Vol. 130, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1985 Pages 249-256

INHIBITION OF INITIATION OF SIMIAN VIRUS 40 CHROMOSOME SYNTHESIS BY DIHYDROXYANTHRAQUINONE

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Received May 20, 1985

SUMMARY: The mode of action of dihydroxyanthraquinone, a new antitumor drug, on eucaryotic chromosome structure and function was investigated using simian virus 40 as a model system. Dihydroxyanthraquinone specifically inhibited initiation of viral replicons. Little or no viral DNA synthesis was recovered in cells after the removal of the drug. Elongation and termination of DNA already initiated could proceed continuously to completion in drug-treated cells. The drug appeared to be stably associated with viral chromosomes in cells. The irreversible inhibition of replicon initiation might contribute to its anti-proliferative and anti-neoplastic activity.

Dihydroxyanthraquinone(DHAQ) is a newly synthesized anticancer agent which has shown potent anti-neoplastic activity in several animal tumor systems [1, 2, 3]. It is currently under clinical study for its therapeutic value in human neoplasms [4,5]. The exact mode of action of DHAQ in mammalian cells is presently not clear. It exhibits an anti-proliferative effect on several cultured cells [6,7] and is capable of inducing a high frequency of chromosome abnormality [8]. The exact step(s) of chromosome replication (i.e. initiation, elongation and termination) which is impaired by DHAQ is unknown. In papovaviruses (simian virus 40 and

Abbreviations: SV40(I)DNA, SV40 covalently closed supercoiled, monomeric DNA; SV40(II)DNA, double-stranded circular SV40 DNA containing an interruption of the phosphodiester bonds in at least one of the two strands; SV40(RI)DNA, SV40 replicative intermediates containing a superhelical region of unreplicated parental DNA and two nonsuperhelical regions of newly replicated DNA; Hepes, N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid; DHAQ, 1,4-dihydroxy-5,8-bis((2-(2-hydroxyethyl amino) ethyl amino)-9,10anthracenedione; Ametantrone(AQ), 1,4-bis((2-(2-hydroxyethyl-aminoethyl) amino)-9,10-anthracenedione; Quinizarin, 1,4-dihydroxy-anthraquinone.

0006-291X/85 $1.50

polyoma), viral chromosomes share many similar structural features with that of mammalian cells [9, 10]. Viral DNA replication, with the exception of initiation of new replicons, relies entirely on host factors. Thus, simian virus 40 (SV40) has been widely used as a model system to elucidate the regulatory mechanism of mammalian chromosome replication [10, 11].

In this study, we present evidence that (a) DHAQ inhibits initiation of viral replicons; (b) replicating SV40 chromosomes can continue elongation and termination into mature chromosomes in treated cells; (c) viral chromosome-DHAQ complexes formed in vivo can be encapsidated into virus particles. This study suggests that the anti-proliferative effect of DHAQ on cells is a result of the irreversible inhibition of replicon initiation by a specific interaction of DHAQ with nuclear chromosomes.

METHODS

Virus and cells: African green monkey kidney (CV-I) cell line and SV40 stocks were prepared as described previously [12]. For all the experiments described, confluent cells were infected with SV40 at a multiplicity of infection of 50.

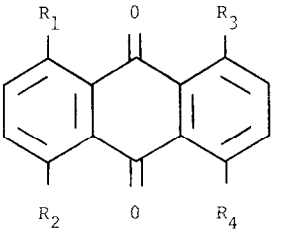
Anthraquinone derivatives: Adriamycin (Sigma) and DHAQ were prepared in 0.2 mM acetic acid at 2 mg/ml. Ametantrone (AQ, NCS No. 196473) was received from Dr. L.H. Kedda. [^{14}C]DHAQ(32.2 μCi/mg) was obtained from Dr. R.R. Engle at National Cancer Institute. Ametantrone and Quinizarin (Aldrich) were prepared in 95% ethanol. The chemical structures of DHAQ, ametantrone and quinizarin are show in Fig. 1.

Sedimentation analysis of viral chromosomes and virions: At 36 hours after infection, cells were incubated in medium containing [^{14}C]DHAQ (5 μCi/ml) for 8 hours. Cells were lysed by hypotonic buffer and sedimented by centrifugation at 16,000 x g for 15 min. at 2°C. Supernatant containing both viral chromosomes and virions was directly layered onto a linear 5-30% sucrose gradient in 10 mM Hepes, pH 7.8, 5 mM KCl, 0.5 mM $MgCl_2$ and centrifuged at 38,000 rpm in a Beckman SW41 rotor for 90 min. at 4°C [12]. Fractions were collected and the amount of acid-insoluble radioactivity was measured with a liquid scintillation counter.

Gel electophoresis: Viral DNA synthesized in DHAQ-treated cells was electrophoresed on 1.4% cylindrical agarose gels (0.6 cm x 15 cm) at 3 V/cm for 15 hours at room temperature. The DNA fragments generated by digestion of DNA with restriction endonucleases Hind III or Hind II + III (P.L. Biochemicals) were analyzed as described previously [15].

RESULTS

We have previously shown that DHAQ inhibits SV40 DNA synthesis in a dose-dependent manner [15]. To determine which of the replication



Quinizarin: $R_1 = R_2 = H$; $R_3 = R_4 = OH$

Ametantrone(AQ): $R_1 = R_2 = H$; $R_3 = R_4 = NHCH_2CH_2NHCH_2CH_2OH$

DHAQ: $R_1 = R_2 = OH$; $R_3 = R_4 = NHCH_2CH_2NHCH_2CH_2OH$

FIGURE 1. Chemical structures of DHAQ, AQ and quinizarin.

processes i.e. initiation, elongation, and termination was inhibited by DHAQ, completion of the SV40(RI)DNA was examined in cells. Essentially, no difference was observed in the amount of supercoiled SV40(I)DNA synthesized in cells treated or untreated with DHAQ(Table 1). The rate of completion of replicating chromosomes into mature chromosomes in the presence and absence of DHAQ was the same. The rate of elongation of newly synthesized daughter strands was very similar in cells treated or untreated with DHAQ as judged by the analysis of the progression of linear-stranded SV40 DNA in alkaline sucrose gradients. The results implied that DHAQ specifically

TABLE 1. COMPLETION OF SV40(RI)DNA SYNTHESIS IN CELLS TREATED WITH DHAQ [a]

DHAQ 10 μM)	Chase time (min.)	SV40 DNA(%) supercoiled SV40(I)DNA	SV40 DNA(%) relaxed SV40(RI)+SV40(II)DNA
Without DHAQ	0	2.2	97.8
Without DHAQ	15	40.1	59.9
With DHAQ	15	38.6	61.4
Without DHAQ	30	89.0	11.0
With DHAQ	30	87.2	12.8

[a] Infected cells were pulse-labelled with [^{3}H]thymidine for 5 min. at 36 hours after infection. Cells were then chased with 10 μM thymidine in DMEM with or without DHAQ. At different times of incubation, cells were lysed by the method of Hirt [13] and viral DNA in the Hirt supernatant was analyzed on 5-20% alkaline sucrose gradients [14]. Each value is the average of values from three independent experiments.

inhibited initiation of SV40 DNA and had no effect on SV40 DNA elongation and termination processes. To examine whether the synthesis of SV40 DNA in treated cells was the result of elongation of DNA already initiated, supercoiled SV40(I)[^{3}H]DNA isolated from cells pre-treated with 10 μM DHAQ was digested with restriction endonuclease Hind III. More than 50% of the radioactivity was found in fragment A where the SV40 DNA termination region has been determined [10] (Fig. 2). A similar result was seen with SV40(I)[^{3}H]DNA digested with Hind II + III where radioactivity was mainly found in fragments B and G (Fig. 2). The results indicated that the majority of DNA molecules completed in treated cells were from the late replicating DNA.

When cell lysates prepared from infected cells that were incubated with medium containing [^{14}C]DHAQ for 5 hours were analyzed on sucrose gradients, [^{14}C]radioactivity was found associated with both viral chromosomes (70S) and mature virions (250S) as judging from sedimentation values and sensitivities to DNase (50 μg/ml)(Fig. 3). [^{14}C]DHAQ associated with viral chromosomes could continuously be chased into mature virions. [^{14}C]radioactivity remained associated with viral chromosomes after mild protease and detergent treatment. It is likely that inhibition of viral initiation is the result of direct interaction of DHAQ with viral chromosomes. However, we do not know the exact nature of this interaction. DHAQ could be removed from chromosomes during deproteinzation by phenol.

DHAQ, like other anthraquinone derivatives, intercalates DNA *in vitro* [16, 17]. To determine whether or not the inhibitory effect of DHAQ or other anthraquinone derivatives such as quinizarin, adriamycin, and ametantrone (Fig. 1) on DNA synthesis was reversible, viral DNA synthesis in cells was measured after the removal of DHAQ or other anthraquinone derivatives. The inhibitory effect of ametantrone and quinizarin on DNA synthesis was reversible since the rate of viral DNA synthesis returned to the same level as that of controls within 24 hours after removal of the

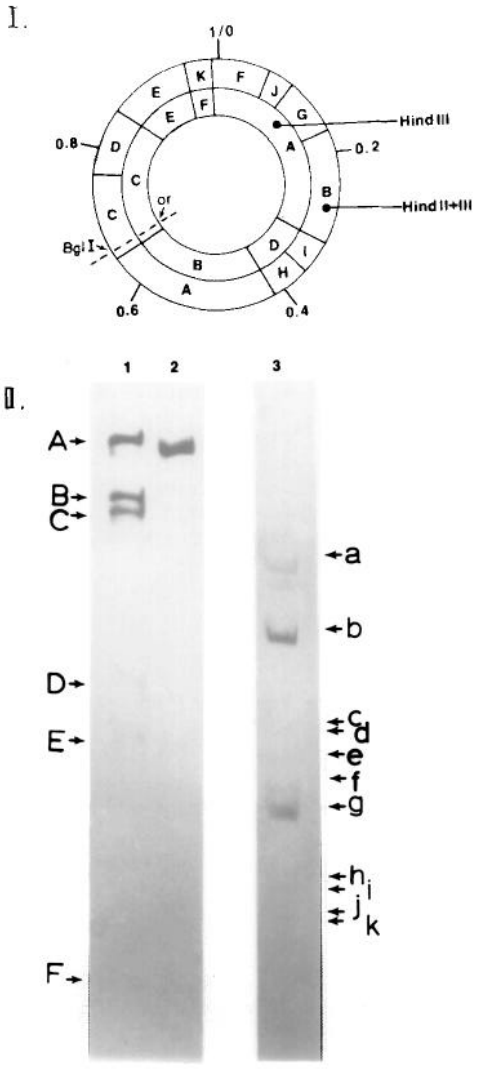


FIGURE 2. Gel electrophoresis of SV40 DNA digested by restriction endonucleases Hind III and Hind II + III. Panel I: The diagram indicates restriction endonuclease cleavage maps of SV40 DNA. Panel II: SV40 DNA was isolated from cells pulse-labelled with [^{3}H]thymidine for 5 min. and subsequently chased for 30 min. in the absence (lane 1) and presence(lane 2, 3) of 10 μM DHAQ. [^{3}H]SV40(I)DNA was purified from 1.4% agarose gels and then digested by restricition endonuclease Hind III (lane 1, 2) or Hind II + III (lane 3). DNA fragments generated by Hind III were separated on a 3.5% acrylamide/0.5% agarose slab gel at 10 mA for 15 hours. DNA fragments generated by Hind II + III were analyzed on a 3.5-7% polyacrylamide gradient gel at 12 mA for 15 hours. The gel was stained, dried, and fluorography was carried out according to the method of Bonner and Laskey [19].

drug (Table 2). However, less than 10% of viral DNA synthesis was detected in cells treated with 2 μM or 10 μM DHAQ. The results indicated that SV40 replicons in DHAQ-treated cells were unable to re-initiate a second

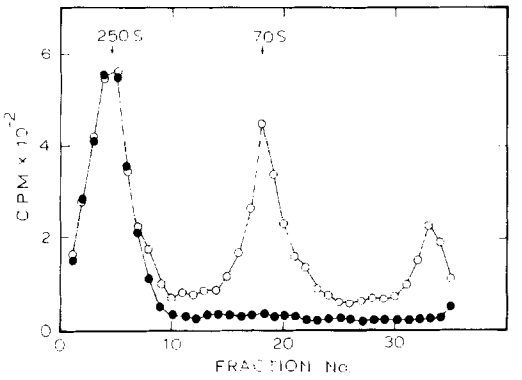


FIGURE 3. Sedimentation analysis of SV40 mature chromosomes and virions synthesized in DHAQ-treated cells. At 36 hours after infection, cells were incubated in medium containing [^{14}C]DHAQ (5 μCi/ml) for 8 hours. Cell lysate was prepared and analyzed on a linear 5-30% sucrose gradient as described in Methods. Sedimentation was from right to left. Fractions were collected from the bottom of the tubes. Acid-insoluble radioactivity in fractions treated (●) or untreated (○) with DNAse I (20 μg/ml) was determined by liquid scintillation counting.

round of replication. Whether the low-level DNA synthesis observed in cells after removal of the drug represents replication of residual replicons or repair synthesis remains to be determined. Comparing the chemical structures among the drugs used, it seemed that the 1, 4-

TABLE 2. RECOVERY OF SV40 DNA SYNTHESIS IN CELLS AFTER REMOVAL OF DRUGS [a]

TREATMENT	%, DNA SYNTHESIS AFTER REMOVAL OF DRUG FOR VARIOUS TIMES			
	0 HR.	6 HR.	12 HR.	24 HR.
CONTROL	100	100	100	100
AMETANTRONE (10.0 μM)	10.1	51.2	76.4	85.9
QUINIZARIN (10.0 μM)	28.4	54.3	77.1	92.5
DHAQ (2.0 μM)[b]	12.8	7.9	6.6	8.2
(2.0 μM)	3.6	7.4	8.4	10.4
(10.0 μM)	1.4	3.6	4.6	5.0
ADRIAMYCIN (20.0 μM)	4.6	72.8	65.5	63.3

[a] At 30 hours after infection, cells were incubated with medium containing the indicated drugs for 90 min. Cells were then incubated with fresh medium in the absence of the drug. At different times after incubation, cells were labelled with [^{3}H]thymidine for 30 min. and viral DNA synthesis was determined as described in Methods. The zero time (0 hr.) represented the beginning of the incubation of cells with fresh medium. In control samples, the amount of SV40 DNA synthesis measured by the incorporation of [^{3}H]thymidine into acid insoluble materials was 82,994 cpm (0 hr.); 85,431 cpm (6 hrs.); 39,395 cpm (12 hrs.); 44,139 cpm (24 hrs.). Each value is the average of triplicated samples.

[b] Infected cells were treated with DHAQ for 30 min.

dihydroxyl groups and the aminoalkylamino side chain on DHAQ were important for it to exert its irreversible inhibition of replicon initiation.

DISCUSSION

This study indicated that DHAQ specifically inhibited the initiation of viral replicons, but not elongation and termination. The inhibition of initiation by DHAQ was not reversible after the removal of drug from culture. Complete inhibition of viral DNA synthesis could be achieved when all the available replicons were inactivated by DHAQ. At 36 hours after infection, all viral replicons were virtually inactivated for re-initiation at 5 μM DHAQ within 40 min. [15]. A similar inhibitory effect of DHAQ on cellular DNA synthesis was observed in normal CV-I cells. Cells treated with 10 μM DHAQ for one hour, were unable to divide, although they were morphologically unaltered. Cells could not be replated after trypsinization from monolayers. Chromosome condensation was, however, not seen under electron microscopy (data not shown). The anti-proliferative effect of DHAQ on cultured cells as shown by others [6, 7] was thus possibly caused by irreversible blockage of chromosome initiation.

None of the anthraquinone derivatives used in this study exert inhibition on SV40 DNA elongation and termination. If the anthraquinone derivatives preferentially inhibit the initiation of replicons, it is conceivable that topological constraints will be generated on the chromosome fiber when two adjacent replicons fail to initiate simultaneously in the S phase. The removal of these constraints may lead to the induction of sister-chromatid-exchange(SCE) and chromosome aberrations, as seen in several cultured cells [6, 8]. DHAQ has been shown to have stronger activity than its analogues in the induction of SCEs in Chinese hamster ovary cells [7]. The reason why DHAQ exhibited permanent inactivation of initiation of a new round of SV40 DNA synthesis is not clear. The finding of radioactive DHAQ in both isolated chromosomes and virus particles suggested that DHAQ could interact directly with viral chromosomes. Whether inhibition of initiation is caused by the physical

alteration of the origin of replication, or by the inactivation of factors required for initiation, remains to be determined.

The results obtained from this study suggest that the biological activity of DHAQ is determined by its interaction with cellular chromosomes. The analysis of the drug-chromosome interactions in vivo and in vitro may be another approach for studying the relationship of chromosome structures and functions [10]. Although the metabolism of DHAQ in cells remains to be determined, the irreversible inhibition of chromosome replication and perhaps gene expression by DHAQ is likely to contribute to its high anti-tumor activity with limited adminstrations [2] and long-term toxicity [18] in animals.

ACKNOWLEDGEMENTS

This work was supported by a research grant CA-31565 from National Institutes of Health and small grants from Mid-America Cancer Center. Ms. Xiaojun Ma was a visiting scientist from Wuhan University, China.

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