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RNA Cleavage and Inhibition of Protein Synthesis by Bleomycin

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Summary

Bleomycin is a clinically used antitumor antibiotic long thought to function therapeutically at the level of DNA cleavage. Recently, it has become clear that bleomycin can also cleave selected members of all major classes of RNA. Using the computer program COMPARE to search the database established by the Anticancer Drug Screening Program of the National Cancer Institute, a possible mechanism-based correlation was found between onconase, an antitumor ribonuclease currently being evaluated in phase III clinical trials, and the chemotherapeutic agent bleomycin. Following these observations, experimentation revealed that bleomycin caused tRNA cleavage and DNA-independent protein synthesis inhibition in rabbit reticulocyte lysate and when microinjected into *Xenopus* oocytes. The correlation of protein synthesis inhibition to the previously reported site-specific RNA cleavage caused by bleomycin supports the thesis that RNA cleavage may constitute an important element of the mechanism of action of bleomycin.

Introduction

Bleomycin (BLM) is an antitumor agent ([1] and references therein) whose ability to bind to and degrade DNA has been studied extensively [2–4]. More recently, cleavage of RNA [5, 6] by BLM has been reported, suggesting that RNA could also be a therapeutically relevant target for BLM [7, 8].

Onconase, a cytotoxic ribonuclease, was isolated from extracts of *Rana pipiens* oocytes and early embryos [9] based upon its antiproliferative/cytotoxic effects toward cancer cells [10, 11]. Onconase displays anticancer activity in animal models [12], and a phase I/II human clinical trial of onconase as a single agent in patients with a variety of solid tumors has been completed [13], with phase III trials currently in progress. To compare the effects of onconase with other drugs, it was submitted to the Cancer Drug Discovery and Development Program of the NCI [14, 15]. The patterns of cell sensitivity of onconase to all the other agents currently in the NCI screen database were computed using COMPARE software [16]. The best correlation coefficients were to other agents known to cause protein synthesis inhibition such as MAP 30 [17] and restrictocin, a fungal RNase [18]. Interestingly, a significant correlation coefficient was found also with BLM.

Since the mechanism of action of onconase in cell-free systems, and when injected into *Xenopus* oocytes [19] as well as in cultured cells [20, 21], involves RNA degradation that results in protein synthesis inhibition, the possibility that protein synthesis inhibition could contribute to the cytotoxicity of BLM was addressed experimentally. Presently, we demonstrate that BLM can cause DNA-independent inhibition of protein synthesis in both a cell-free system and in intact cells. The relationship of these observations to site-specific RNA cleavage by onconase and BLM is discussed.

Results

Cytotoxic Effects of Onconase on a Panel of Human Tumor Cell Lines in the NCI Anticancer Drug Screen Led to a Comparison with BLM

Five 10-fold dilutions of onconase were applied to a total of 60 human tumor cell lines derived from 9 cancer types (lung, colon, melanoma, renal, ovarian, CNS, leukemia, breast, and prostate) for 2 days before analyzing the results with SRB, a dye that measures the protein content of cultured cells [22]. A standard dose-response curve for onconase was generated for each cell line that was evaluated with respect to the concentration that caused 50% inhibition of growth in all cell lines (GI_{50}). CNS cell lines were the most sensitive with regard to the number of cell lines that were more sensitive than the average GI_{50} (83%; 5 out of 6 sensitive cell lines). Nonsmall-cell lung, ovarian, melanoma, renal, and breast cancer cell lines were also very sensitive (33%, 33%, 50%, 43%, and 42% sensitive cell lines, respectively), while leukemia (25%), colon (10%), and prostate cancer (0%) cell lines were less responsive. Subsequently, the patterns of cell sensitivity of onconase to all of the other agents currently in the NCI screen database were computed using COMPARE software [16]. Correlation coefficients of 0.87 and 0.83 were found for MAP 30 [17] and restrictocin, a fungal RNase [18], respectively; these agents are known to exert their cytotoxicity by inhibiting cellular protein synthesis. Intriguingly, a correlation coefficient of 0.71 was found for BLM, an anticancer antibiotic thought to exert its cytotoxic effects by degrading DNA [2–4] and possibly also RNA [5–8]. It may be noted that the database used for this correlation contained more than 30,000 compounds, and the next best correlation other than BLM was 0.56.

BLM Inhibits Cellular and Cell-free Protein Synthesis

To determine whether protein synthesis inhibition was at least partly responsible for inhibition of cell survival

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Table 1. Sensitivity of Protein Synthesis and Cell Survival to Onconase and BLM

Compound	Day	Protein Synthesis Inhibition (IC_{50}) (nM)[a]	Survival (IC_{50}) (nM)[b]
Onconase	1	1,800 ± 140	>10,000 ± 275
	2	1,000 ± 1,220	1500 ± 30
	3	350 ± 20	800 ± 20
	7	4 ± 0.3	25 ± 0.7
BLM	1	55,000 ± 4,300	100,000 ± 1,820
	2	18,000 ± 980	45,000 ± 90
	3	7,500 ± 250	30,000 ± 500
	7	100 ± 3	100 ± 2

SF539 human glioma cells were plated in 96-well microtiter culture plates and treated with varying concentrations of BLM (0.1–100 μM) or onconase (0.0005–8 μM) for 1–7 days. On the indicated days, two plates were removed and treated as noted below in footnotes a and b. The IC_{50}, defined as the concentration of test sample which inhibited protein synthesis or cell viability by 50%, was determined from semilogarithmic plots in which protein synthesis or cell viability as a percentage of control (buffer-treated cells) was plotted versus test protein concentration. The data ± SEM shown are representative of three experiments with each point in triplicate.

[a] One plate was labeled with [^{14}C]leucine for 1–2 hr and protein synthesis was determined as described in Experimental Procedures.

[b] The second plate was treated with WST as described in Experimental Procedures to determine cell viability.

Table 3. Inhibition of Protein Synthesis in Rabbit Reticulocyte Lysate by BLM

	% Control	
Treatment	− RNasin[a]	+ RNasin[a]
750 μM BLM	77.3 ± 7.5	77.3 ± 0.04
750 μM Fe(II)•BLM	54.8 ± 4.6	58.0 ± 2.0
750 μM Fe^{2+}	100.6 ± 3.7	90.0 ± 1.3
70 nM angiogenin	19.0 ± 1.4	98.4 ± 0.8

In vitro rabbit reticulocyte assays were performed as described in Experimental Procedures. The results from three separate experiments were combined and are expressed as the percentage of mock-treated controls containing mRNA (100%). The data ± SEM are shown. BLM treatment was not significantly different from Fe^{2+} treatment. Treatment with Fe(II)•BLM was significantly different from Fe^{2+} treatment ($p = 0.02$).

[a] RNasin, 40 units/ml.

by BLM, human SF539 glioma cells were treated with onconase or BLM, and both protein synthesis inhibition and cell survival were determined in parallel experiments. Measurements were made on days 1, 2, 3, and 7 after addition of the drugs; in every instance protein synthesis inhibition preceded effects on cell survival (Table 1). A correlation analysis between protein synthesis inhibition and cell survival showed that at 50% inhibition of protein synthesis onconase killed 55% of the cells (Table 2). This was similar to results obtained previously for onconase [21]. Similarly, at 50% inhibition of protein synthesis inhibition BLM killed 60% of the cells. These results show that the effects of both onconase and BLM on protein synthesis and cell survival are very similar.

Both rabbit reticulocyte lysate [19] and *Xenopus* oocytes [23, 24] have been used extensively as model systems to study agents that inhibit cellular protein synthesis. Thus, both of these systems were used to determine whether BLM could inhibit protein synthesis directly. Subsequent results showed that lower concentrations of BLM inhibited cell-free protein synthesis. However, initially it was observed that 750 μM Fe(II)•BLM decreased cell-free, DNA-independent protein synthesis to 55% of that obtained with mock-treated lysates (Table 3). BLM requires a metal ion such as Fe^{2+} to cause oxidative cleavage of DNA [2–4] as well as RNA [5–8]. The results in Table 3 show that BLM alone had a much smaller effect than Fe(II)•BLM, and Fe^{2+} alone did not significantly affect protein synthesis. Therefore, inhibition of protein synthesis can reasonably be attributed to Fe(II)•BLM. Since protein synthesis inhibition by Fe(II)•BLM could in principle have been caused by trace contamination with RNases, the results were reproduced in the presence of RNasin, an RNase inhibitor. Again, 750 μM Fe(II)•BLM decreased protein synthesis to 58% of that produced by mock-treated lysates, even in the presence of enough RNasin to completely inhibit angiogenin (70 nM; Table 3), a human plasma RNase [25, 26] and potent inhibitor of protein synthesis in rabbit reticulocyte lysate [26] (Table 3). Inhibition of protein synthesis by Fe(II)•BLM was concentration dependent, causing a decrease in protein synthesis over a range of 50 to 1000 μM from 78% to 42% of mock-treated lysates (data not shown). Additionally, the decrease in CPM was reflected in a uniform decrease in all of the labeled protein when the newly synthesized [^{35}S]methionine proteins were analyzed by SDS gel electrophoresis (data not shown).

Inhibition of protein synthesis in the reticulocyte lysate

Table 2. Correlation Between Onconase- and BLM-Induced Inhibition of Protein Synthesis and Cell Survival

Compound	Protein Synthesis Inhibition (IC_{50}) (nM)[a]	IC_{50} Survival (IC_{50}) (nM)[b]	Killing at 50% Inhibition of Protein Synthesis[c]
Onconase	1,800	1,500	55%
BLM	55,000	45,000	60%

SF539 human glioma cells were plated in 96-well microtiter culture plates and treated with varying concentrations of BLM or onconase as described in Experimental Procedures.

[a] One day after treatment, half of the plates were labeled with [^{14}C]leucine for 1–2 hr, and protein synthesis was determined as described in Experimental Procedures.

[b] Two days after treatment, the remaining plates were treated with WST as described in Experimental Procedures to determine cell viability.

[c] Both protein synthesis at day 1 and cell survival at day 2 were plotted as functions of the concentration of onconase or BLM, and IC_{50} values for each were calculated. The percentage of cells killed at the concentration of onconase or BLM that caused 50% inhibition of protein synthesis was determined from the graph.

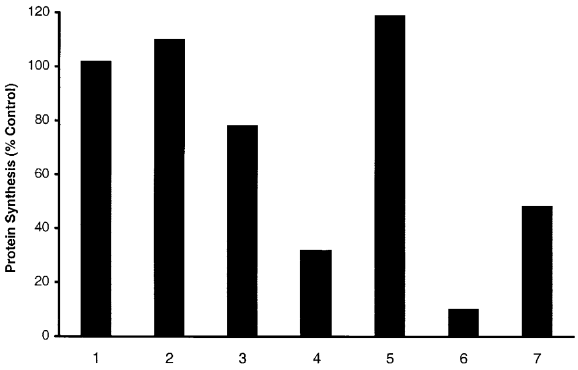

Figure 1. Effect of tRNAs on Luciferase Synthesis Following Treatment with Fe(II)•BLM A_5

Reticulocyte lysate was treated with Fe(II)•BLM A_5 at the indicated concentration in the presence of 40 U of RNasin at 20°C for 15 min. After sufficient time had elapsed for bleomycin inactivation, protein synthesis was carried out as described in Experimental Procedures, either without or with the prior addition of a mixture of calf liver tRNAs. Lane 1, untreated lysate; lane 2, lysate treated with 100 μM Fe^{2+}; lane 3, 100 μM BLM A_5; lane 4, 50 μM Fe(II)•BLM A_5; lane 5, 50 μM Fe(II)•BLM A_5, then 40 μg/ml calf liver tRNAs; lane 6, 100 μM Fe(II)•BLM A_5; lane 7, 100 μM Fe(II)•BLM A_5, then 40 μg/ml calf liver tRNAs.

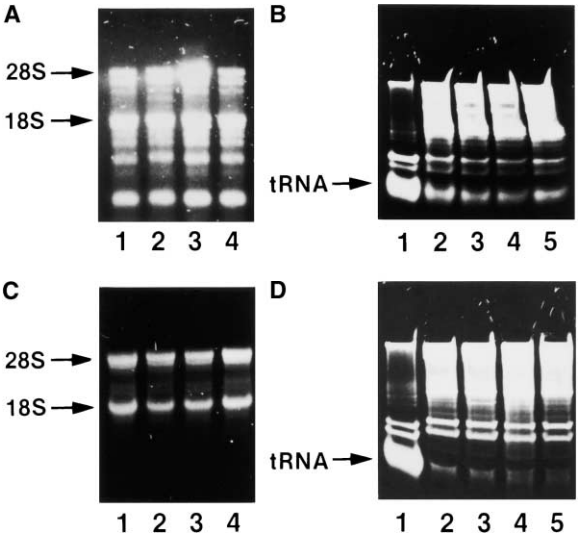

Figure 2. BLM Did Not Cause Extensive Degradation of RNA in Reticulocyte Lysates or *Xenopus* Oocyte

Total RNA was isolated from lysates of the experiment described in Table 3 ([A] and [B]) or from oocytes (Table 4; [C] and [D]) and analyzed on 1.4% formaldehyde agarose gels to assess rRNA degradation ([A] and [C]) or 10% TBE gels to monitor tRNA degradation ([C] and [D]).

(A) Lane 1, control; lane 2, BLM; lane 3, Fe(II)•BLM; lane 4, Fe^{2+}.

(B) Lane 1, tRNA standard; lane 2, untreated control; lane 3, BLM; lane 4, Fe(II)•BLM; lane 5, Fe^{2+}.

(C) Lane 1, untreated control; lane 2, BLM; lane 3, Fe(II)•BLM; lane 4, Fe^{2+}.

(D) Lane 1, tRNA standard; lane 2, untreated control; lane 3, BLM; lane 4, Fe(II)•BLM; lane 5, Fe^{2+}.

was also studied in the presence of firefly luciferase mRNA. As shown in Figure 1, treatment of the lysate with 50 μM Fe(II)•BLM A_5 resulted in a 66% decrease in the production of luciferase, while 100 μM BLM A_5 alone had only a modest effect (22% inhibition, presumably due to the presence of adventious metal ions in the lysate), and luciferase synthesis was actually slightly increased in the presence of 100 μM Fe^{2+}. As shown in lane 5, admixture of tRNAs to the 50 μM Fe(II)•BLM A_5-treated lysate completely restored the ability of the lysate to synthesize luciferase. In contrast, the addition of ribosomes to the Fe(II)•BLM A_5-treated lysate had no restorative effect on luciferase synthesis (data not shown). When treated with 100 μM Fe(II)•BLM A_2, luciferase synthesis by the lysate was reduced further to 14% of control but could not be restored completely by tRNA addition. Thus, it appeared that oxidative destruction of one or more tRNAs by Fe(II)•BLM was primarily responsible for the diminution of the luciferase synthesis by the luciferase mRNA-programmed reticulocyte lysate.

While the experiments carried out in Figure 1 argue that Fe^{2+} or some other metal ion may be required for protein synthesis inhibition by bleomycin, the cytotoxic effects of the drug obtained on cultured SF539 cells (Table 1) were elicited following admixture of metal-free BLM. Presumably, this resulted from the presence of intracellular metal ions sufficient to support oxidative damage by BLM, as suggested in lane 3 of Figure 1. To assess this possibility, we measured protein synthesis in *Xenopus* oocytes following administration of BLM by microinjection.

As shown in Table 4, the injection of 125 μM BLM into *Xenopus* oocytes reduced protein synthesis to 15% of control. BLM coinjected with Fe^{2+} was only slightly more inhibitory (7% of control), and this incremental effect may have been due to the metal ion itself since Fe^{2+} alone decreased oocyte protein synthesis to 59% of that obtained with gelatin-injected oocytes (Table 4). Therefore, although less potent than onconase, BLM was nearly as effective as onconase (33 nM) in decreasing the magnitude of protein synthesis in *Xenopus* oocytes (Table 4). To ensure that protein synthesis inhibition in the oocyte could be independent of DNA cleavage, the translation of an exogenous mRNA was examined. Luciferase mRNA was coinjected into the oocyte with 250 μM BLM (Table 4), and translation of the exogenous mRNA was decreased to 29% of control.

Characterization of Reticulocyte and Oocyte RNA after BLM Treatment

RNA was isolated from BLM-treated lysates or oocytes described in Tables 3 and 4 and was examined by formaldehyde agarose gel electrophoresis to assess the major rRNAs (Figures 2A and 2C) or by electrophoresis on 20% TBE gels to assess tRNA (Figures 2B and 2D). Extensive degradation of RNA was not discernible in the BLM-treated lysates (Figures 2A and 2B) or oocytes (Figures 2C and 2D) at concentrations that had inhibited protein synthesis in each system, although some diminution in tRNA levels was apparent. In contrast, onconase visibly degraded tRNA in both reticulocyte lysates and oocytes [19].

The ability of Fe(II)•BLM to effect the cleavage of some tRNA isoacceptors was studied in additional experiments. Onconase was also employed, albeit at lower

Table 4. Inhibition of Protein Synthesis in *Xenopus* Oocytes by Onconase and BLM

Treatment	Response	% Control
Experiment A	CPM	
Control, uninjected	269,000 ± 19,000	100
Control injected with 0.01% gelatin	258,000 ± 12,000	96
125 μM BLM	41,000 ± 11,000	15
125 μM Fe(II)•BLM	20,000 ± 4,000	7
125 μM Fe^{2+}	152,000 ± 6,000	57
33 nM onconase	12,000 ± 100	4
Experiment B	Fluorescence	
Control, uninjected	24,000 ± 2,000	100
250 μM BLM	7,000 ± 2,000	29

The indicated agents were injected into *Xenopus* oocytes. Inhibition of protein synthesis and luciferase activity were determined as described in Experimental Procedures. Data is representative of two different experiments with each determination performed in triplicate. Data shown are ± SEM. In Experiment A, injected oocytes were labeled with [^{35}S]methionine and analyzed for the incorporation of the labeled methionine into protein. All injections included 80 U/ml RNasin. BLM, Fe(II)•BLM, and onconase were not significantly different from each other ($p = 0.2$ and 0.12, respectively). These treatments were significantly different from the control, control + gelatin, and Fe^{2+} ($p < 0.01$). In Experiment B, luciferase mRNA was microinjected into the oocytes and luciferase activity was determined. BLM was shown not to inhibit light production by luciferase directly. BLM-treated oocytes were significantly different from control oocytes ($p = 0.004$).

concentration (0.15 nM) than those employed in Table 4 (33 nM) and in earlier experiments [19] that resulted in total degradation of tRNA. Rabbit reticulocyte lysate was treated with 100 μM Fe(II)•BLM A_5 or 0.15 nM onconase, and the RNAs in the lysate were then radiolabeled by an exchange reaction [27] in the presence of [γ-^{32}P]ATP, polynucleotide kinase, and excess unlabeled ADP. As shown in Figure 3, the ribosomal RNAs at the top of the gel were largely unaffected under these conditions, but several tRNA isoacceptors were degraded both by Fe(II)•BLM A_5 and by onconase. Commercial rabbit reticulocyte lysate is supplemented with calf liver tRNAs; an isoacceptor susceptible to Fe(II)•BLM A_5 and onconase was purified from a mixture of calf liver tRNAs by preparative gel electrophoresis and is shown in lane 6 of Figure 3A.

The 5′-^{32}P end-labeled tRNA shown in lane 6 of Figure 3A was treated with Fe(II)•BLM A_5 and onconase to determine whether the sites of cleavage mediated by both agents were the same. As shown in Figure 3B, this tRNA isoacceptor was cleaved at several sites by Fe(II)•BLM A_5, but none of the major sites of cleavage corresponded to the sites cleaved by onconase.

Discussion

The National Cancer Institute Anticancer Drug Screening Program has tested thousands of compounds for antitumor activity against a panel of 60 human tumor cell lines, thereby establishing an extensive set of reference data [14, 15]. Compounds with similar mechanisms of action have reproducibly shown similar patterns of cytotoxicity, and the COMPARE computer program [16] was developed to search the database of agents already tested against the cytotoxicity pattern of newly tested compounds. Although not infallible in its predictive capabilities, this type of analysis has been valuable in predicting mechanism-based correlations from the NCI anticancer screen database [28]. Therefore, based on the correlation of the BLM cytotoxicity profile in the NCI anticancer screen with agents known to act by inhibiting cellular protein synthesis, as well as on the proposal that site-specific cleavage of RNA could be a therapeutically relevant target for BLM [7, 8], we reasoned that degradation of cellular RNA by BLM could also result in protein synthesis inhibition. Presently, we describe an experimental test of that hypothesis. It may be noted that we undertook this analysis in spite of early studies that failed to document RNA cleavage [29–32], in the belief that the highly selective nature of BLM-mediated RNA cleavage [6–8] had precluded the observation of key RNA cleavage events.

Since the assessment of cytotoxicity in the NCI drug screen was obtained by applying dilutions of each test compound to the cells for 2 days before analyzing the results with SRB, a pink anionic dye that measures cellular protein content [22], it was necessary to demonstrate that protein synthesis inhibition could be used as an indication of BLM cytotoxicity in cultured cells. Protein synthesis inhibition preceded effects on cell survival after treatment with either onconase or BLM. A correlation analysis between protein synthesis and cell survival was similar for both drugs (observed cell kill at 50% inhibition of protein synthesis was 55% and 60% for onconase and BLM, respectively [Table 2]). Emetine is a potent and irreversible inhibitor of ribosomal translocation and kills cells by inhibiting protein synthesis. A similar analysis between inhibition of protein synthesis inhibition and survival showed that at 50% inhibition of protein synthesis only 15% of the cells were killed [21]. Therefore, both onconase and BLM are considerably more cytotoxic than emetine, suggesting that mechanisms in addition to protein synthesis inhibition contribute to the cell death actions of onconase and BLM through their effects on damaging tRNA, consistent with the predicted similarities in their actions.

More direct assessments of the effect of BLM on DNA-independent protein synthesis were obtained using the rabbit reticulocyte lysate as well as a *Xenopus* oocyte microinjection system (Figure 1, Tables 3 and 4). In those assay systems, BLM inhibited protein synthesis independent of any effect on DNA. BLM was particularly

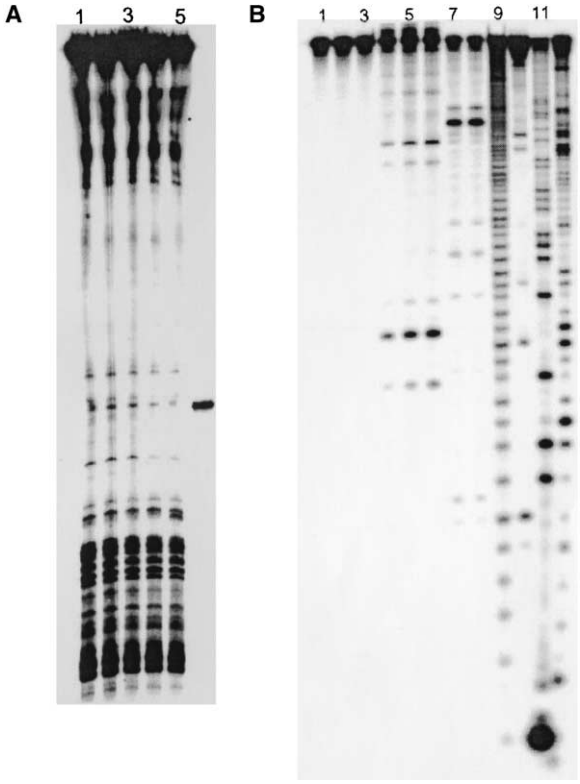

Figure 3. Transfer RNA Isoacceptor and Site Selectivity of Cleavage by BLM and Onconase

(A) Degradation of individual rRNAs and tRNA isoaceptors by Fe(II)•BLM A_5 and onconase. Rabbit reticulocyte lysate was treated with Fe(II)•BLM A_5 or onconase, then 5′-^{32}P labeled in the presence of [γ-^{32}P]ATP + ADP + polynucleotide kinase and analyzed by polyacrylamide gel electrophoresis. Ribosomal RNAs migrated at the top of the gel; tRNAs in the middle and lower portions of the gel. Lane 1, untreated control; lane 2, 100 μM Fe^{2+}; lane 3, 100 μM BLM A_5; lane 4, 100 μM Fe(II)•BLM A_5; lane 5, 0.15 nM onconase, lane 6, isolated calf liver tRNA found to be susceptible to cleavage both by Fe(II)•BLM A_5 and by onconase.

(B) Sites of tRNA cleavage by Fe(II)•BLM A_5 and onconase. A 5′-^{32}P end-labeled tRNA isolated from a mixture of calf liver tRNAs by preparative gel electrophoresis was treated with Fe(II)•BLM A_5 or onconase and then analyzed by polyacrylamide gel electrophoresis. Lane 1, untreated tRNA; lane 2, 100 μM Fe^{2+}; lane 3, 100 μM BLM A_5; lane 4, 25 μM Fe(II)•BLM A_5; lane 5, 50 μM Fe(II)•BLM A_5; lane 6, 100 μM Fe(II)•BLM A_5; lane 7, 0.15 nM onconase; lane 8, 0.30 nM onconase; lane 9, aq. NaOH; lane 10, C + U lane; lane 11, G lane; lane 12, A + G lane.

effective when injected into oocytes without any added metal ions, presumably due to the presence of adventitious metal ions within the cell as well as conditions compatible with oxidative degradation of RNA within the cell. However, it is conceivable that the effects mediated following application of metal-free BLM could also reflect an effect of metal-free BLM in the oocyte, since sequence-specific hydrolysis of RNA by BLM in the absence of added metal ions has been reported [33]. In the oocyte, protein synthesis is encoded almost entirely by maternal transcripts [34], but, to underscore DNA independence in the oocyte, the effect of BLM on protein synthesis was also determined under conditions in which the translation of exogenous mRNA was measured.

The ability of individual RNAs to restore protein-synthesizing capability to a rabbit reticulocyte lysate system was studied following treatment with bleomycin A_5. As shown in Figure 1, treatment with 50 μM Fe(II)•BLM A_5 diminished the synthesis of luciferase to about one third of the original value. Following BLM self-inactivation [35–37], admixture of unfractionated calf liver tRNAs completely restored the protein-synthesizing capability of the treated lysate. Analogous addition of rabbit reticulocyte ribosomes to the BLM-treated lysate failed to restore protein synthesis, arguing that one or more tRNAs constitutes a critical target for BLM. It may be noted, however, that following treatment with a higher (100 μM) concentration of Fe(II)•BLM A_5, the addition of calf liver tRNAs effected only partial restoration of protein synthesis.

In parallel with assessing the effect of BLM on protein synthesis, both lysate and oocyte RNA were examined to determine whether RNA cleavage could be correlated to the inhibition of protein synthesis, as had been shown previously for onconase [19]. Using the same methods of detection that had revealed tRNA degradation by onconase, only limited tRNA cleavage was seen in BLM-treated samples (Figure 2). In view of these results, the ability of Fe(II)•BLM to cleave tRNA using assay conditions previously reported [6] was confirmed by using low-voltage, low-resolution electrophoresis [38] to look at RNA fragments rather than specific cleavage sites (data not shown). Interestingly, it was also found that the cleavage of a ^{32}P-labeled tRNA by Fe(II)•BLM was diminished in the presence of rabbit reticulocyte lysate. It has been reported that RNA cleavage by Fe(II)•BLM can be inhibited in the presence of Mg^{2+} [39]; the presence of Mg^{2+} in the lysate and oocyte systems may well have resulted in the diminution of observed RNA cleavage in Figure 2.

Holmes et al. [40] have also noted that RNA cleavage by Fe(II)•BLM is more selective in the presence of physiological concentrations of Mg^{2+} and Na^{+} and have argued that this may reflect an important source of selectivity in the action of the drug as a therapeutic agent. Accordingly, we sought to determine whether there were specific tRNA isoacceptors affected by Fe(II)•BLM. Following treatment of lysate with Fe(II)•BLM A_5, RNAs present in the lysate were 5′-^{32}P end labeled and analyzed by polyacrylamide gel electrophoresis. Several tRNAs were found to decrease in abundance following treatment with Fe(II)•BLM A_5. Intriguingly, some of these tRNAs were also degraded selectively by low concentrations of onconase (Figure 3A), suggesting a basis for the similar behavior of these two agents in the 60-cell-line panel. As shown in Figure 3B, however, Fe(II)•BLM A_5 and onconase cleaved one susceptible tRNA isoacceptor at different sites.

It may be noted that while the concentrations of BLM employed in the experiments carried out here were relatively high, this simply reflects the absence of added reducing agents, which greatly potentiate DNA [2–4] and RNA [6–8, 40] cleavage by BLM. The reductants typically employed in biochemical experiments contain functional groups that can participate in metal ion coordination, thereby potentially altering the structure and behavior of the antitumor agent under study. The pur-

poseful omission of reductants here is intended to facilitate mechanistic analysis of the observations that have been made. The omission of reductants also facilitated a better understanding of the intrinsic potency of BLM in mediating single biochemical transformations, although the control of key experimental parameters to assure quantitative reproducibility of experiments can require careful attention to issues such as the order and timing of addition of reagents [41], such that some variability is typically observed nonetheless.

Further, in comparison with a study by Poddevin et al. [42], the implications of which are that BLM is not taken up readily by cultured cells, recent confocal microscopy experiments that monitored the concentration of bleomycin in the cytoplasm of cultured HeLa cells by virtue of the intrinsic fluorescence of the bithiazole moiety actually suggest that BLM is concentrated selectively within these cells. Specifically, incubation of HeLa cells in the presence of 50 μM BLM resulted in an equilibrium intracellular concentration of approximately 3 mM BLM within 2 min; the use of 2 mM BLM in the incubation medium produced an intracellular concentration of the drug estimated at 40 mM in the same time frame.

The present study provides further support for the suggestion [7, 8] that tRNA cleavage may constitute an important locus of action for bleomycin. Studies that may help to define the spectrum of tRNA isoacceptors susceptible to cleavage by bleomycin are underway. In addition to its possible action on tRNA, it must be acknowledged that cellular processes such as uptake and detoxification can be important in the expression of cell toxicity by an applied agent, and these may affect the behavior of BLM as well.

In addition to the well-known cytotoxic effects on bacterial cells resulting from ribosomal RNA targeting by agents such as the aminoglycosides, erythromycin, and colicin E3, the cytotoxic ribonuclease colicin E5 has been shown to function by inhibiting protein synthesis at the level of tRNA cleavage [43, 44]. While the mechanisms of cell death induced by agents such as onconase are clearly complex [21], sustained tRNA degradation with concomitant inhibition of protein synthesis undoubtedly contributes to the observed cytotoxicity. More generally, there is accumulating evidence that targeting RNA can lead to apoptotic cell death by activation of cellular stress response mechanisms [45]. Thus, the effects noted for BLM in the present study support the thesis that RNA may constitute a reasonable locus of action for this antitumor agent.

Significance

In previous experiments, it has been shown that BLM is capable of cleaving some members of all classes of RNAs, including tRNAs, rRNAs, mRNAs, and the RNA strands of DNA-RNA heteroduplexes. The present experiments demonstrate for the first time that BLM can mediate (transfer) RNA cleavage and consequent inhibition of protein synthesis in an intact biological system (*Xenopus* oocytes) as well as in a cell-free system. Further, the finding that BLM exhibits a strong correlation coefficient with the antitumor RNase onconase (which does not cleave DNA at all) in the COMPARE algorithm and cleaves the same tRNAs as onconase argues strongly that RNA may be a therapeutically relevant target for BLM.

Experimental Procedures

Materials

BLM, a clinically used mixture of congeners consisting primarily of BLM A_2 and BLM B_2, was obtained from the National Cancer Institute (NCI) anticancer drug screen and was fractionated as described [46]. Onconase is a registered trademark of Alfacell Corporation (Bloomfield, NJ) and was obtained as lyophilized protein. Angiogenin was purified as described previously [23]. Prepoured TBE gels were obtained from Novex (San Diego, CA). Human placental ribonuclease inhibitor (RNasin), Brome Mosaic Viral mRNA, luciferase mRNA, and Flexi rabbit reticulocyte lysate translation system were purchased from Promega (Madison, WI). The tRNA translation mix and T4 polynucleotide kinase were from GIBCO-BRL (Grand Island, NY), and yeast $tRNA^{Phe}$ was obtained from Sigma Chemicals (St Louis, MO). L-[^{35}S]methionine (1,134 Ci/mmol) and [^{14}C]leucine (310 mCi/mmol) were purchased from DuPont-New England Nuclear (Beverly, MA); [γ-^{32}P]ATP (6000 Ci/mmol) was from Amersham (Arlington Heights, IL).

Cell Culture

SF539 human glioma cells were obtained from and grown as specified by the American Type Culture Collection (Rockville, MD) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, and 10 μg/ml gentamycin. All cells were grown at 37°C in 5% CO_2 in a humidified atmosphere.

Cancer Drug Discovery and Development Program of the National Cancer Institute

The experimental and analytical details of the NCI cancer screen have been described [14, 15]. Onconase was submitted to the screen twice. The relative sensitivity of each cell line was compared to the average sensitivity of all the cell lines with respect to the concentration causing 50% inhibition of growth (GI_{50}). The patterns of cell sensitivity of onconase to all the other agents in the NCI screen database were computed using COMPARE software [16].

Cytotoxicity Assays

Protein synthesis was measured as described previously [23, 24]. Briefly, cells were plated (at concentrations of 2.5×10^4 cells/ml) in 96-well microtiter plates in 100 μl of Dulbecco's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, sodium pyruvate, and nonessential amino acids; additions were made in a total volume of 10 μl (10% of the final volume), and the plates were incubated at 37°C for the times indicated. Phosphate-buffered saline (PBS) containing 0.1 mCi of [^{14}C]leucine was added for 2 hr, and the cells were harvested onto glass fiber filters using a PHD cell harvester from MEPCO Scientific (Arnold, MD), lysed, and washed with water to remove unincorporated [^{14}C]leucine, then dried with ethanol and counted. Cells (2500 in 0.1 ml) were placed in each well of a 96-well plate 24 hr before treatment. On the day of treatment, 10 μl test samples were added to the appropriate wells, and the cells were incubated for 1–7 days at 37°C in a humidified CO_2 incubator. A colorimetric assay based on cleavage of the tetrazolium salt WST (Roche Molecular Biochemcials, Indianapolis, IN) to a soluble formazan salt was used to determine cell viability following the manufacturer's instructions. WST (10 μl) was added directly to each well, and the plates were incubated for a further 60–90 min. Absorbance at 460 nm was determined in a MR4000 microtiter plate reader (Dynatech Laboratories, Chantilly, VA). Each experiment was performed at least twice with triplicate determinations for each point. The IC_{50}, defined as the concentration of test sample which inhibited protein synthesis by 50%, was determined from semilogarithmic plots in which protein synthesis or cell viability as a percentage of control (buffer-treated cells) was plotted versus test protein concentration.

In Vitro Translation Assays

The in vitro translation assay was performed as described previously [47] with the following exceptions: the concentration of magnesium was optimized (2.5 mM) using magnesium acetate and the Flexi Translation system in a total volume of 12.5 μl containing 0.1 M HEPES (pH 7.5). When used, a freshly prepared 0.45 mM $Fe(NH_4)_2(SO_4)_2$ solution was added in three equal portions as described [6] at 0, 20, and 40 min during a total reaction time of 60 min at 30°C. The amount of protein synthesized was determined by the incorporation of [^{35}S]methionine into products precipitable by 10% trichloroacetic acid (TCA), following the protocol recommended by Promega and by SDS-gel electrophoresis. In some cases, the results were presented as percentage of the mock-treated, mRNA-containing control reactions.

Oocyte Microinjection and Protein Synthesis Determination

Twenty-three nanoliter samples were microinjected into the vegetal pole of stage VI oocytes as described [48]. When used, freshly prepared 0.45 mM $Fe(NH_4)_2(SO_4)_2$ was injected alone or coinjected with BLM [6]. For each protein-synthesis determination, duplicate wells containing three injected oocytes were labeled with [^{35}S]methionine (0.2 mCi/ml) in Oocyte Ringer (OR) medium for 20 hr at 18°C. After labeling, the medium was removed and the oocytes were rinsed with an excess of OR, homogenized, and analyzed for incorporation of [^{35}S]methionine into protein as described [49]. In some cases, the results were presented as percentage of the 0.01% gelatin-injected control. For determination of luciferase mRNA translation, 9 oocytes per point were used for injection into the vegetal pole of the oocyte with 23 ng of mRNA, as described above. The oocytes were homogenized as described above, and the extract was mixed with 100 μl of luciferase assay reagent (Promega) and placed in a scintillation counter to determine luciferase activity. Calculation of molar concentrations was accomplished using an approximate oocyte volume of 500 nl, as described previously [50].

Characterization of Reticulocyte and Oocyte RNA

RNA from control or treated lysates or oocytes was prepared as described [38]. Electrophoretic analysis was accomplished by the use of formaldehyde/agarose gels or 10% TBE gels.

BLM Cleavage of RNA

The tRNA translation mix or yeast $tRNA^{Phe}$ was 5′-^{32}P end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase as described by Maniatis et al. [51]. The reaction mixtures contained 1–2 pmol of tRNA with or without other agents in 0.1 M HEPES buffer (pH 7.5; total volume 12.5 μl). Labeled tRNA was also added to the Mg^{2+} containing rabbit reticulocyte lysate, and translation was initiated as described above. When used, a freshly prepared 0.45 mM $Fe(NH_4)_2(SO_4)_2$ solution was added at 0, 20, and 40 min, and the reaction was allowed to proceed for a total of 60 min at 22°C or 30°C (buffer and lysate translation systems, respectively). RNA was recovered by ethanol precipitation (buffer system) or isolated from the lysate as described above. Electrophoretic analysis was accomplished using 8% acrylamide-7% urea gels.

Cellular Uptake of Bleomycin

HeLa cells (that had been grown as monolayers and then harvested by treatment with trypsin) were placed on the stage of a microscope, and individual cells were selected using the bright field optics. BLM was added to the coverslips at the indicated concentrations, and the observed cells were monitored as a function of time by confocal microscopy (irradiation at 290 nm; observation at 420 nm). Values were obtained after 2 min of incubation. Quantification was accomplished by using a draw tool to circle the (intra- or extra-) cellular areas of interest; the gray level intensity units determined were compared to those obtained in the absence of any cells for known concentrations of BLM itself.

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