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Mechanism of action of dichloro-β-D-ribofuranosylbenzimidazole: Effect on *in vitro* transcription

(whole cell extract/RNA polymerase II)

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ABSTRACT **The adenosine analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and its mono- and triphosphate derivatives inhibit RNA polymerase II-specific transcription in an extract of whole HeLa cells. The analog does not inhibit RNA polymerase III-specific adenovirus VA RNA transcription in the whole cell extract. With purified RNA polymerase II under nonspecific transcription conditions, no effect of DRB could be detected. DRB is equally effective in inhibiting *in vitro* transcription from several of the adenovirus promoters and the human ε-globin gene. The inhibitory effects are in the order DRB > DRB monophosphate > DRB triphosphate. Thus DRB acts *in vitro* presumably on systems in which specific RNA polymerase II initiation of transcription occurs and with no detectable effect on premature termination. This will provide a suitable model for study of the molecular mechanism of action of DRB on transcription.**

The nucleotide analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) is a widely used inhibitor of mRNA synthesis in eukaryotic cells (for review, see ref. 1). The action of DRB is selective for RNA polymerase II transcripts; RNA polymerase III 5S and tRNA-specific transcripts and the rates of RNA polymerase I-mediated rRNA synthesis are all not affected (1). Initial reports (2, 3) indicated that DRB acted at or very close to the site of initiation of transcription, on the basis of incorporation of [^{3}H]uridine into specific-size mRNAs of Balbiani rings in *Chironomus* (2) and of the labeling kinetics of HeLa cell mRNA precursors (3). The effect of adding DRB to HeLa cells is very rapid (<5 min). Even in the presence of DRB, however, 30% of the HeLa cell large nuclear RNA can be labeled (3–5), but the role of this RNA, which is not transported to the cytoplasm, remains unclear. By contrast, short, capped RNAs transcribed by RNA polymerase II continue to be made in DRB-treated HeLa cells (4, 6).

Synthesis of specific low molecular weight DRB-resistant RNAs was also described for the globin gene of Friend erythroleukemia cells (7), adenovirus-specific RNA in infected HeLa cells (8, 9), and simian virus 40-specific RNA in infected monkey cells (10). In the case of the adenovirus major late promoter RNA, these short RNA species are initiated at the same site as the viral mRNA precursor transcripts of normal length and are capped (11). Some of these low molecular weight RNAs therefore could represent prematurely terminated mRNA precursors.

DRB has also been shown to inhibit the methylation of 5′-end caps of mRNAs larger than 18 S without affecting the methylation of shorter mRNA-like molecules (5). Transcription of the nuclear low molecular weight RNAs with trimethylated caps shows DRB sensitivity identical to that of the large mRNA precursor RNAs (5). Thus, transcription of short or prematurely terminated capped mRNA-like molecules seems to be resistant to DRB. It has been suggested (6) that DRB acts to enhance premature termination, but from the evidence presented it is difficult to establish whether this phenomenon, which occurs spontaneously in the absence of DRB, is enhanced by the drug or whether these short RNA molecules are just more easily detected due to the reduction in the background and the increased availability of the labeled nucleotide precursors.

Attempts at using *in vitro* systems to analyze the mechanism of action of DRB have indicated that the analog was inactive on isolated nuclei (6) or showed only partial effects (12). Evidence has been presented that DRB triphosphate (DRB-TP) at high concentrations (1 mM) can inhibit purified RNA polymerase II transcription *in vitro* on either calf thymus DNA or poly[d(A,T)] templates (13). However, extensive evidence against phosphorylation of DRB beyond the monophosphate level *in vivo* has been presented (13–15). Because isolated nuclei are not very efficient in *in vitro* initiation of transcription (16, 17), the development of new cell-free transcription systems (18, 19) capable of faithful initiation prompted us to reanalyze the problem of the action of DRB *in vitro*. We have found that DRB and its phosphorylated derivatives [DRB monophosphate (-P) and DRB-TP] can effectively inhibit specific RNA polymerase II-mediated initiation in a whole cell extract at concentrations similar to the concentrations of DRB that inhibit *in vivo* transcription. *In vitro* transcription by RNA polymerase III was not affected.

MATERIALS AND METHODS

HeLa cells were maintained in suspension in Joklik's modified minimal essential medium with 7% (vol/vol) donor horse serum at densities of 3–8 × 10^5 cells per ml. [5-^{3}H]Uridine was obtained from ICN; [α-^{32}P]GTP and [α-^{32}P]UTP were from Amersham.

DRB, initially obtained from A. Egyhazi and N. Fraser and later from Calbiochem–Behring, was dissolved in ethanol at 2 mg/ml and stored at 4°C. DRB-P and DRB-TP (Calbiochem) were stored as aqueous solutions at −20°C.

Cell extracts were prepared for *in vitro* transcription as described (18, 19). Transcription reactions were performed in a volume of 25 μl with DNA concentrations of 15–25 μg/ml and whole cell extracts at 10–15 mg of protein per ml. The template was a *Bam*HI fragment of human ε-globin DNA (20, 21) inserted into the *Bam* site of pBR322. Nucleotide triphosphates were used at 50 μM, except for ATP which was at 100 μM and the labeled precursor, [α-^{32}P]GTP or [α-^{32}P]UTP, which was at 10 μM (10–400 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels). For RNA polymerase III-mediated VA RNA transcription, whole-cell extract was used as a source of enzyme and 5′dl +10 cloned VA gene (22) was used as a supercoiled template. Reaction

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Abbreviations: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; DRB-P, DRB monophosphate; DRB-TP, DRB triphosphate.

mixtures were processed and analyzed on polyacrylamide/urea gels as described (22).

RESULTS

Although extensively analyzed *in vivo*, the effect of the drug DRB has not been elucidated *in vitro*. Whole cell extracts from HeLa cells are able to initiate *in vitro* transcription correctly at the same sites used to initiate *in vivo* transcription (18, 19). To establish the relationship between the inhibitory activity of DRB *in vivo* and its effect in the *in vitro* transcription system, we assayed the inhibitory effect on rates of RNA transcription in the same tissue culture cells from which the transcription extracts are prepared. RNA was labeled in whole tissue culture cells preincubated for 60 min with various concentrations of DRB. Aliquots of the cells were taken 15, 30, and 60 min after addition of [^{3}H]uridine, RNA was extracted, and incorporation measured in a scintillation counter. Effects on transcription were detected at all DRB concentrations (Fig. 1). No corrections for effects of DRB on the uridine pool were made in this case (6–12) because they represent a difference of only 20–30%. These results agree well with the reported (1, 4) inhibitory effects of DRB on RNA synthesis in HeLa cells. Levels of incorporation of [^{3}H]uridine after 60 min of incubation, which show a larger inhibitory effect of DRB, probably reflect a combination of effects on transcription, processing, and transport rates and probably overestimate the inhibition.

We also tested a partially purified RNA polymerase II, from HeLa cells, under conditions of nonspecific transcription using denatured calf thymus DNA as a template. Table 1 clearly indicates that DRB has no effect on transcription under these conditions. Thus, we conclude that DRB is a specific inhibitor for RNA polymerase II-mediated transcription only under conditions of specific initiation.

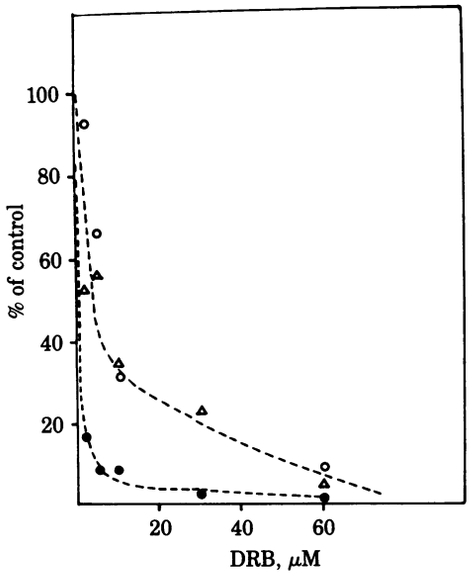

FIG. 1. Effect of DRB on [^{3}H]uridine incorporation in HeLa cells. HeLa cells were preincubated for 60 min with the indicated concentrations of DRB. [5-^{3}H]Uridine (13 Ci/mmol) was added to each culture and aliquots were taken at 15 (○), 30 (△), and 60 (●) min. The cells were lysed with 0.5% $NaDodSO_4$ and treated with proteinase K (25 μg/ml), and aliquots were spotted on DEAE-paper. After the unincorporated nucleotides were washed five times in 0.5 M KH_2PO_4 and twice in water, the filters were dried and assayed for radioactivity in a Beckman LS-8100 liquid scintillation counter. The control values were (per 9×10^4 cells): 9.3×10^3 cpm after 15 min, 43×10^3 cpm after 30 min, and 625×10^3 cpm after 60 min of labeling with [^{3}H]uridine. The 50% inhibition point for the short incubation times (15 and 30 min) was 4–6 μM DRB.

Table 1. Effect of DRB on RNA synthesis by purified HeLa cell RNA polymerase II

DRB, μM	Incorporation cpm	Incorporation % of control
0	52,257 ± 3,926	100
30	53,213 ± 2,132	101.8
60	55,606 ± 5,038	106.4
90	52,779 ± 2,799	100.9

Reaction mixtures (final volume, 20 μl) contained 5 μl of RNA polymerase II purified through DEAE-cellulose, denatured calf thymus DNA (80 μg/ml), 100 μM ATP, 100 μM CTP, 100 μM GTP, 11.7 μM [α-^{32}P]UTP (20 Ci/mmol), 4 mM creatine *P*, 7 mM $MgCl_2$, 70 mM ammonium sulfate, and 0.5 mM dithiothreitol. Incubation was for 60 min at 30°C. Triplicate reaction mixtures were spotted on DEAE-paper, washed, and assayed. Controls containing α-amanitin (1 μg/ml) were included for each reaction. The control contained 2.4% ethanol, as did all the DRB concentrations used. Incorporation into the RNA polymerase II-mediated transcript was 0.8 pmol of UMP per 20-μl reaction mixture per hr. Background, 200 cpm; α-amanitin-resistant incorporation (subtracted for each point), 3,600 cpm.

The development of *in vitro* transcriptional systems able to correctly initiate and transcribe RNAs (18, 19) prompted us to analyze the effect of DRB in these systems. The RNAs synthesized *in vitro* are analyzed on polyacrylamide/urea gels as size-specific run-off products from DNA templates cut with restriction enzymes. With a whole-cell extract as a source of RNA polymerase II and factors and human ε-globin DNA cut with *Bam*HI (20, 21) as template, RNA initiated at the cap site produces a run-off product 460 nucleotides long (20, 21) (Fig. 2, lane 1). Analysis with various concentrations of DRB, DRB-P, and DRB-TP is shown in lanes 2–9. The inhibitory activity of DRB (lanes 2–4) suggests that the inhibition detected in the whole cell extract is similar to the one previously detected in HeLa cell pulse-labeled RNA *in vivo* (4). At high DRB concen-

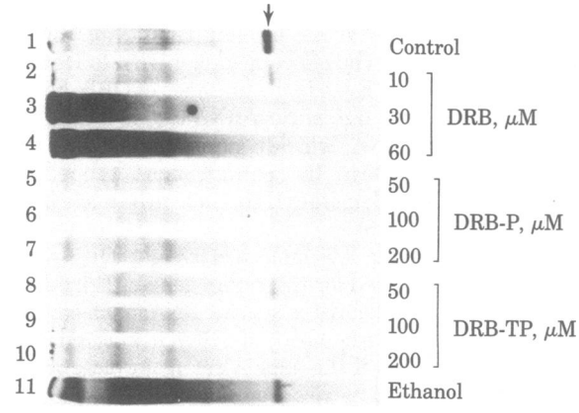

FIG. 2. Effect of DRB derivatives on *in vitro* transcription by RNA polymerase II. Whole-cell extract (19) (10 μl) was incubated with human ε-globin plasmid DNA (20, 21) cut with *Bam*HI in 25-μl reaction mixtures. In addition to the normal components of the reaction, DRB and its phosphorylated derivatives were added at the concentrations indicated on the right for lanes 2–10. Lane 1 is a complete reaction and lane 11 contained the same amount of ethanol (2% final concentration) as lane 4. Reaction mixtures were incubated for 60 min at 30°C with 10 μM [α-^{32}P]UTP (40 Ci/mmol) as the labeled precursor. The extracted RNAs were analyzed on 4% polyacrylamide/urea gels, with electrophoresis from left to right. Autoradiography was overnight at −70°C with Lightning Plus (Du Pont) screens and XRP-1 film (Kodak). The size of the 460-nucleotide run-off product (indicated by the arrow) was determined by comparison with *Hae* III-digested end-labeled φX174 DNA fragments (not shown).

trations, more of the larger RNA transcripts were detected, an effect attributable to the solvent concentration alone (lane 11). Higher concentrations of DRB-P or of DRB-TP were required to attain comparable levels of inhibition of the specific RNA run-off product (lanes 5–10). Because DRB-P and DRB-TP are readily soluble in water, no ethanol effects were seen. When DRB-TP was substituted for ATP, no RNA polymerase II run-off RNA was detected (results not shown). Addition of the inhibitor of RNA polymerase II, α-amanitin, at 1 μg/ml resulted in complete inhibition of the 460-nucleotide RNA transcript but not of the RNAs of the upper bands (results not shown).

We also analyzed the effect of DRB and its phosphorylated derivatives on RNA polymerase III. Transcription systems containing a crude form of RNA polymerase III and supercoiled DNA template faithfully initiate and terminate *in vitro* transcription (18, 23). A cloned adenovirus VA RNA gene (22) was utilized as template, and the whole-cell extract (19) was the source of RNA polymerase III and factors. DRB, DRB-P, and DRB-TP had no inhibitory effect on the RNA polymerase III-mediated transcription of the VA gene, except at the highest concentrations (140 μM DRB) (Fig. 3). DRB-TP had a slight but consistent stimulatory effect on the RNA polymerase III-mediated VA RNA transcription. *In vivo* experiments also indicate that 5S RNA and tRNA transcription are not affected by DRB (14).

The effect of DRB and the phosphorylated derivatives *in vitro* can thus be compared to the effect *in vivo*. Fig. 4*B* provides a summary of several experiments similar to the one in Fig. 4*A*. In one particular experiment (Fig. 4*A*) the ethanol concentration was maintained at 0.5% for all DRB concentrations, and less of the larger RNA transcripts was detected than when a higher concentration of solvent was used (compare Fig. 2, lane 11). Remarkably, no discrete lower molecular weight RNAs were detected in the presence of DRB (compare with Fig. 2). The 50% inhibition values are between 2 and 6 μM DRB, in good agreement with the *in vivo* value..Note that higher levels of inhibition were attained with DRB than with DRB-P. This, together with the *in vivo* evidence, would suggest that the active compound is the nucleoside, DRB. In all cases, a small fraction of residual DRB-resistant transcription was observed.

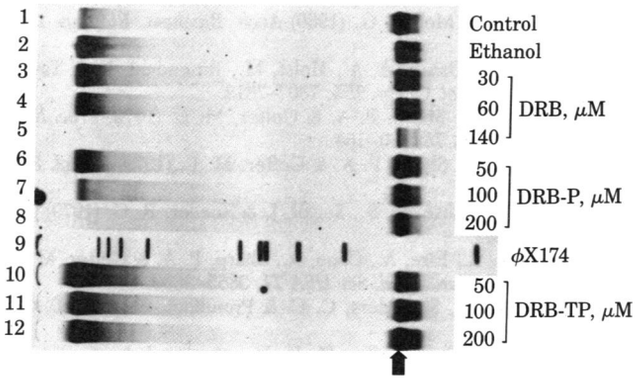

FIG. 3. Effect of DRB and its derivatives on *in vitro* transcription by RNA polymerase III. Incubation conditions as in Fig. 1 were used to transcribe the adenovirus VA RNA-containing (22) supercoiled plasmid with [α-^{32}P]GTP (40 Ci/mmol) as the labeled RNA precursor. Lane 1 contained a complete reaction mixture and lane 2 was an ethanol (2%) control. The concentrations of DRB and its derivatives are indicated on the right for lanes 3–12. Lane 9 shows the position of the end-labeled *Hae* III-digested ϕX174 DNA marker; the fragments are 1,353, 1,078, 872, 603, 310, 281, 271, 194, and 118 nucleotides long. Electrophoresis (from left to right) was in a 4% polyacrylamide/urea gel. The position of the VA RNA (150–160 nucleotides) is indicated by the arrow.

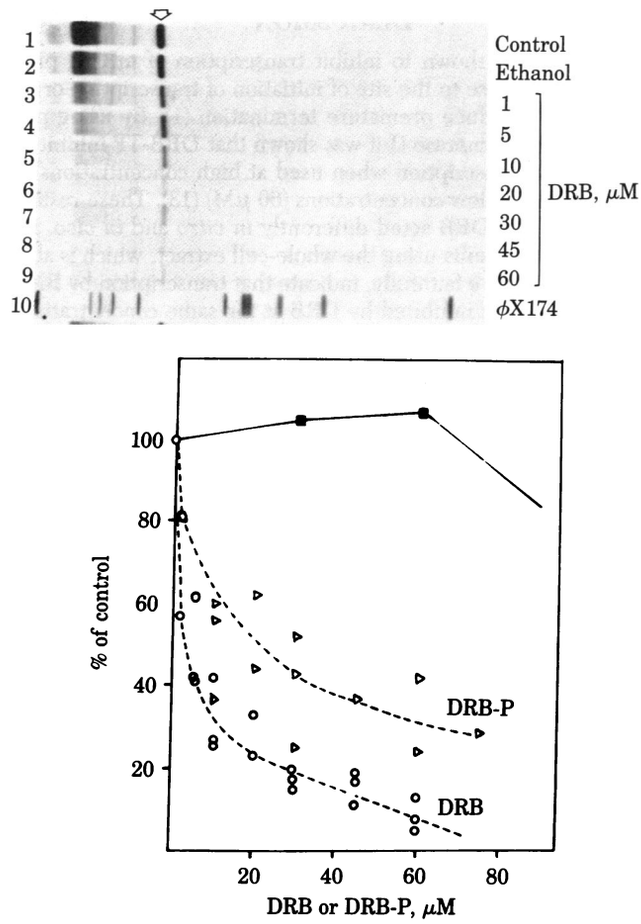

FIG. 4. Effect of DRB and DRB-P on RNA synthesis by RNA polymerases II and III in cell-free systems. (*A*) Autoradiogram for a typical transcription experiment for RNA polymerase II, in which DRB concentration in the *in vitro* incubation reaction was varied. Lane 1 shows the control and lane 2 a control with the constant ethanol concentration used in this experiment (0.5% final concentration). The DRB concentrations for lanes 3 to 9 are given on the right. Lane 10 contains the *Hae* III-digested ϕX174 DNA marker, with bands 1,353, 1,078, 872, 603, 310, 281, 271, 194, and 118 nucleotides long. The arrow indicates the 460-nucleotide ε-globin run-off RNA band. (*B*) Summary of the experiment in *A* and similar experiments. The globin-specific 460-nucleotide run-off RNA band was cut out of the gel and the percentage of inhibition of this band was plotted against the ethanol control in the DRB experiments (○) or against the normal reaction in the case of DRB-P (▷). Also shown are results with RNA polymerase III (■) taken from the experiment in Fig. 3. Background values for gel slices taken from adjacent regions of the gel were subtracted. Incorporation in the control 460-nucleotide RNA band (lane 1) corresponds to 56 fmol/25-μl reaction mixture per hr, as monitored by the incorporation of [α-^{32}P]GTP (40 Ci/mmol). The complete autoradiogram up to the 118-nucleotide DNA fragment of the ϕX174 marker is shown in *A*.

No discrete premature termination RNA products larger than 100 nucleotides could be detected in this *in vitro* reaction. RNAs smaller than 100 nucleotides cannot be distinguished due to the background inherent in this *in vitro* transcriptional system (Fig. 4*A*). It should be noted that premature termination products of sizes ranging from 460 to 100 nucleotides in length cannot be excluded at this time because they would produce a smear not detectable in our assay. In addition, smaller discrete RNA bands would be of decreased intensity. We could not detect these even when autoradiographic exposure times were increased 10-fold.

DISCUSSION

DRB has been shown to inhibit transcription of mRNA precursors at or close to the site of initiation of transcription or to enhance or produce premature termination (1). By using purified RNA polymerase II it was shown that DRB-TP inhibited nonspecific transcription when used at high concentrations (1 mM) but not at low concentrations (60 μM) (13). These results suggested that DRB acted differently *in vitro* and *in vivo*. By contrast, our results using the whole-cell extract, which is able to initiate *in vitro* faithfully, indicate that transcription by RNA polymerase II is inhibited by DRB at the same concentrations that inhibit hnRNA synthesis *in vivo*. No specific effects on termination or premature termination were detected *in vitro* although a small fraction of the transcripts remained DRB-resistant. The effect of DRB is probably reversible because extracts prepared from cells treated with DRB for 1 hr were active in transcription and showed similar DRB sensitivities to untreated HeLa cell extracts (results not shown). This is probably due to the fact that DRB was washed out (either with the ammonium sulfate precipitation or with the dialysis step) during the preparation of the extract. The use of the cell-free extract excludes membrane-mediated transport mechanisms as being solely responsible for the effect of DRB on transcription.

Some investigators (13–15) have reported that DRB does not become phosphorylated *in vivo*. This would suggest that DRB-TP is not the actual effector of the phenomenon obtained by adding DRB to cells *in vivo*. We tested DRB, DRB-P, and DRB-TP in the *in vitro* initiating system and found an appreciable effect of all analogs when ATP was present. Higher concentrations of DRB-P or DRB-TP were required to attain similar levels of inhibition of synthesis of specific RNA run-off products. Although it is possible that only small amounts of DRB are phosphorylated *in vivo* but are nonetheless sufficient to inhibit hnRNA initiation, the rapid effect of inhibition of transcription upon addition of DRB does not support this idea (1). No effect of adenosine, AMP, or ADP could be detected in the *in vitro* transcriptional system (results not shown). Thus, we conclude that DRB acts in the *in vitro* whole-cell transcription extract in a manner that could explain some of its *in vivo* effects. This is not an effect limited to the human ε-globin gene; adenovirus promoters from early region EIa and the major late promoter showed identical sensitivities to DRB (results not shown).

The results presented here are difficult to reconcile with the proposed role of DRB in enhancing premature termination (3–8, 10, 11). Failure to detect the previously described effects on termination could be due to an artifact of the *in vitro* system because specific *in vitro* termination for RNA polymerase II-specific transcripts has not yet been described. However, it is also possible that the DRB-resistant RNAs described *in vivo* are transcribed by a different form of RNA polymerase II, which is inactive or undetectable in the whole-cell extract. A large excess (5-fold) of prematurely terminated RNA molecules is synthesized even in the absence of DRB (8, 11). This form of DRB-insensitive RNA polymerase II may lack the "DRB factor" and thus terminate spontaneously (with or without DRB), never yielding full-length mRNA precursor transcripts. A precursor–product relationship between these short RNA molecules and mature mRNA after DRB release was never demonstrated. Recently we found an ATP requirement for initiation of RNA polymerase II-mediated specific transcription (unpublished data). It seems plausible then that DRB is acting not at the normal RNA elongation site of the RNA polymerase II-initiation complex but rather at the site of this ATP requirement, be it the RNA polymerase II itself or one of the specific transcriptional factors (24) required for the faithful *in vitro* initiation described previously.

In summary, we have described a cell-free *in vitro* transcription system that is inhibited by physiological concentrations of DRB. The coincidence of the DRB inhibition curve for the intact HeLa cells with our *in vitro* transcription inhibition curve suggests that most of the DRB effects found *in vitro* account for the inhibition of transcription previously described *in vivo*. The mechanism of action of DRB can now be analyzed at the molecular level *in vitro*, thus facilitating our understanding of the phenomena of initiation and premature termination.

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