer$^-$ phenotype. With this drug the sensitivity of the viral capacity assay in distinguishing sensitive from resistant cells was less than that of clonogenic survival, but for 6-TG the reduction in sensitivity was only about two fold.

Ara-C strongly inhibited the viral capacity of the sensitive MM69L but had much less affect on three resistant lines (Table 3). The viral capacity assay identified a group of cell lines which were very resistant to this agent (MM418, MM466, MM470), but no relationship with the Mer$^-$ phenotype was observed. The cell line MM470 showed almost no inhibition of viral capacity with even the highest doses of this drug, a similar response to that observed for this cell line with MTX.

For all three drugs, inhibition of DNA or RNA synthesis 1–72 hr after commencement of treatment correlated poorly with cellular sensitivity (Fig. 5).

DISCUSSION

The present data showed that inhibition of adenovirus replication in the viral capacity assay correlated qualitatively with cellular sensitivity to the antimetabolites methotrexate, 6-thioguanine and cytosine arabinoside in four human tumour cell lines for which clonogenic survival data were available. It should therefore have sufficient sensitivity to identify tumours likely to give a significant response *in vivo*. The application of this assay to primary cultures of tumour biopsy samples [10] may therefore provide a rapid indication of the outcome of chemotherapy with these agents, and avoid the problems of clonogenic assays with agents such as MTX [14, 15].

The aim of the additional studies was to determine whether the viral capacity assay (i) was superior to nucleoside incorporation for predicting chemosensitivity, (ii) could contribute to an understanding of the mechanism of action of these drugs, and (iii) could elucidate the relationship between DTIC and MTX sensitivity.

Cellular sensitivity to the three antimetabolites correlated poorly with short term (≤48 hr) inhibition of DNA and RNA synthesis compared with inhibition of viral capacity, presumably due to complex interactions between altered pool sizes and inhibited DNA synthesis. For the purpose of predicting sensitivity to MTX and 6-TG isotopically, treatment times of 24–48 hr would be required to discriminate sensitive from resistant cells, at which time thymidine and uridine incorporation by primary cultures of tumours is extremely low (unpublished results). Viral capacity as judged by synthesis of complete virus particles therefore appears to be a more comprehensive test of cytotoxity than cellular DNA or RNA synthesis.

MTX is thought to exert its cytotoxic effect by inhibiting dihydrofolate reductase and other enzymes, leading to decreased purine and thymidylate synthesis [8]. Viral capacity was much more sensitive to the action of this drug than the other two antimetabolites studied, the D_{37} values being lower than those required to similarly affect cell survival. This may reflect a reliance of adenovirus on *de novo* nucleotide synthesis by host cells because 6-TG and ara-C, inhibitors of the salvage pathway, congruently had much less effect on viral capacity than on cell survival. One cell line (MM470) was found to be extremely resistant to MTX and ara-C and therefore warrants investigation of both pathways of nucleotide synthesis.

The inhibition of viral DNA synthesis by MTX declined when the drug was added less than 6 hr before infection. One explanation is that despite MTX effects on *de novo* purine synthesis being essentially complete 5 hr after addition of the agent [25], viral DNA synthesis, like cellular DNA synthesis, is not inhibited until 24 hr after exposure. Alternatively, adenovirus induction of cellular thymidine kinase, for example [26], may initially counteract the inhibitory effect of MTX on thymidylate synthetase by increasing thymidine salvage efficiency.

6-TG is phosphorylated by enzymes of the salvage pathway of nucleotide synthesis and incorporated into DNA, thereby causing cell death [27]. Virus replication was not only less sensitive to 6-TG than to MTX but required a much longer exposure to be effective, in contrast to cellular DNA and RNA synthesis which was inhibited within 24 hr. The latter properties were poor determinants of cell sensitivity and it can therefore be concluded that some other effect not directly dependent on nucleic acid synthesis is associated with resistance to 6-TG in these cell lines. The resistance to 6-TG of the cell lines MM96L and MM466 may be related to a decreased

activity of the salvage enzyme purine nucleoside phosphorylase responsible for phosphorylating this analogue.

Ara-C toxicity relies on the salvage enzyme deoxycytidine kinase for conversion to Ara-CTP, a potent inhibitor of DNA polymerase activity [28], leading to inhibition of chain elongation and chain termination [29]. Virus replication was relatively unaffected by this drug unless a long pretreatment was used, whereas cellular and RNA synthesis was inhibited rapidly. These observations may result from a reliance of viral DNA synthesis on *de novo* generation of nucleosides, virus and cell replication being inhibited by events other than nucleic acid synthesis. Alternatively, the viral DNA polymerase may be intrinsically resistant to incorporating ara-CTP during replication. The MM96L cell line, although relatively resistant to both MTX and 6-TG proved to be extremely sensitive to the action of ara-C. This may be due to decreased intracellular deoxycytidine triphosphate pools [30] resulting from purine nucleoside phosphorylase deficiency [31] because the MM96L line was previously found to be sensitive to deoxyadenosine. In order to define the molecular basis of the patterns of related resistance and sensitivity observed, a complete activity profile of the key enzymes of nucleotide synthesis and of nucleotide pool sizes is now being undertaken.

The relationship between MTX sensitivity and the Mer^- phenotype has been confirmed by these results, and extended to more lines of known Mer status. Mer^- cells were not deficient in DHFR and were not sensitive to 6-TG or ara-C. Overall, the viral capacity results suggest that the cross-sensitivity between MTX and DTIC is linked to a mechanism involving *de novo* nucleotide synthesis. Ribonucleotide reductase (and perhaps other enzymes involved in nucleotide synthesis) was induced in yeast by a methylating agent [32]. The apparent reliance of the Mer^- phenotype on *de novo* nucleotide synthesis suggests an inverse relationship with synthesis of the methyl transferase repair protein, the latter being depleted by methylating agents and being low in Mer^- cells constitutively. Alternatively, Mer^- cells may be deficient in the ligation of strand breaks in parental DNA [23, 24, 33] or of accumulated, newly synthesized fragments [34] which may eventually lead to breaks in the parental DNA strand.

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