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# A 1:2-DIHYDRO-*s*-TRIAZINE WITH MARKED ACTIVITY AGAINST CERTAIN PATHOGENIC BACTERIA AND SYNERGISTIC WITH A SULPHONAMIDE IN EXPERIMENTAL BACTERIAL INFECTIONS

M. W. FISHER and L. DOUB
Research Laboratories, Parke, Davis and Company,
Detroit, Michigan

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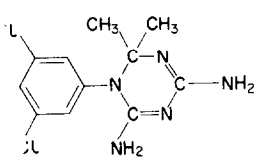
**Abstract**—A dihydro-*s*-triazine (1-(3′:5′-dichlorophenyl)-2:2-dimethyl-4:6-diamino-1:2-dihydro-*s*-triazine) is described which has marked selective activity *in vitro* and *in vivo* against certain pathogenic bacteria. The most susceptible bacteria were streptococci of Lancefield Groups A, C, E, F and G, a type 2 pneumococcus, and a strain of *Streptococcus salivarius*. This triazine was also highly synergistic with sulfamethoxypyridazine when both were given orally in experimental infections caused by *Streptococcus pyogenes Diplococcus pneumoniae* and *Streptococcus salivarius*. The implications of these observations are discussed.

THIS is a partial and preliminary report on a study of a series of 1:2-dihydro-*s*-triazines concerned with their activity *in vitro* and *in vivo* against bacterial pathogens of medical importance. More specifically, this communication deals with representative experiments related to the antibacterial activities of 1-(3′:5′-dichlorophenyl)-2:2-dimethyl-4:6-diamino-1:2-dihydro-*s*-triazine, (here called "ABT-15251") the structure of which is given below:




In addition, a description is presented of the synergizing effects of this compound with the sulphonamide, sulphamethoxypyridazine (Midicel*), when given together in certain experimental bacterial infections in mice. Further studies on similar triazines, the synergism of this and other triazines with various sulphonamides, and additional details of antibacterial activity will be reported elsewhere.

Although other compounds related to the one reported here have been described with activity against certain protozoa[1, 2, 3, 4] and some strains of nonpathogenic bacteria,[5] no previous publication has described such activity against pathogenic bacteria, particularly *in vivo*. Indeed, there is indirect evidence, to be inferred from

*The trade name of Parke, Davis and Company for sulphamethoxypyridazine (3-sulphanilamido-6-methoxypyridazine) is "Midicel".

previous reports,[1, 6] that compounds of this type should not be effective *in vivo* against bacterial pathogens. However, it will be shown here that compound ABT-15251 exerts very marked selective inhibitory activity against certain of the pathogenic bacteria *in vitro*, and marked chemotherapeutic effects against infections in mice with some of these bacteria.

Of even greater importance, from the viewpoint of potential applications in the chemotherapy of bacterial infections, is the striking synergism effected by ABT-15251 when it is administered with sulphamethoxypyridazine in infected mice. The degree of this combined action resembles that previously reported by one of us (M.W.F.) of the synergistic combined action of human $\gamma$-globulin and chloramphenicol (Chloromycetin*) in experimental bacterial infections in mice.[7] Moreover, this triazine-sulphonamide synergism *in vivo* also has not been described in any previous publication with respect to pathogenic bacteria.

## MATERIALS AND METHODS

*Dihydrotriazine.* ABT-15251 was first described by Crowther[8]; it was prepared for this work by the general method outlined in references 1 and 5.

*In vitro.* Stock solutions of ABT-15251 were autosterilized in 70% ethanol, and were then serially diluted in tubed trypticase soy broth (Baltimore Biological Laboratories) containing 10% bovine serum (v/v). Twofold decrements were prepared from an initial concentration of 100 $\mu$g/ml. Each 5 ml of medium was inoculated with 0·05ml of a $10^{-6}$ dilution of a fully grown broth culture of bacteria, these tubes were incubated for 18–24 hr at 37 °C, and then an estimate made of complete growth-inhibition end-points. Tests involving tubercle bacilli were performed in a like manner, with the exception that a defined basal medium was employed (consisting essentially of asparagine, glycerol and a few mineral salts) and incubation was for 7 days. All determinations *in vitro* were performed in duplicate and on separate occasions.

*In vivo.* Webster strain, Albino Swiss mice of either sex, weighing 18–22 g, were used throughout these studies. Acute and subacute infections were induced with the C203 strain of *Streptococcus pyogenes*, employing dosages of from 100 to 1000 $LD_{50}$. For the acute infection, 0·5 ml of a $10^{-5}$ dilution in broth of a 6 hr broth culture was administered intraperitoneally; this was uniformly lethal in untreated mice within 24 hr. The subacute infection was similarly prepared, with the exception that the mice were injected subcutaneously; without treatment the median survival time of mice so infected was approximately 5 days. Experiments with the SVI strain of *Diplococcus pneumoniae* involved the intraperitoneal injection of 0·5 ml of broth containing a $10^{-6}$ dilution of a 6 hr broth culture. This dose, 100 $LD_{50}$, effected a 90–100 per cent mortality in untreated mice within 3 days. Infection with the 04150 strain of *Streptococcus salivarius* was accomplished by the intraperitoneal injection of 0·5 ml of a $10^{-1}$ dilution of a 6 hr broth culture suspended in 5% hog gastric mucin, representing 100 $LD_{50}$. All untreated mice infected with this organism died within 48 hr.

All drug treatment was peroral, with various dose levels administered by intubation of 0·5 ml of a 5% acacia solution of each compound. In all instances, the treatment, which usually consisted of a single dose, was initiated within a few minutes after the infection was induced. Groups of from fifteen to twenty mice were employed per

*The trade name of Parke, Davis and Company for chloramphenicol is "Chloromycetin".

regimen, and all experiments were performed at least twice. Surviving mice were observed for 7–21 days after infection, depending on the acuteness of the infection.

In testing ABT-51251 alone, such dosage levels were selected from preliminary tests as would provide survival percentages ranging between 0 and 100. This permitted the estimation of the 50 per cent curative dose, employing the Miller–Tainter method.[9]

TABLE 1. *In vitro* ANTIBACTERIAL SPECTRUM OF ABT-15251

| Organism | Strain | MIC* ($\mu$g/ml) |
|---|---|---|
| *Aerobacter aerogenes* | 0126 | >100·0 |
| *Brucella suis* | H-1772 | 100·0 |
| *Corynebacterium diphtheriae* | 036 | 1·56 |
| *Diplococcus pneumoniae*, type 1 | SVI | 100·0 |
| *Diplococcus pneumoniae*, type 1 | D-1 | 100·0 |
| *Diplococcus pneumoniae*, type 2 | D-2 | 0·012 |
| *Klebsiella pneumoniae* | AD | 100·0 |
| *Mycobacterium tuberculosis* | H37Rv | 50·0 |
| *Neisseria catarrhalis* | 03447 | 25·0 |
| *Neisseria meningitidis* | B | 50·0 |
| *Neisseria meningitidis* | C | >100·0 |
| *Neisseria meningitidis* | D | >100·0 |
| *Neisseria meningitidis* | CDC | 100·0 |
| *Pasteurella multocida* | 310 | 6·25 |
| *Proteus vulgaris* | 1810 | 100·0 |
| *Pseudomonas aeruginosa* | 28 | 50·0 |
| *Salmonella paratyphi* | 02156 | 50·0 |
| *Salmonella typhimurium* | V31 | 50·0 |
| *Salmonella typhosa* | 02481 | 50·0 |
| *Shigella sonnei* | 04628 | 50·0 |
| *Staphylococcus aureus* | Smith | 100·0 |
| *Staphylococcus aureus* | UC76 | 100·0 |
| *Staphylococcus aureus* | 04945 | 100·0 |
| *Staphylococcus aureus* | 04985 | 100·0 |
| *Streptococcus pyogenes*, grp. A† | C203 | 0·02 |
| *Streptococcus pyogenes*, grp. A | 04772 | 0·1 |
| *Streptococcus pyogenes*, grp. A | N-19 | 0·05 |
| *Streptococcus pyogenes*, grp. A | GLN-55 | 0·02 |
| *Streptococcus pyogenes*, grp. A | 04715 | 3·13 |
| *Streptococcus pyogenes*, grp. A | 04364 | 3·13 |
| *Streptococcus pyogenes*, grp. A | Rammelkamp | 0·003 |
| *Streptococcus pyogenes*, grp. A | Quinn | 0·000002 |
| *Streptococcus agalactiae*, grp. B | RI-090R | 6·25 |
| *Streptococcus agalactiae*, grp. B | IbH36B | 100·0 |
| *Streptococcus sp.*, grp. C | Loosli | 0·2 |
| *Streptococcus sp.*, grp. C | 674 | 0·012 |
| *Streptococcus sp.*, grp. C | CDC-SS-188 | 0·006 |
| *Streptococcus faecalis*, grp. D | Viger | 100·0 |
| *Streptococcus faecalis*, grp. D | C-L | >100·0 |
| *Streptococcus faecalis*, grp. D | CDC-SS-498 | >100·0 |
| *Streptococcus faecalis*, grp. D | MGH-1 | >100·0 |
| *Streptococcus faecalis*, grp. D | MGH-2 | >100·0 |
| *Streptococcus faecalis*, grp. D | MGH-3 | >100·0 |
| *Streptococcus sp.*, grp. E | K129 | 0·098 |
| *Streptococcus sp.*, grp. E | K131 | 1·56 |
| *Streptococcus sp.*, grp. F | C628 | 0·024 |
| *Streptococcus sp.*, grp. F | B647 | 0·003 |
| *Streptococcus sp.*, grp. G | RI-D166B | 0·006 |
| *Streptococcus sp.*, grp. G | RI-F68A | 0·098 |
| *Streptococcus salivarius* | 04150 | 0·01 |

*Minimal inhibitory concentration.
†Designates Lancefield group.

Combination therapy experiments with ABT-15251 and sulphamethoxypyridazine were designed so that minimally effective dose levels, of each compound alone, were employed. In these studies, simultaneous tests were conducted with the compounds separately and given as a mixture. The type of combination therapy experiments reported here involved the use of a constant dose of ABT-15251 given with varying dosages of sulphonamide. Similar effects were obtained in these infections when therapy was conducted with a fixed sulphonamide dose given with varying dosages of ABT-15251, but these will not be reported here.

## RESULTS

The range of activity of ABT-15251 against various pathogenic bacteria *in vitro* is presented in Table 1. It is apparent that the most marked activity was shown against the six strains of *Streptococcus pyogenes*, one strain, "Quinn", being completely inhibited by as little as 0·000002 μg/ml. Good to marked inhibition was also observed with streptococcal species of Lancefield groups C, E, F and G, and with a strain of *Streptococcus salivarius*. Little or no activity was obtained against group D streptococci (six strains of *Streptococcus faecalis*), and a substantial difference in susceptibility occurred between the two strains of group B streptococci. An additional provocative observation was that a strain of type 2 pneumococcus was completely inhibited by 0·012 μg/ml, whereas two strains of type 1 required almost 10,000 times more compound for inhibition. Finally, less than 10 μg/ml was effective against *Corynebacterium diphtheriae* and *Pasteurella multocida*.

The nature of the activity of ABT-15251 given alone *in vivo* is illustrated by the data given in Tables 2, 3 and 4, involving infections with *Streptococcus pyogenes* (C203) and *Streptococcus salivarius* (04150). Although the experiments are not described here, it was observed that this compound was considerably more active when given subcutaneously in these infections. The fact that single doses of ABT-15251 were about equally effective in the acute and subacute streptococcal infections suggests that this compound either had a significant bactericidal action *in vivo* or persisted for prolonged periods in these mice.

TABLE 2. ACTIVITY OF ABT-15251 IN AN ACUTE INFECTION IN MICE INDUCED WITH *Streptococcus pyogenes* (C203)*

| Dose of ABT-15251 (mg/kg)† | Survivors/total | Survivors % |
|---|---|---|
| 0 | 0/20 | 0 |
| 0·625 | 1/20 | 5 |
| 1·25 | 2/20 | 10 |
| 2·5 | 3/20 | 15 |
| 5·0 | 9/20 | 45 |
| 10·0 | 14/20 | 70 |

50% curative dose: 6·0 ± 1 mg/kg.
*Induced intraperitoneally; median survival time untreated controls less 24 hr.
†Single oral dose given at time infection was induced.

TABLE 3. ACTIVITY OF ABT-15251 IN A SUBACUTE INFECTION IN MICE INDUCED WITH *Streptococcus pyogenes* (C203)*

| Dose of ABT-15251 mg/kg† | Survivors/total‡ | Survivors % |
|---|---|---|
| 0 | 3/40 | 7·5 |
| 3·13 | 6/20 | 30·0 |
| 6·25 | 17/40 | 42·5 |
| 12·5 | 29/40 | 72·5 |
| 25·0 | 26/40 | 65·0 |
| 50·0 | 37/40 | 92·5 |
| 100·0 | 20/20 | 100·0 |

50% curative dose: 8·0 ± 2·3 mg/kg.
*Induced subcutaneously; median survival time of untreated controls approximately 120 hr.
†Single oral dose given at time infection was induced.
‡Data pooled from two experiments.

TABLE 4. ACTIVITY OF ABT-15251 IN AN ACUTE INFECTION IN MICE INDUCED WITH *Streptococcus salivarius* (04150)

| Dose of ABT-15251 (mg/kg per day)* | Survivors/total | Survivors % |
|---|---|---|
| 0 | 0/15 | 0 |
| 9·4 | 1/15 | 7 |
| 18·8 | 4/15 | 27 |
| 37·5 | 12/15 | 80 |
| 75·0 | 13/15 | 87 |

50% curative dose: 27·0 ± 5 mg/kg per day.
*Given in two divided doses, orally, on day infection was induced.

TABLE 5. SYNERGISM BETWEEN ABT-15251 AND SULPHAMETHOXYPYRIDAZINE IN AN ACUTE INFECTION IN MICE INDUCED WITH *Streptococcus pyogenes* (C203)

| Dose of Sulphamethoxypyridazine (mg/kg)* | | Dose of ABT-15251 (mg/kg)* | Survivors/total | Survivors (%) |
|---|---|---|---|---|
| 0 | | 0 | 0/20 | 0 |
| 0 | | 0·5 | 2/20 | 10 |
| 3·13 | | 0 | 0 20 | 0 |
| 6·25 | | 0 | 0 20 | 0 |
| 12·5 | | 0 | 0 20 | 0 |
| 25·0 | | 0 | 2,20 | 10 |
| 3·13 | | 0·5 | 9/20 | 45 |
| 6·25 | + | 0·5 | 12,20 | 60 |
| 12·5 | + | 0·5 | 15/20 | 75 |
| 25·0 | + | 0·5 | 17/20 | 85 |

*Single oral dose given at time infection was induced.

TABLE 6. SYNERGISM BETWEEN ABT-15251 AND SULPHAMETHOXYPYRIDAZINE IN A SUBACUTE INFECTION IN MICE INDUCED WITH *Streptococcus pyogenes* (C203)

| Dose of Sulphamethoxypyridazine (mg/kg per day) | | Dose of ABT-15125 (mg/kg per day) | Survivors/total | Survivors (%) |
|---|---|---|---|---|
| 0 | | 0 | 0/20 | 0 |
| 0 | | 0·25 | 0/20 | 0 |
| 3·13 | | 0 | 0/20 | 0 |
| 6·25 | | 0 | 0/20 | 0 |
| 12·5 | | 0 | 2/20 | 10 |
| 25·0 | | 0 | 6/20 | 30 |
| 3·13 | + | 0·25 | 8/20 | 40 |
| 6·25 | + | 0·25 | 10/20 | 50 |
| 12·5 | + | 0·25 | 13/20 | 65 |
| 25·0 | | 0·25 | 16/20 | 80 |

*Given orally once daily for two consecutive days.

TABLE 7. SYNERGISM BETWEEN ABT-15251 AND SULPHAMETHOXYPYRIDAZINE IN AN ACUTE INFECTION IN MICE INDUCED WITH *Streptococcus salivarius* (04150)

| Dose of Sulphamethoxypyridazine (mg/kg)* | | Dose of ABT-15251 (mg/kg)* | Survivors/total | Survivors (%) |
|---|---|---|---|---|
| 0 | | 0 | 0/20 | 0 |
| 0 | | 5 | 0/20 | 0 |
| 6·25 | | 0 | 0/20 | 0 |
| 12·5 | | 0 | 1/20 | 5 |
| 25·0 | | 0 | 2/20 | 10 |
| 6·25 | + | 5 | 12/20 | 60 |
| 12·5 | + | 5 | 13/20 | 65 |
| 25·0 | + | 5 | 18/20 | 90 |

*Single oral dose given at time infection was induced.

TABLE 8. SYNERGISM BETWEEN ABT-15251 AND SULPHAMETHOXYPYRIDAZINE IN AN ACUTE INFECTION IN MICE INDUCED WITH *Diplococcus pneumoniae* (SVI)

| Dose of Sulphamethoxypyridazine (mg/kg)* | | Dose of ABT-15251 (mg/kg)* | Survivors/total | Survivors (%) |
|---|---|---|---|---|
| 0 | | 0 | 1/20 | 5 |
| 0 | | 50 | 1/20 | 5 |
| 25 | | 0 | 0/20 | 0 |
| 50 | | 0 | 0/20 | 0 |
| 100 | | 0 | 5/20 | 25 |
| 25 | + | 50 | 15/20 | 75 |
| 50 | + | 50 | 17/20 | 85 |
| 100 | + | 50 | 19/20 | 95 |

*Single oral dose given at time infection was induced.

The synergistic combined action of ABT-15251 and sulphamethoxypyridazine is clearly evident from the results given in Tables 5, 6, 7 and 8. There are a number of instances where dosages of either compound alone provided no activity whatsoever, but in which these same dosages given together caused the survival of 50 per cent or more of the mice. It is also apparent that in all instances minimally active dose levels of ABT-15251 alone, when given with sulphamethoxypyridazine, caused a many-fold increase in sulphonamide activity.

## DISCUSSION

With regard to the pathogens tested herein, the high degree of selectivity of this triazine suggests that those organisms most susceptible, i.e. streptococci of groups A, C, E, F and G, may possess a vulnerable metabolic site that is distinctive and peculiar to these bacteria. Intraspecies differences, such as those exhibited by *Diplococcus pneumoniae*, are even more provocative, and merit further study.

Although it is not within the scope of this communication to discuss mechanisms of action, substantial evidence has been obtained that this and similar triazines do not act directly on known PABA–folic–folinic acid systems[10] in so far as a representative strain of *Streptococcus pyogenes* is concerned. These observations do not alone rule out the possibility that the triazine acts on the same metabolic sequence as sulphonamides. However, until some interrelationship is shown between triazines and sulphonamides with respect to sites of inhibition among these bacteria, there is no basis for explaining or predicting synergism.

In general, the susceptibility of an organism to either agent alone does not appear wholly to determine its response to combined treatment. For example, a strain of *Diplococcus pneumoniae* which was essentially insusceptible to the triazine both *in vitro* and *in vivo* was quite vulnerable to a synergistic combination of triazine and sulphonamide *in vivo*. As a rule, however, infections with bacteria which are highly senstive *in vitro* to the triazine alone will also respond synergistically *in vivo* to triazine-sulphonamide treatment. In this regard, the triazine-sulphonamide combination was synergistic *in vivo* against *Streptococcus pyogenes*, but such combined action was not observed *in vitro*. The use of a highly enriched culture medium may have served to suppress such effects.

The potential medical applications of a triazine-sulphonamide combination are quite evident from these experiments, particularly in streptococcal infections caused by Lancefield group A strains. The high degree of activity, effectiveness by oral administration, and specificity serve as desirable qualities. Additional studies related to chemotherapy are in progress and will be described when completed.

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