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Light-Dependent Activity of the Antitumor Antibiotics Ravidomycin and Desacetylravidomycin

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The antitumor antibiotics ravidomycin and desacetylravidomycin were studied by the biochemical λ prophage induction assay. In this assay, induction of the enzyme β-galactosidase is measured as a specific indication of the ability of an agent to directly or indirectly damage DNA. Induction was observed only when these two antibiotics were irradiated with light in the presence of the indicator organism. Drug treated with light followed by incubation with the indicator organism in the dark did not cause induction. Light in both the near UV and visible wave length ranges activated these antibiotics; near UV and visible blue wavelengths were most effective, while 597-nm light was totally ineffective. The amount of induction caused by these drugs varied directly with the dosage of light provided. Bacterial growth inhibition, as well as cytotoxicity for a human colon carcinoma cell line, was also dramatically enhanced by light. These data suggest that ravidomycin and desacetylravidomycin are potent photosensitizing, DNA-damaging agents.

Ravidomycin (8, 20, 21), desacetylravidomycin (20), the gilvocarcins (1, 15, 25), and the chrysomycins (24, 29) constitute a recently described class of structurally related antitumor antibiotics. These compounds (Fig. 1) possess distinctive sugar moieties coupled to a chromophore common to all members of the class; vinyl, methyl, or ethyl side chains are linked to this chromophore at carbon 8. The vinyl species demonstrate the greatest antimicrobial and antitumor activities (1, 15, 16, 29).

Results of mode of action studies have indicated that these antibiotics interfere primarily with DNA synthesis (22, 26, 27). Examination of gilvocarcin V and chrysomycin A binding to covalently closed circular DNA in a cell-free system revealed that DNA strand interruption by these drugs was dependent on exposure to light (27). The activity of both drugs in the biochemical λ prophage induction assay (BIA) (7), which was the original detection system for the gilvocarcin complex in a natural products screening program (28), was subsequently shown to be promoted by light (6). In this report, we describe the effect of light on the BIA response, inhibition of bacterial growth, and cytotoxicity against cultured cells for ravidomycin and desacetylravidomycin.

MATERIALS AND METHODS

Bacterial strains and cell lines. *Escherichia coli* BR513 (λ*lacZ* Δ*uvrB envA*) was provided by R. K. Elespuru, Frederick Cancer Research Facility, Frederick, Md. All other bacteria were from the Lederle Laboratories culture collection. The human colon carcinoma cell line WiDR was established at Lederle from a clinical specimen.

Media. LBE broth was prepared as described previously (7).

Antibiotics and chemicals. Ravidomycin and desacetylravidomycin were isolated and purified from microbial fermentation broths by Guy Carter at our research facility. Gilvocarcin V was provided by Kevin Byrne, Frederick Cancer Research Facility. Concentrated solutions of these antibiotics were prepared in dimethyl sulfoxide and were diluted sufficiently in the various assays to avoid solvent interference.

O-Nitrophenyl-β-D-galactopyranoside, chloramphenicol, and ampicillin were obtained from Sigma Chemical Co., St. Louis, Mo.

BIA. The BIA was conducted by a modification of the method of Elespuru and Yarmolinsky (7). Briefly, a culture of *E. coli* BR513 grown to log phase (A_{600} = 0.4) in LBE broth was diluted 1:10 in fresh LBE supplemented with 10 μg of ampicillin per ml. This cell suspension (100 μl) was added to 10-μl fractions of the drug solutions to be tested in a 96-well microtiter plate (Corning Glass Works, Corning, N.Y.). A control well containing 10 μl of water instead of drug was included on each plate. After incubation of this induction mixture at 37°C for 3 h (induction period), the A_{405} of each sample well was determined with an automated microtiter plate reader (Artek Systems Corp., Farmingdale, N.Y.) to provide a background value. A 50-μl fraction of a solution containing *O*-nitrophenyl-β-D-galactopyranoside (2 mg/ml), a chromogenic β-galactosidase substrate, and chloramphenicol (50 μg/ml) was then added to each well. After incubation for 30 min at 37°C, 1 M $NaCO_3$ (100 μl per well) was added, and the A_{405} of each sample well was again determined. This final reading was corrected by subtraction of the background value. All data are expressed as a β-galactosidase induction ratio, which was calculated as the ratio of the A_{405} in a drug-treated sample to that in an untreated control sample. Values greater than 1.2 indicate the presence of inducing activity, while values of 0.8 to 1.2 represent an absence of activity. Ratios less than 0.8 result from toxicity in the assay system, i.e., interference with the expression of the *lacZ* gene.

The effect of light on the activities of ravidomycin and desacetylravidomycin in the BIA was evaluated by exposing the assay plates to light from selected sources throughout the 3-h induction period. All operations prior to the induction period, such as the addition of indicator bacteria to drug, were conducted in a darkened room or under yellow lights. Dark controls were shielded from light throughout these experiments with aluminum foil.

Antibacterial assay. Antibiotic susceptibility testing was performed by the conventional agar plate dilution method for

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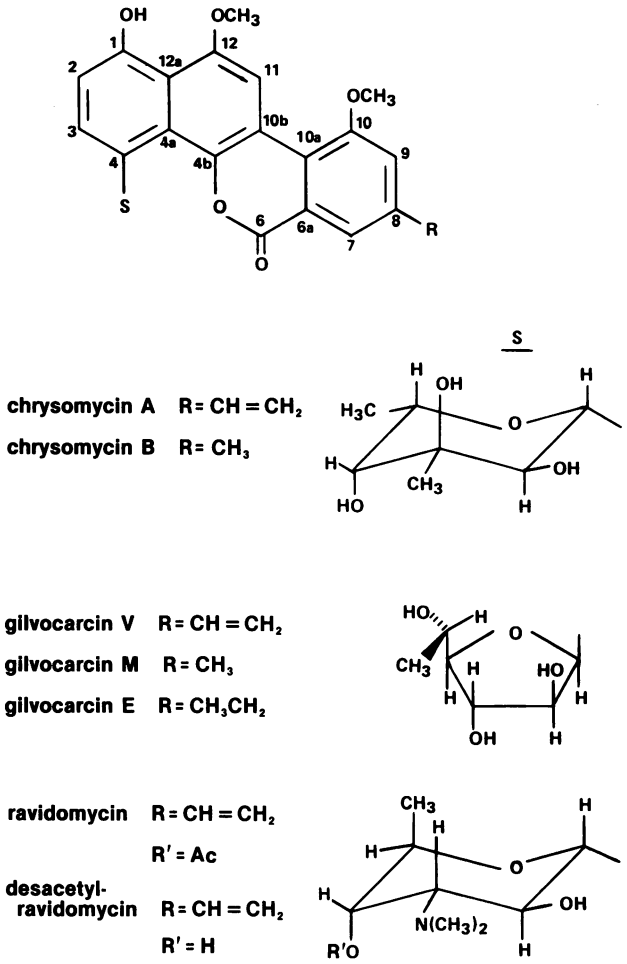

FIG. 1. Structures of the gilvocarcins, the chrysomycins, ravidomycin, and desacetylravidomycin. The chromophore common to all members of this antibiotic class is at the top. Presented beneath it are the distinctive sugars (S) and side chains (R, R') of the individual antibiotics within this group.

determining MICs. Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) containing various antibiotic concentrations was employed for all test organisms. Inocula containing 10^4 to 5×10^4 CFUs of various bacteria were spotted onto the plate surfaces in a darkened room with a Steers multiple inoculating device. To study the effects of light, one set of plates was incubated in the dark for 18 h at 35°C, while a duplicate set was irradiated with a fluorescent light at a fluence rate of 1.8 Wm^{-2} during incubation. The lowest antibiotic concentration inhibiting the growth of a culture was designated as the MIC.

Clonogenicity assay for cytotoxic activity. Human colon carcinoma cells (strain WiDR) were seeded into 35-mm-diameter culture dishes in modified RPMI 1640 medium (GIBCO Laboratories, Chagrin Falls, Ohio) at 10^3 cells per dish. After incubation for 24 h at 37°C, antibiotics were added to the dishes in a darkened room. One set of dishes was exposed to a fluorescent light at 4.8 Wm^{-2} for 15 min and incubated in the dark (covered with aluminum foil) at 37°C. An identical set of plates was protected from light throughout the experiment. After 10 days of incubation the cell colonies were counted.

Light sources and irradiation conditions. In BIAs of the effects of light on drug activity, polychromatic visible light was provided by two 15-W F15T8/D fluorescent bulbs (Sylvania GTE Products Corp., Danvers, Mass.) or one 24-W No. 1133 incandescent bulb (General Electric Co., Schenectady, N.Y.). Monochromatic light was obtained by inserting narrow-band interference filters (2 by 2 inch [2.54 by 2.54 cm]; Oriel Corp., Stratford, Conn.; Schott Glass Technologies, Inc., Duryea, Pa.) into the slide compartment of a Kodak Carousel projector equipped with a 250-W tungsten-halogen lamp. Samples to be irradiated were placed in front of the projector lens. The source of polychromatic visible light employed in studies of the antibacterial and cytotoxic activities of drugs was a single Panasonic F15T8/CW fluorescent bulb (Panasonic Industrial Co., Secaucus, N.J.). In all cases, light dosage was regulated by adjusting the distance of samples from the light sources. Light fluence rates were determined with a YSI-Kettering

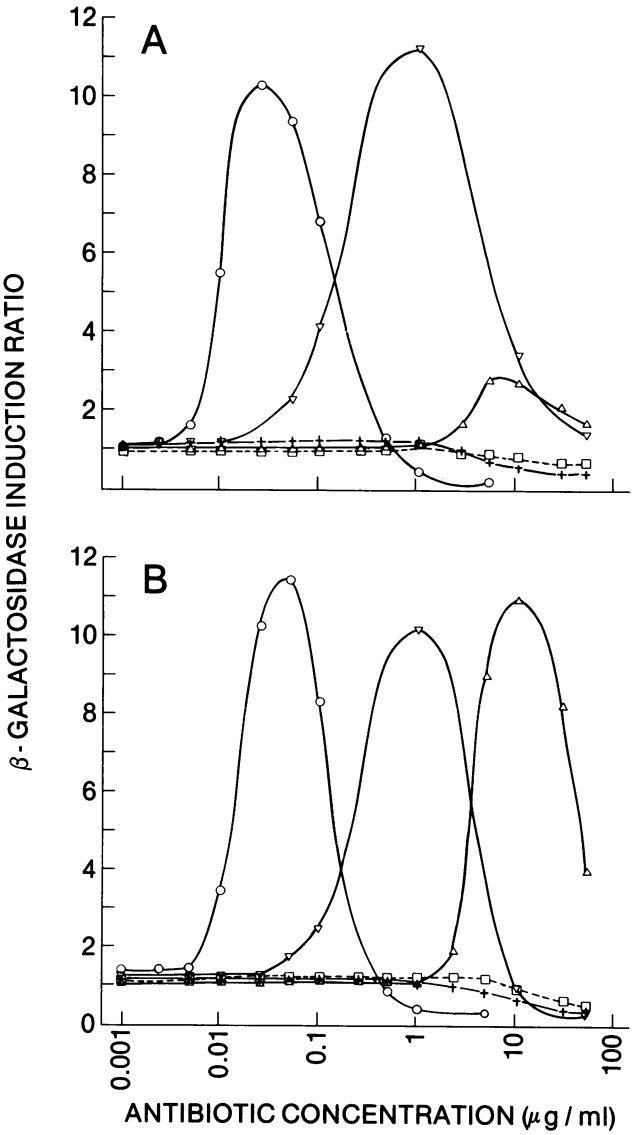

FIG. 2. Activation of ravidomycin (A) and desacetylravidomycin (B) in the BIA by light of specific wavelengths. Irradiation conditions were 362 nm, 0.1 Wm^{-2} (▽); 400 nm, 0.95 Wm^{-2} (○); 497 nm, 1.0 Wm^{-2} (△) ; 597 nm, 5.0 Wm^{-2} (+); dark control (□).

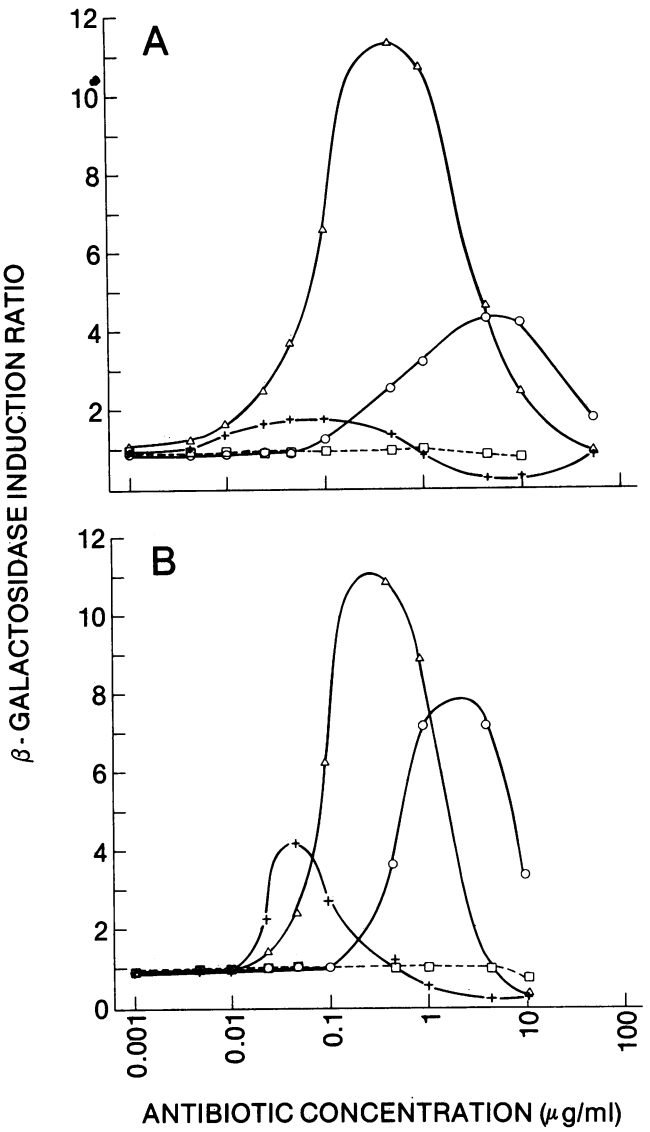

FIG. 3. Effect of light dosage on the BIA activities of ravidomycin (A) and desacetylravidomycin (B). Irradiation with visible light at the following fluence rates was tested: 1.0 Wm^{-2} (○), 10 Wm^{-2} (△), 100 Wm^{-2} (+), dark control (□).

model 65A radiometer (Yellow Springs Instruments, Inc., Yellow Springs, Ohio).

RESULTS

Enzyme induction in the BIA. Specific wavelengths of light were evaluated for their effects on the ability of ravidomycin and desacetylravidomycin to induce β-galactosidase in the BIA (Fig. 2). When induction mixtures were incubated in the dark, neither antibiotic caused enzyme induction; a decline in enzyme activity below spontaneous, uninduced levels (toxicity) was observed at antibiotic concentrations exceeding 5 μg/ml. When induction mixtures were irradiated with visible light at a wavelength of 400 nm and a fluence rate of 0.95 Wm^{-2}, both ravidomycin and desacetylravidomycin induced significant enzyme activity with approximate peak-inducing concentrations of 0.025 and 0.045 μg/ml, respectively. Light at 362 nm and 0.1 Wm^{-2} initiated peak enzyme induction at 20- to 30-fold-higher antibiotic concentrations, while light at 497 nm and 1.0 Wm^{-2} was effective at 200-fold-higher drug levels. When light at 597 nm and 5 Wm^{-2} was tested, no induction was observed (equivalent to incubation in the dark). The concentration of a reference antitumor antibiotic, bleomycin, required for enzyme induction was unaffected by light under our assay conditions (data not shown). No enzyme induction was observed when the assay organism was treated with light in the absence of antibiotics.

The relationship between BIA response and light dosage (Fig. 3) was determined by irradiating drug-cell mixtures with visible light at various fluence rates. As light intensity was increased, the concentrations of both drugs required to give peak enzyme induction and toxicity decreased. The reduction in actual peak enzyme activity at 100 Wm^{-2} compared with that at the lower fluence rates probably reflects enhanced antibiotic toxicity at this highest level of light treatment. These data suggest that the activities of ravidomycin and desacetylravidomycin in the BIA vary directly with the dosage of light that is introduced.

The activity of desacetylravidomycin treated with light before analysis in the BIA was examined (Fig. 4). Solutions of desacetylravidomycin at various concentrations were pretreated with visible light at 10 Wm^{-2} for 3 h. These were then tested with or without equivalent light exposure during the BIA induction period. Control antibiotic solutions, which were not pretreated with light, were similarly evaluated. It is clear that drug pretreated with light is not active when incubated with cells in the BIA in the dark. Enzyme induction only occurred when cells and drug combined were exposed to light during the induction period.

Antibacterial activity. The influence of light on the antibacterial activities of ravidomycin, desacetylravidomycin, and gilvocarcin V was evaluated (Table 1). The MIC of each antibiotic was determined in the presence and absence of

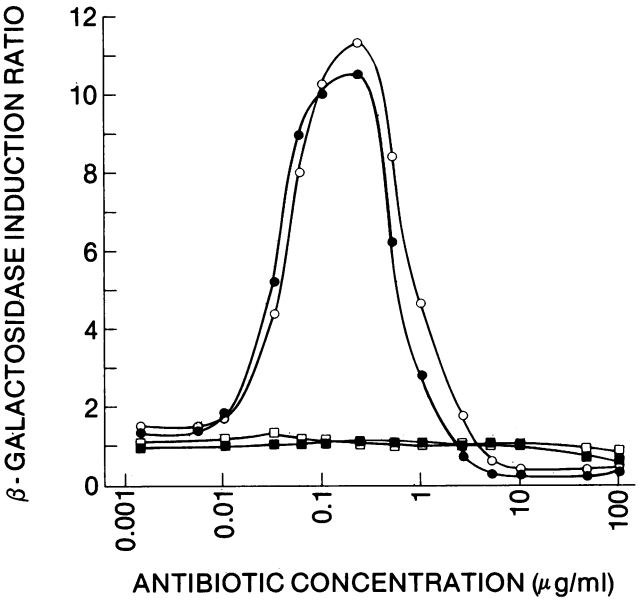

FIG. 4. Activity of desacetylravidomycin treated with light prior to analysis in the BIA. Antibiotic solutions were treated with visible light (open symbols), while duplicates were maintained in the dark (closed symbols). These were evaluated in the BIA with (circles) or without (squares) light treatment during the induction period.

TABLE 1. Effect of light on the antibacterial activity of ravidomycin, desacetylravidomycin, and gilvocarcin V

Organism	MIC (μg/ml) for the following antibiotics:					
	Ravidomycin		Desacetylravidomycin		Gilvocarcin V	
	Dark	Light	Dark	Light	Dark	Light
Gram-positive bacteria						
Staphylococcus aureus						
Smith	1	≦0.06	0.5	≦0.06	2	≦0.06
LL14	1	≦0.06	0.5	≦0.06	2	≦0.06
LL45	1	≦0.06	0.5	≦0.06	2	≦0.06
LL27	1	≦0.06	0.5	≦0.06	2	≦0.06
SSC-80-11	1	≦0.06	0.5	≦0.06	1	≦0.06
ATCC 25923	2	≦0.06	1	≦0.06	4	≦0.06
Enterococcus sp. strain						
SSC-80-62	0.5	≦0.06	0.25	≦0.06	2	≦0.06
SSC-80-63	0.5	≦0.06	0.25	≦0.06	2	≦0.06
Bacillus subtilis LL17	0.5	≦0.06	0.25	≦0.06	1	≦0.06
Gram-negative bacteria						
Escherichia coli						
311	>128	2	>128	1	>128	>128
Stfd-79-20	>128	2	>128	1	>128	>128
Klebsiella pneumoniae AD	128	1	128	1	>128	16
Serratia sp. strain TUV-78-15	128	64	>128	64	>128	>128
Serratia marcescens QHC-72-2	128	32	128	32	>128	>128
Citrobacter freundii K-81-28	>128	8	>128	1	>128	>128
Providencia stuartii SSC-80-78	128	2	64	1	>128	2
Proteus morganii K-79-25	64	2	>128	0.5	>128	4
Proteus vulgaris SM-77-1	>128	2	>128	0.5	NT[a]	NT
Proteus rettgeri K-77-6	>128	2	128	0.5	>128	2
Acinetobacter calcoaceticus K-77-1	128	2	128	0.5	>128	1
Pseudomonas aeruginosa						
12-4-4	128	64	128	16	>128	>128
SSC-78-13	128	64	128	64	>128	>128

[a] NT, Not tested.

visible light during culture incubation. In the dark, all three antibiotics were inhibitory for the gram-positive organisms (MIC range, 0.12 to 4 μg/ml), while little activity was demonstrated against the gram-negative organisms (MIC range, 64 to >128 μg/ml). When the plates used for the assays were irradiated with visible light during the incubation period, however, a dramatic increase in activity was observed for all of the gram-positive bacteria (MICs, ≦0.06 μg/ml) and for most of the gram-negative bacteria (MICs, ≦0.5 μg/ml). A decrease in MICs for *Proteus morganii* and *Proteus vulgaris* of greater than 256-fold was observed for desacetylravidomycin as a function of light treatment. The relative antimicrobial activities of the three drugs were in the following order: desacetylravidomycin > ravidomycin > gilvocarcin V.

TABLE 2. Effect of light on the cytotoxic activities of ravidomycin and desacetylravidomycin in the human colon carcinoma clonogenicity assay

Antibiotic dose (μg/ml)	Average no. of surviving colonies/culture for the following antibiotics:			
	Ravidomycin		Desacetylravidomycin	
	Light	Dark	Light	Dark
None	460	472	460	472
0.1	0	478	0	458
0.05	6	472	0	458
0.01	61	458	0	474
0.005	258	456	10	453
0.001	442	438	20	454
0.0002	466	460	28	467

Human colon carcinoma cytotoxicity. The killing of cultured human colon carcinoma cells by ravidomycin and desacetylravidomycin was measured as a function of visible light exposure (Table 2). In a clonogenicity assay, treatment with either antibiotic did not result in a decrease in the number of surviving colonies at concentrations as high as 0.1 μg/ml when cultures were held in the dark. However, in the light both drugs promoted a decline in surviving colonies. Desacetylravidomycin, the more active of the two compounds, demonstrated total inhibition at 0.01 μg/ml and a significant reduction even at the lowest concentration tested (0.0002 μg/ml).

DISCUSSION

In this study is presented a comprehensive analysis of the photoactivation properties of ravidomycin and desacetylravidomycin, results of which suggest that there is a correlation between their light-dependent initiation of DNA damage and the dramatic enhancement by light of their antimicrobial and cytotoxic activities. Photoactivation of these antibiotics in the BIA varies directly with the dosage of light and appears to be maximal at wavelengths in the visible blue and near UV (UVA) ranges. Application of light to drug prior to addition to cells did not result in induction; therefore, it appears that drug must be in contact with its cellular target for the light-dependent reaction to occur. It seems likely that these data are related to an earlier observation of photoactivated DNA damage by the antibiotics gilvocarcin V and chrysomycin A in a cell-free assay (27) and the subsequent indication of their light-initiated activities in the BIA (6).

Other natural products likewise have been reported to affect DNA via photodynamic reactions. The plant-derived furocoumarins (23), or psoralens, and the furoquinolines (18, 19) form covalent mono- or diadducts with DNA only when UVA light is introduced. Similarly, the psoralens require UVA treatment for their activity in the BIA (data not shown [6]). Camptothecin, a cytotoxic antitumor alkaloid, initiates single-stranded breaks in supercoiled DNA only when the drug-DNA mixture is irradiated with UVA light (14). Light-activated DNA damage promoted by camptothecin, which was observed in the absence of enzymes or other cellular constituents, presumably occurs by a mechanism that is distinct from its induction of protein-linked DNA breaks via mammalian DNA topoisomerase I (12). Recently, daunomycin has been observed to initiate DNA cleavage when visible light is applied to drug-DNA mixtures (10), while bleomycin, which initiates DNA damage without light, demonstrates enhanced activity if light at 300 to 350 nm is introduced (5). The poorly active cobalt (III) bleomycins efficiently introduce single-stranded breaks in DNA in the presence of UV or visible radiation (2).

As was observed with other photosensitizers (13), activation of ravidomycin and desacetylravidomycin seems to be optimal at wavelengths (362 and 400 nm) close to their absorption maxima (350 and 392 nm) (8). The same absorption peaks were retained when these drugs were tested in the presence of DNA (data not shown). It is interesting, however, that both compounds still induced in the BIA, although only at very high drug concentrations, when light at 497 nm was applied (Fig. 2). Absorption of light within this wavelength region was not observed with these drugs in the presence of absence of DNA (data not shown), nor has it been reported for any of the antibiotics within this class. It is possible that these potent photosensitizers can even be activated by the absorption of light energy at levels that cannot be detected by conventional analytical methods.

Although our data suggest that ravidomycin and desacetylravidomycin only initiate DNA damage with light exposure, the extent to which this light reaction may contribute to their in vivo antitumor activities remains uncertain. While irradiation with light was absolutely required for induction in the BIA, a distinct toxic reaction was still observed for both drugs in the dark at concentrations exceeding 5 μg/ml (Fig. 2). Antimicrobial activity likewise was detected for these antibiotics and gilvocarcin V in the absence of light (Table 1). These activities may reflect blocks in macromolecular synthesis because of intercalative drug binding to DNA, which has been observed to occur in the dark for members of this antibiotic class (26, 27). The failure of gilvocarcin M, which does not respond to light in the BIA (6), to demonstrate significant antitumor activity (1, 15) has been cited as evidence that the in vivo antitumor properties of gilvocarcin V may be related to light-mediated activation (6). However, other properties of the methyl component that are unrelated to light activation, such as a reduced capacity to intercalate into DNA (11, 26) or possibly altered pharmacological behavior, may be responsible for its reduced in vivo activity. Further studies are necessary to establish conclusively whether ambient light or another endogenous activating mechanism might have contributed to any extent to the reported in vivo antitumor activity of the active antibiotic species.

The significance of the light reaction is demonstrated by the potent in vitro photosensitizing activities of ravidomycin and desacetylravidomycin. The enhanced cytotoxicity of these compounds for human colon carcinoma cells because of a brief exposure to visible light, which was at least 500-fold for ravidomycin and even greater for desacetylravidomycin (Table 2), dramatically illustrates this point. This suggests that one could introduce light directly into a tumor in an animal treated with levels of these drugs that are well below those that would normally produce any therapeutic or generalized toxic effect and could target a favorable, toxic response solely to the tumor site. Such tumor-localized phototherapy has been practiced with hematoporphyrin derivative for a variety of cancers (3, 4) and with the psoralens in the treatment of cutaneous T-cell lymphomas (9) and the extracorporeal therapy of leukemias (R. L. Edelson, U.K. patent application 2,100,143, December 1982). We realize that the UVA-visible blue wavelengths required for optimal activation of ravidomycin and desacetylravidomycin are more limited in tissue penetration than are longer wavelengths (17, 30). However, the actual potential of these highly potent photosensitizers in phototherapy will only become apparent through direct in vivo experimentation.

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