Vol. 117, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1983 Pages 30-36

COORDINATE INHIBITION OF DNA SYNTHESIS AND THYMIDYLATE SYNTHASE ACTIVITY FOLLOWING DNA DAMAGE AND REPAIR

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Received October 12, 1983

Two agents, 3-aminobenzamide (3-AB) and beta lapachone, that inhibit repair of mammalian cell DNA damaged by methyl methane sulfonate (MMS), also coordinately blocked both DNA replication (incorporation of ^{3}H-thymidine) and thymidylate synthase (TS) activity. Aphidicolin also inhibited both ^{3}H-TDR incorporation and TS in damaged cells, the former more strongly than the latter, in a manner not coordinated with lethality. It is proposed that the DNA lesions created by MMS and modified by repair inhibit semiconservative DNA synthesis by allosterically interacting with the DNA replication replitase complex, so as to block its overall function and also the activity of TS, one of its enzymes.

DNA damage and semi-conservative DNA replication are interrelated. Agents such as MMS decrease synthesis of new DNA (1), and DNA synthesis is necessary for killing and mutation in damaged cells (2,3). Recent work from our laboratory shows that DNA replication is necessary for the lethality caused by 3-AB in MMS treated cells. In addition, both 3-AB and beta-lapachone, which inhibited DNA repair, also inhibited or delayed replicative DNA synthesis in MMS damaged cells (Boorstein and Pardee, unpublished observations). The experiments presented here illustrate interactions between damaged DNA and the DNA replicative system.

Interactions in mammalian cells of the enzymes of deoxynucleotide biosynthesis and of DNA replication in a multienzyme complex which has been named replitase have been reported (4,5). One enzyme of this complex, thymidylate synthase, which is responsible for the conversion of dUMP to TMP, is func-

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Abbreviations: 3-aminobenzamide, 3-AB; methyl methane sulfonate, MMS; thymidine, TDR; thymidylate synthase, TS; phosphate buffered saline, PBS; trichloracetic acid, TCA.

0006-291X/83 $1.50

tional in vivo only in S phase due to its association with replitase; although in vitro it is active at all stages of the cell cycle (6). Inhibition in vivo of any of three enzymes of this complex--DNA polymerase, ribonucleotide reductase, or topoisomerase--produces coordinate inhibitions of thymidylate synthase and DNA biosynthesis (7). It was thus of interest to see whether repair inhibitors also coordinately inhibited DNA replication and enzymes of this complex.

MATERIALS AND METHODS

Cell culture methods. The normal human foreskin fibroblast strain GM 3652 was purchased from the Human Genetic Mutant Cell Repository, Camden, New Jersey.

Methods for cell culture, synchronization with hydroxyurea, and colony formation have been described (Boorstein and Pardee, submitted).

Assay of DNA biosynthesis. Cells were treated with 2.5 μCi/ml carrier free 6-^{3}H-dUrd (24.5 Ci/mmol, NEN) or ^{3}H-TdR (78.9 Ci/mmol, NEN) during the indicated interval, in addition to other treatments. Cells were washed three times with ice cold PBS, incubated at 4oC for 20 min with 5% TCA, then washed with 5% TCA, and the TCA insoluble material was dissolved in 0.2 N NaOH. 0.5 ml aliquots were counted to determine ^{3}H incorporation.

Measurement of thymidylate synthase activity in intact cells. This activity was measured by release of ^{3}H-water from 5-^{3}H-dUrd according to the methods of Reddy (6), based on Tomich, et al. (8). Background was determined by incubating an equal amount of 5-^{3}H-dUrd in the absence of cells. Control experiments have been done to demonstrate that this assay measures in vivo thymidylate synthase activity and not nucleotide pool or nucleoside transport effects (Reddy and Pardee, in preparation).

Drugs. 3-AB was purchased from Pfalz and Bauer. Aphidicolin was provided by the National Cancer Institute. MMS was purchased from Aldrich. Beta-lapachone was a gift of Ciba-Geigy of India, Ltd. Other drugs and chemicals were purchased from Sigma or Aldrich.

RESULTS

Beta-lapachone inhibits DNA synthesis and TS activity preferentially in MMS damaged cells. In cells released from a hydroxyurea G_1/S block and treated with MMS, beta-lapachone inhibited DNA synthesis and in vivo thymidylate synthase activity to a greater extent than when added to untreated cells (Figure 1). The dose responses were similar for both activities and for cell survival (Table 1). DNA synthesis was inhibited similarly, regardless of whether ^{3}H-thymidine or ^{3}H-deoxyuridine incorporation was measured. Thus in addition to enhancing lethality, this combination of drugs also inhibited two S phase specific activities.

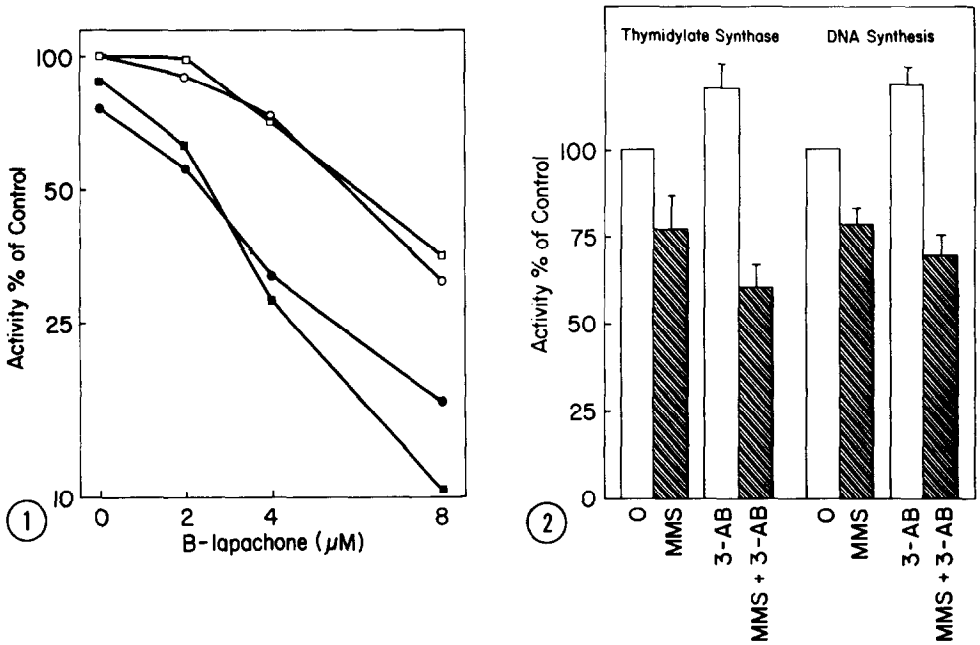

Figure 1. Effect of beta-lapachone on thymidylate synthase activity and DNA synthesis in MMS treated human cells. Normal human fibroblasts GM3652 were released from a G_1/S block and treated with 0.5mM MMS for 1 hour (closed symbols) or were untreated (open symbols). The cells were then washed and treated with beta-lapachone for three hours, after which thymidylate synthase activity (○ , ●) and DNA biosynthesis (□ , ■) were measured.

Figure 2. Effect of 3-AB on thymidylate synthase activity and on DNA biosynthesis. Cells were synchronized at G_1/S and treated with MMS (shaded bars) or were untreated (open bars) as in Figure 1. Cells were then incubated with or without 4 mM 3-AB for 3 hours as indicated. Thymidylate synthetase activity and DNA biosynthesis were measured after this interval. Data are the average of four experiments for thymidylate synthetase and three for DNA biosynthesis. Error bars represent the standard deviation of the mean of the duplicate determinations.

3-AB inhibits both DNA synthesis and thymidylate synthase activity in MMS damaged cells. 3-AB, an inhibitor of DNA repair, also decreased both thymidylate synthase activity and DNA synthesis in MMS treated cells as compared with untreated cells (Figure 2). 3-AB alone slightly stimulated both activities, but MMS alone inhibited both activities. Thymidylate synthase activity was reduced 25 $\pm$ 10% by MMS treatment alone; MMS treatment followed by 3-AB reduced this activity by 48 $\pm$ 6% relative to undamaged cells incubated with 3-AB. For DNA synthesis, these reductions averaged 21 $\pm$ 5% in the absence of 3-AB, and 44 $\pm$ 2% in the presence of 3-AB. Thus, with 3-AB (mM) as with beta-lapachone (μM), DNA synthesis and thymidylate synthase activity were inhibited in parallel.

TABLE 1

Thymidylate synthase activity, DNA biosynthesis and cell survival in cells treated with MMS, 3-AB and beta-lapachone.

MMS	3-AB	Beta-lapachone	Thymidylate Synthase	DNA Biosynthesis	Survival
-	-	-	100	100	100
+	-	-	77	78	81
-	+	-	119	118	99
+	+	-	63	69	53
-	-	+	72	71	92
+	-	+	32	29	37
-	+	+	80	91	80
+	+	+	34	33	10

Cells at the G_1/S boundary were treated with 0.5 mM MMS as indicated for one hour and then with 4 mM 3-AB or 4 μM beta-lapachone for three hours. Thymidylate synthase or DNA synthesis were measured during this interval. To assess lethality, cells after the three hour incubation were replated at 500 per 60mm dish and colony formation after two weeks incubation, with a media change after two days, was determined. For all measurements, data are presented as percent of untreated controls.

Correlation of thymidylate synthase activity, DNA synthesis, and cellular lethality. Cells were treated with MMS, and then with 3-AB, beta-lapachone, or both. Thymidylate synthase activity, DNA synthesis, and cell viability were then measured (Table 1). DNA synthesis correlation with thymidylate synthase activity is shown in Figure 3 (slope = 0.95 and $r^2 = 0.97$). Lethality

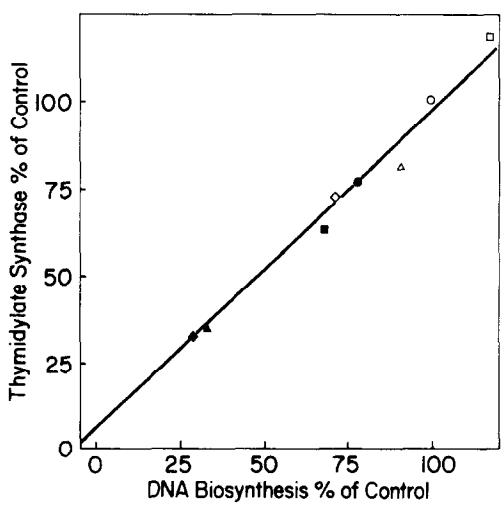

Figure 3. Correlation of thymidylate synthase activity and DNA biosynthesis in cells damaged by combinations of agents. Data from columns 4 and 5 of Table 1 are plotted. Solid symbols represent MMS treated cells and open symbols represent cells without MMS. Treatments after removal of MMS were: none (○ , ●), 3-AB (□ , ■), beta-lapachone (△ , ▲) or both 3-AB and beta-lapachone (◇ , ◆). The line drawn was determined by linear regression.

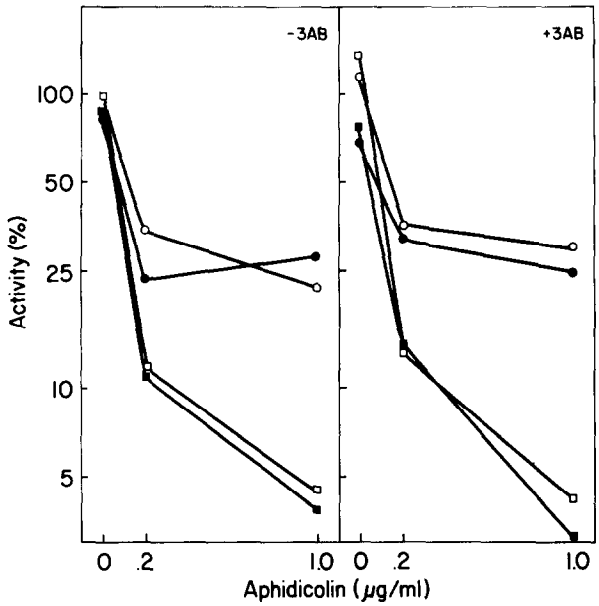

Figure 4. Aphidicolin inhibition of DNA synthesis or thymidylate synthesis. 4A. Cells were synchronized at G_1/S and treated with MMS (closed symbols) or untreated (open symbols) as previously. They were then incubated with indicated doses of aphidicolin for 3 hours and thymidylate synthase (○ , ●) or DNA synthesis (□ , ■) were measured. 4B. Cells were treated as above except that 4 mM 3-AB was present concurrently during the aphidicolin treatment. Data represent averages of two experiments each done in duplicate.

was also well correlated with both thymidylate synthase activity (slope = 0.98, r^2 = 0.81) and with DNA synthesis activity (slope = 0.93, r2 = 0.78).

Aphidicolin inhibition of DNA synthesis and thymidylate synthase activity. In contrast with beta-lapachone and 3-AB, aphidicolin inhibited DNA synthesis to a greater degree than it did TS activity. Pretreatment of cells with MMS did not change inhibition of either of these activities (Figure 4A). 3-AB alone produced greater inhibition of both these activities in MMS treated cells, relative to undamaged cells; this difference was largely eliminated by aphidicolin (Figure 4B). Aphidicolin did not increase lethality of MMS, plus or minus 3-AB (Boorstein and Pardee, submitted).

DISCUSSION

Interfering with repair of MMS damage similarly inhibited colony formation, DNA synthesis and TS activity in human cells. Damage alters DNA struc-

ture, which could decrease DNA polymerase (alpha) activity as long as it remains. Other enzymes in the replitase complex such as TS could be inhibited by allosteric interactions in the replitase complex. The tight relationship between TdR incorporation and thymidylate synthase activity strongly suggests that these two effects are linked.

Alternative explanations for the effects on thymidylate synthase activity include direct inhibition by the repair inhibitors, or feedback inhibition by a metabolite. It is unlikely that either 3-AB or beta-lapachone would inhibit both DNA synthesis and thymidylate synthase independently, and to the same extent in untreated or MMS treated cells. Furthermore, drugs which inhibit thymidylate synthase in vivo, most likely by an allosteric mechanism, do not inhibit this solubilized enzyme in extracts (7). It is also unlikely that thymidylate synthase is inhibited by feedback inhibition, since supraphysiological levels of dTMP or TTP (9,10) do not inhibit its activity either in permeabilized cells (6) or in cell extracts (Reddy, personal communication).

The close correlation between lethality and inhibition of DNA synthesis or thymidylate synthase activity suggests that the same event, unrepaired DNA damage is responsible. The DNA replitase complex could be inhibited in proportion to the amount of remaining damage, as could cell survival. 3-AB and beta-lapachone appear to block repair and convert non-lethal MMS damage to lethal damage (Boorstein and Pardee, submitted).

Aphidicolin inhibited in vivo DNA synthesis more strongly than thymidylate synthase activity in human cells. Similar results have been demonstrated in CHEF/18 cells (6). Furthermore, this drug eliminated the differential effects on these activities caused by MMS and 3-AB (Figure 4); it similarly protected cells from 3-AB lethality after MMS damage (Boorstein and Pardee, submitted). While 3-aminobenzamide and beta-lapachone appear to act on replitase indirectly through damaged DNA, aphidiciolin acts directly on an enzyme of the complex.

ACKNOWLEDGEMENTS: We thank Rose Kindy for technical assistance and Marjorie Rider for preparing the manuscript. This work was supported by Grants CA 22427 and NIGMS T32 GM 7753 from the U.S. Public Health Service.

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