Antitumor Activity and Some Pharmacologic Properties of Anthramycin Methyl Ether¹

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SUMMARY

The antitumor antibiotic, anthramycin methyl ether (AME), was shown to be active in increasing survival time in mice bearing various experimental leukemias. Like actinomycin D, it was active against P388 but inactive against P388/38280. AME also was active against the plasma cell tumor LCP, while having no activity against the plasma cell tumor YPC-1. AME had an ID₅₀ of 0.02 µg/ml against L1210 cells in tissue culture.

The effects of AME were studied on other pharmacologic parameters. Hexobarbital sleeping times were doubled in mice receiving daily injections of AME for 4 days, while single injections at times varying from 30 minutes to 4 hours prior to hexobarbital had no effect on sleeping time. AME did not affect the blood pressure, respiration, or EKG in anesthetized dogs, nor did it alter the blood pressure responses to various autonomic stimulants.

AME markedly inhibited uptake of tritiated uridine in both L1210 and P388 leukemic cells in vivo. AME also interfered with uptake of tritiated thymidine by L1210 cells. Spectral studies indicated that the ultraviolet absorption maximum of AME was shifted to longer wave lengths following interaction with DNA, but deoxyguanosine had no effect on the absorption maximum of AME. The anthranilic acid moiety did not form complexes with DNA or deoxyguanosine nor did it have cytotoxic effects in tissue culture in doses up to 1000 times that of the ID₅₀ for AME.

INTRODUCTION

In 1963 it was observed that a thermophilic actinomycete, Streptomyces ruineus, produced in a fermentation beer a substance possessing antibiotic properties and exhibiting antitumor activity in three mouse tumor systems (11). This preparation contained approximately 0.5% of an antibiotic, anthramycin, which has been purified and is responsible for the antitumor activity (9). Anthramycin has been isolated in the form of its crystalline methyl ether which is comparatively more stable than anthramycin (8, 9).

¹A preliminary report of this study was presented at the Federation of American Societies for Experimental Biology, Chicago, Illinois, 1967.

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MATERIALS AND METHODS

Antitumor Activity. Four doses of AME were used in each experiment: 0.05, 0.1, 0.15, and 0.2 mg/kg (10 mice/dose). The drug was dissolved in DMSO and diluted with physiologic saline so that the final concentration of DMSO was not greater than 2%. AME was administered i.p. daily from Day 1 (24 hours after the tumor transplant) through Day 10. The 2 reticulum cell sarcomas were inoculated s.c.; the other tumors used in this study were inoculated i.p. The ascitic form of leukemia P388 was also inoculated intracerebrally. All of these...
tumors are carried in our laboratory and we have previously
tested other compounds in these systems (1, 2, 4). The optimal
dose (mg/kg) for increasing the median survival time of each
tumor is reported. The approximate optimal dose in mg/sq m
was calculated using the $K_m$ factor of 3 (5). The survival time
of mice treated with 0.25–2% DMSO did not differ from un-
treated controls.

L1210 cells (originally obtained from Southern Research
Institute, Birmingham, Alabama) were maintained in static cul-
ture in R.P.M.I. #1630 medium (20% fetal calf serum) (3,
10). Stock bottles, after five days growth, were counted and
sorted so that there were approximately 1 x 10^6 cells/ml and a
total of 5 ml in each experiment. Drugs were filtered through
a Swinney filter immediately before using. A total of 0.1 ml of
the drug solution or the solvent alone and 0.1 ml of physiologic saline was added to 5 ml of these cells. Cell counts were taken
every 24 hours for three days using a Coulter counter.

**Pharmacology.** The effects of AME on the blood pressure of the
anesthetized dog were studied. Two male Beagle dogs were
anesthetized by the i.v. administration of pentobarbital, 30 mg/
kg, and barbital, 220 mg/kg. A femoral artery was cannulated and
connected to a pressure transducer for recording blood
pressure. An endotracheal tube was inserted and connected
to a pressure transducer for recording respiration. Skin elec-
trodes were used to monitor the EKG. Recordings for blood
pressure and respiration were made on a Sanborn Model No.
350 polygraph. After respiration and arterial blood pressure had
stabilized, pharmacological responses to the following i.v. injec-
tions were obtained for comparative purposes: epinephrine,
norepinephrine, acetylcholine, histamine, and DMPP. The con-
tral blood pressure response from cross-clamping both carotid
arteries for 45 seconds or peripheral vagal stimulation also
was recorded. AME was administered in doses up to 1 mg/kg.

The effect of AME on hexobarbital sleeping time in mice
(loss of righting reflex) was studied. CDF1 male mice were
injected i.p. with 0.15 mg/kg AME at either 30 minutes, 1
hour, 4 hours, or daily for 4 days prior to i.p. hexobarbital,
150 mg/kg. The effect of AME on phenobarbital induction of
hexobarbital sleeping time was also studied. In these studies,
groups of mice were injected for 4 days with one of the fol-
lowing: DMSO-saline (solvent), AME (0.15 mg/kg), solvent
30 minutes prior to saline, solvent 30 minutes prior to pheno-
barbital (80 mg/kg in saline) or AME (0.15 mg/kg) 30 minutes
prior to phenobarbital (80 mg/kg). Twenty-four hours after
the last injection, mice were given hexobarbital, 150 mg/kg
i.p., and the sleeping times were observed.

**The Effect of AME on Uptake of Tritiated Thymidine and
Uridine.** The effect of AME on uptake of tritiated thymidine
and uridine *in vivo* by P388 and L1210 leukemia cells in the
ascites form was studied using a radioautographic technic. Iso-
topic uptake was studied on the sixth day after tumor inocula-
tion. Untreated mice and mice treated with actinomycin D
were used as controls. Untreated mice weighing 25 gm each
were injected i.p. with 10 μc of TdR-3H, specific activity 2
c/mole, or UdR-3H, specific activity 2 c/mole; ascites fluid
was sampled at 1 hour for TdR-3H-treated mice and at 30
minutes for mice receiving UdR-3H. In the treated group for
each tumor a group of 8 mice was used. Four were treated 1
hour prior to the injection of isotope with AME, 0.15 mg/kg
i.p., and 4 with actinomycin D, 0.1 mg/kg i.p. Two mice in each
drug group received UdR-3H and 2 received TdR-3H in the
same dose as the controls by the i.p. route. Ascites fluid was
sampled as in the control group.

Cells were removed by abdominal puncture in 0.1 ml quan-
tities and suspended in 1% citrate for five minutes, centrifuged
at 800 rpm, and resuspended in a 4:1 mixture of methyl alcohol
and glacial acetic acid for fixation. The cells were then cen-
trifuged and resuspended in enough fixative to give a cloudy
suspension, dropped on previously gelatinized slides, and
allowed to dry. Kodak AR10 stripping film was applied and the
slides were exposed for one week in light-tight boxes, developed,
and stained with giemsa for reading. The labeling index, the
percent of cells labeled in a 200 cell count, and the mean grain
count, the average number of grains per cell (50–100 cell count),
determined on each of 2 slides for each animal and the
results averaged.

**Effect of DNA on Spectrum of AME and Related Com-
pounds.** Purified calf thymus DNA dissolved in 1:10 SSC
(0.015 M NaCl, 0.0015 M sodium citrate) was added to a cu-
vette containing AME, anthranilic acid, or 3-hydroxyanthranilic
acid, and the spectral changes were recorded on a Beckman DB
Spectrophotometer for 60 minutes. A total of 0.5 ml DNA (500
μg/ml) was added to 1 ml of AME, 5 μg/ml, or 1 ml of an-
thranilic acid or 3-hydroxyanthranilic acid (33 μg/ml). The
effect of deoxyguanosine, 0.5 ml (500 μg/ml), on the spectra
of the 3 compounds was also observed.

### RESULTS

Results presented in Table 1 show that AME is active against
various mouse lymphocytic leukemias, including leukemia
L1210, leukemia K1946, leukemia P388, and the variant of P388
resistant to vincristine (P388/VCR). The percent increase in
survival time of AME-treated mice as compared to controls
ranged from 50 percent (leukemia L1210) to 104 percent (leu-
kemia 5178Y). However, AME had little or no activity against
the variant of P388 resistant to NSC 38280, a terephthalanilide
derivative. It is of interest that actinomycin D, which is active
against leukemia P388, is also cross-resistant to P388/38280

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Daily optimal dose mg/kg</th>
<th>% increase in median survival over control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukemia L1210</td>
<td>0.2</td>
<td>50</td>
</tr>
<tr>
<td>Leukemia K1946</td>
<td>0.05-0.15</td>
<td>71</td>
</tr>
<tr>
<td>Leukemia 5178Y</td>
<td>0.2</td>
<td>104</td>
</tr>
<tr>
<td>Leukemia P388</td>
<td>0.15-0.2</td>
<td>95</td>
</tr>
<tr>
<td>Leukemia P388/VCR</td>
<td>0.15</td>
<td>71</td>
</tr>
<tr>
<td>Leukemia P388/38280</td>
<td>0.15</td>
<td>17</td>
</tr>
</tbody>
</table>

Activity of anthramycin methyl ether (AME) against various
mouse leukemias.

*a* Tumors inoculated i.p., AME given daily i.p. Days 1–10 after
inoculation, at 0.05, 0.1, 0.15, and 0.2 mg/kg.
Antitumor Activity of Anthramycin Methyl Ether

Table 2

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Median Optimal daily dose (mg/kg)</th>
<th>Median survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P388</td>
<td>0.05</td>
<td>23</td>
</tr>
<tr>
<td>Controls</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>P388/38280</td>
<td>0.0125-0.05</td>
<td>8.5</td>
</tr>
<tr>
<td>Controls</td>
<td>8.5</td>
<td></td>
</tr>
</tbody>
</table>

Activity of actinomycin D against leukemias P388 and P388/38280.

* Tumors inoculated i.p., actinomycin D given daily i.p., Days 1-10 after inoculation, at 0.0125, 0.025, 0.05, and 0.1 mg/kg.

Table 3

<table>
<thead>
<tr>
<th>Dose of AME* (mg/kg)</th>
<th>Median survival time (days)</th>
<th>% increase in median survival over control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>10.5</td>
<td>5</td>
</tr>
<tr>
<td>0.15</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>0.2</td>
<td>11.5</td>
<td>15</td>
</tr>
<tr>
<td>Controls</td>
<td>10</td>
<td>--</td>
</tr>
</tbody>
</table>

Activity of anthramycin methyl ether (AME) against leukemia P388 after intracerebral inoculation.

* AME given daily i.p., Days 1-10 after inoculation.

Table 4

<table>
<thead>
<tr>
<th>Tumor*</th>
<th>Daily optimal dose (mg/kg, mg/sq m)</th>
<th>% increase in median survival over control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mast cell P815</td>
<td>0.15/0.45</td>
<td>23</td>
</tr>
<tr>
<td>Plasma cell LCP</td>
<td>0.1/0.3</td>
<td>104</td>
</tr>
<tr>
<td>Plasma cell YPC-1</td>
<td>0.15/0.45</td>
<td>30</td>
</tr>
<tr>
<td>Reticulum cell sarcoma (L)</td>
<td>0.1/0.3</td>
<td>0</td>
</tr>
<tr>
<td>Reticulum cell sarcoma (O)</td>
<td>0.05/0.15</td>
<td>0</td>
</tr>
</tbody>
</table>

Activity of anthramycin methyl ether (AME) against various mouse tumors.

* Reticulum cell sarcomas inoculated s.c., other tumors i.p., AME given daily i.p.

Table 5

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Sleeping time, minutes* ^a,c</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO-saline (solvent)</td>
<td>66.7 ± 3.2</td>
</tr>
<tr>
<td>AME in solvent</td>
<td>124.7 ± 11.0</td>
</tr>
<tr>
<td>Solvent 30 minutes prior to saline</td>
<td>64.4 ± 11.0</td>
</tr>
<tr>
<td>Solvent 30 minutes prior to phenobarbital</td>
<td>23.7 ± 4.9</td>
</tr>
<tr>
<td>AME 30 minutes prior to phenobarbital</td>
<td>31.1 ± 2.9</td>
</tr>
</tbody>
</table>

Effect of anthramycin methyl ether (AME) on phenobarbital induction of hexobarbital sleeping time.

* 10 mice in each group

* Average ± standard error.

* Experiment repeated with similar results.

Table 6

<table>
<thead>
<tr>
<th>Tumor*</th>
<th>Median optimal daily dose (mg/kg)</th>
<th>Median survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210 leukemia</td>
<td>Thymidine-3H Li MGC</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Uridine-3H Li MGC</td>
<td>60</td>
</tr>
<tr>
<td>P388 leukemia</td>
<td>Thymidine-3H Li MGC</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Uridine-3H Li MGC</td>
<td>17</td>
</tr>
<tr>
<td>Control</td>
<td>58</td>
<td>60</td>
</tr>
<tr>
<td>AME</td>
<td>52</td>
<td>13</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>55</td>
<td>58</td>
</tr>
</tbody>
</table>

Effect of AME on uptake of thymidine-3H and uridine-3H by leukemia L1210 and P388 in vivo. AME, anthramycin methyl ether; Li, labeling index (percent of cells labeled) 200 cell count per slide; and MGC, mean grain count per cell average of 50-100 cells counted per slide.

(Table 2). AME activity in the L1210 and P388 systems was optimal at the dose of 0.2 mg/kg, an LD50 dose; in the other leukemias, optimal activity was generally shown at less than the LD50 dose.

A heptane-buffer or heptane-water partition coefficient revealed that less than 0.1% of AME was in the heptane layer. Therefore, one would not expect AME to cross the blood-brain barrier to any great extent. Data presented in Table 3 show that AME was inactive at all dose levels against leukemia P388 inoculated intracerebrally. This is in contrast to the marked increase in survival time of mice inoculated with leukemia P388 i.p. (Table 1).

AME was also active against the plasma cell tumor LCP but was inactive against another plasma cell tumor YPC-1. Anthramycin methyl ether was inactive in the mast cell P815 and 2 reticulum cell sarcomas (Table 4).

AME also was effective against L1210 cells grown in tissue culture; the dose necessary for 50 percent inhibition was approximately 0.02 µg/ml.

AME, when injected in mice 30 minutes, 1 hour, or 4 hours prior to hexobarbital, did not significantly affect hexobarbital sleeping time. The effect of AME on phenobarbital induction of hexobarbital sleeping time is difficult to evaluate (Table 5) because of the effect of AME on sleeping time itself after 4 days' treatment. However, the results indicate AME has little or no effect in blocking the phenobarbital induction of sleeping time.

Doses of AME up to 1 mg/kg did not affect the blood pressure, respiration, or EKG of anesthetized dogs. Also, blood pressure responses to epinephrine, norepinephrine, acetylcholine, histamine, DMPP, bilateral carotid occlusion, or peripheral vagal stimulation were not altered after administration of AME.

Effect of AME on the Uptake of Tritiated Thymidine and Uridine. The results of these studies are illustrated in Table 6. AME markedly inhibited the uptake of Udr-3H in both L1210 and P388 leukemia cells. The effect was more striking in L1210 leukemia, where the rate of uptake of Udr-3H is high in the untreated controls. The uptake of Udr-3H was also inhibited by pretreatment with actinomycin D in both tumor systems. However, the effect in L1210 leukemia cells was not as marked as that produced by AME. AME caused a 95 percent reduction in the number of cells utilizing Udr-3H and a 67 percent reduction in the mean grain count per cell, which serves as a gross indication of the quantity of isotope incorporated per
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cell. This effect was expected with actinomycin D since this
drug is thought to inhibit DNA-directed RNA synthesis.

In the L1210 leukemia cells, AME did not change the la-
beling index for TdR-3H but did markedly reduce the mean
grain count from 60 to 13/cell, indicating interference with the
incorporation of TdR-3H into DNA, an effect not noted with
actinomycin D. This effect was not apparent in the P388 leu-
kemia cells pretreated with AME.

Effect of DNA on Spectrum of AME and Related Com-
ounds. It has previously been observed that DNA reacts with
AME, resulting in a shift of the absorption maximum from 334
to 343 ma after a period of 30 minutes (Kohn and Spears,
unpublished observations). We have confirmed this observa-
tion and have also studied the effect of DNA and deoxyguano-
sine on the absorption maximum of anthranilic acid and 3-
hydroxyanthranilic acid. Under our experimental conditions,
neither DNA nor deoxyguanosine affect the absorption maxi-
num of anthranilic acid or 3-hydroxyanthranilic acid after
observation for 60 minutes. Furthermore, unlike actinomycin
D, the absorption maximum of AME is not altered by deoxy-
guanosine.

DISCUSSION

On the basis of studies reported here and elsewhere (6), it is
evident that anthramycin methyl ether has a broad spectrum of
activity against a number of experimental neoplasms in
rodents. We have found AME to be especially effective against
several leukemias. Studies of the heptane-water partition of
AME showed that it was very soluble in water. It is not sur-
prising therefore that AME was active against P388 inoculated
i.p. but inactive against P388 inoculated intracerebrally.

AME did not affect hexobarbital sleeping time in mice when
given 30 minutes, 1 hour, or 4 hours prior to hexobarbital.
However, after 4 days of treatment with AME the hexobarb-
ital sleeping time of mice was doubled. This effect of AME
was probably not due to its synergizing the central depressant
effects of hexobarbital because, on awakening, the mice were
again injected with AME and none lost their righting reflex.
The lack of acute effects by AME on sleeping times also sup-
ports this viewpoint. Phenobarbital induction of hexobarbital
sleeping time apparently was not effected by treatment with
AME. Therefore, it appears likely that the prolongation of
sleeping time after repeated doses of AME is due to its inhi-
bitory effects on nucleic acid synthesis and not to any central
action of the drug.

Acute, AME is devoid of adrenergic blocking, ganglionic
blocking, anticholinergic activity, and antihistaminic proper-
ties, as evidenced by dog blood pressure studies.

In studies on the mechanism of action of AME, it has been
postulated that this antibiotic is transformed in vivo to
"actinomycin analogs" which are actually responsible for the
observed antitumor activity (8). That interference with nucleic
acid synthesis is a mechanism by which AME is active is sug-
bested by the results of our studies on the uptake of uridine
and thymidine into L1210 and P388 leukemia cells after treat-
ment with AME or actinomycin D. AME inhibited the incor-
poration of uridine into both of the latter leukemias. However,
with the L1210 cells this effect was even more pronounced than
that observed with actinomycin D. On the other hand, inhibi-
tion of thymidine incorporation into L1210 cells was only ob-
erved with AME-treated cells, while no inhibition of uptake
was noted with the 2 antibiotics in P388 leukemia.

It is also of interest that AME exhibits cross-resistance to
the terephthalanilide 38280-resistant variant of P388, as does
actinomycin D. Spectral studies show that this terephthalanilide
derivative [4',4"-bis (2-imidazolin-2-yl) 2-chloroterephthalanilide
dihydrochloride] interacts in vitro with both DNA and RNA
(7). Spectral studies also indicate that, although the ultraviolet
absorption maxima of both AME and actinomycin D are
shifted to longer wave lengths following interaction with DNA
in vitro, deoxyguanosine has no effect on the absorption max-
imum of AME.

Finally, neither anthranilic acid or 3-hydroxyanthranilic acid,
integral parts of the anthramycin molecule, form complexes
with DNA or deoxyguanosine, as evidenced by spectral studies;
also, these compounds have no cytotoxic effects against L1210
in tissue culture in doses up to 100 times that of the ID50
for anthramycin methyl ether. Therefore, the anthranilic acid
moiety alone is not responsible for the biologic effects observed
with AME.

Thus, there are similarities as well as dissimilarities be-
 tween AME and actinomycin D in the above-mentioned
studies, all of which indicate an interference with nucleic acid
synthesis. However, the manner in which this interference is
manifested remains unclear and requires further study.

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methyl ether used in these studies.

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