Brit. J. Pharmacol. (1951), **6**, 643.

A CINNOLINE COMPOUND ("528") FOR THE TREATMENT OF *TRYPANOSOMA CONGOLENSE* INFECTIONS

BY

E. M. LOURIE, J. S. MORLEY, J. C. E. SIMPSON, AND J. M. WALKER

*From the Department of Pharmacology, Oxford University, and the Department of Chemistry, Manchester University**

(Received June 8, 1951)

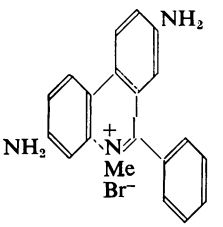
The most common pathogenic trypanosome of cattle throughout tropical Africa is *Trypanosoma congolense*. This species is relatively resistant to the arsenicals, diamidines and suramin, which are so useful against *T. gambiense*, the cause of the most common form of the disease in man. It was therefore not until compounds of the phenanthridinium group were introduced by Browning, Morgan, Robb, and Walls (1938) that the chemotherapy of *T. congolense* infections began to assume a favourable aspect. Of this chemical group "dimidium" bromide, 2 : 7-diamino-9-phenyl-10-methylphenanthridinium bromide (see formula I), first used in cattle by Carmichael and Bell (1944), has achieved considerable success in field practice; and another member of the group, 2 : 7-diamino-9-*p*-aminophenyl-10-methylphenanthridinium chloride, has recently been claimed by Brownlee, Goss, Goodwin, Woodbine, and Walls (1950) as an improvement on dimidium bromide in *T. congolense* infections in mice.

The first type of compound to be successful against *T. congolense* infections on a wide scale was therefore one in which the hetero-N is quaternized. This is interesting because no such type has found a place in the chemotherapy of human trypanosomiasis. It is a development that was foreshadowed many years earlier by the limited success of another group of quaternary ammonium compounds, viz., the styryl-quinolinium substances of Browning, Cohen, Ellingworth, and Gulbransen (1926). Quaternary nitrogen, this time at two points on the molecule, is again a feature of the most recent compound to be used successfully against *T. congolense* on a wide scale, namely "antrycide" (Curd and Davey, 1949, 1950), a salt of substituted quinaldinium-pyrimidinium residues connected by an imino linkage (see formula II).

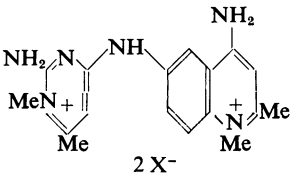
THEORETICAL AND EARLY EXPERIMENTAL BACKGROUND

A preliminary note on the initial work which eventually led to "528" has already appeared (Keneford *et al.*, 1948). The work began before antrycide was introduced, and

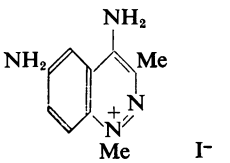
* This work was started in the Warrington Yorke Department of Chemotherapy at the Liverpool School of Tropical Medicine. Its later chemical development was facilitated by the creation of a Medical Research Council Group for Research in Chemotherapy in the Department of Chemistry at Manchester University.



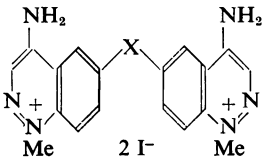
(I)



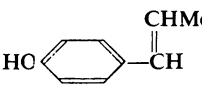
(II)



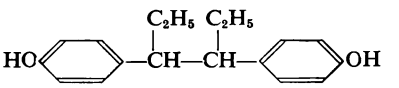
(III)



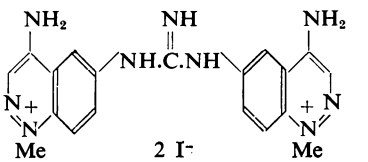
(IV)



(V)



(VI)



(VII)

two of the considerations by which it was influenced were (1) the probability that N-quaternization would favour activity against *T. congolense*, as testified by the success of the phenanthridinium compounds, and (2) the possibility that the phenanthridinium configuration might include redundant features, unnecessary for activity against *T. congolense*.

We therefore synthesized, and sought trypanocidal activity in, representatives of what might be regarded as a simplification of the phenanthridinium type, namely quaternary quinoline compounds, extending our studies at the same time to members of the closely related quaternary cinnolines and quinazolines. One obvious advantage of exploring these simplifications in preference to the original phenanthridinium type seemed to be that they might offer greater scope for introducing amino-groups into selected combinations of positions (amino-groups apparently being essential for trypanocidal activity in this class of compound) ; and we felt ourselves to be in a specially advantageous position for including cinnoline compounds in the investigation because of experience already gained with this group while examining its potentialities for the chemotherapy of malaria (Keneford and Simpson, 1947).

The chemical part of the work will be described elsewhere, together with notes on the degree of trypanocidal activity observed in individual compounds. Here it will suffice to give no more than an outline of the steps leading towards " 528," with a detailed consideration of the results obtained with that substance.

The first few pure preparations of the mono- and diamino-quaternary quinolines, cinnolines, and quinazolines examined showed little or no activity. An important clue was, however, afforded by finding that one of these compounds, though inactive in the pure state, was significantly active when tested as a crude preparation before being finally crystallized. This was 4 : 6-diamino-1 : 3-dimethylcinnolinium iodide (formula III). The impure (and trypanocidally active) preparation of this substance was a crude reduction product of the corresponding 6-nitro salt, an intermediate in one of the routes of synthesis towards the eventual 6-amino compound. Since a pure preparation of this nitro-analogue was also devoid of trypanocidal activity, the obvious interpretation was that the activity observed in the impure preparation must, in fact, have been a property of one of the impurities rather than of the actual compound towards which the synthesis was directed. It seemed that the hypothetical trypanocidal impurity might indeed be highly active, since the crude preparation of which it necessarily formed only a fraction was capable of clearing trypanosomes from the blood of infected mice not only temporarily but permanently. The strain of trypanosome used for this early work was Strain II of Browning and Calver (1943), which had been maintained in mice for many years in Liverpool, and which normally produced acute infections ending fatally about five to seven days after inoculation. For preliminary evaluation of trypanocidal activity the drugs were given in a single dose intraperitoneally, on a twofold dosage-scale, in aqueous solution in 0.5 ml. per 20 g. body weight ; and the very rough measures of activity used (admittedly without any pretensions to precision, though very useful for indicating a compound's potentialities) were the Maximum Tolerated Dose, the highest dose which failed to kill any of five mice, and the Minimum Curative Dose, the lowest dose which cured at least four of five mice. The very approximate therapeutic index (MTD/MCD) obtained from these values was about 2 for the most active of the impure preparations tested. Bearing in mind that the impurity formed only a part of the preparation tested, this figure suggested a high degree of activity in the hypothetical impurity, because the therapeutic indices obtained in the same way for presumably pure specimens of the two most active of the phenanthridinium compounds known at that time, dimidium bromide and " phenidium " chloride (Browning *et al.*, 1938), were only 15 and 3 respectively.

It seemed possible that the hypothetical impurity responsible for trypanocidal activity in the crude preparation of III might be a symmetrical binuclear compound of the type shown in formula IV, with CH_3 in positions 3 and 3′, formed as a side-reaction during conversion of the nitro- to the amino-quaternary salts. This seemed to be a possibility not only for chemical reasons, the likelihood being that the linkage *X* was the azo group -N : N-, but also because of the known composition of certain non-arsenical trypanocidal substances. Examples are trypan red, suramin, the aromatic diamidines, and bis-quinolyl compounds such as Surfen C (Iensch, 1937) and its analogues. Compounds conforming to formula IV would have molecular weights of the same general order as those of the substances named, and these weights would therefore satisfy the hypothetical requirement of falling within a narrow critical range which might be necessary for trypanocidal activity. Perhaps more significantly, compounds of formula IV would share also with the trypanocides named the general pattern of two identical, or nearly identical, substituted cyclic units with a simple connecting linkage. A further chemical analogy, not known at the time, became available with the announcement of antrycide (Curd and Davey, 1949) after our original working hypothesis had been published (Keneford *et al.*, 1948). Antrycide also, as shown in formula II, consists of two cyclic residues joined by a simple linkage, but in this case the cyclic components are not identically constituted as in the trypanocides just mentioned. The feature they do share with our envisaged type (formula IV) and not shown by the other trypanocides is bis-quaternization, to which attention has already been drawn in our opening paragraphs.

The variety of these analogies strengthened the case for examining a range of compounds of type IV with linkages of various other types as well as the azo, which chemical reasons suggested as the most likely one in the active impurity of the crude compound III. An obvious further extension of the investigation would be to examine a similar range of bis-quinolinium and related symmetrical heterocyclic compounds.

There are interesting analogies not only between the chemical structure of our envisaged type and that of known trypanocides, but also between the sequence of discovery leading to " 528 " and that which led to certain other therapeutic substances. Both in our own work and in an entirely different field, that of the search for synthetic sex hormones, the sequence was: (i) Exploration of simplifications of a relatively complex chemical configuration known to be associated with biological activity ; (ii) activity found to be present in a crude preparation of one of these simple derivatives but absent from pure specimens ; and (iii) identification of the active impurity as a member of a chemical type in which the cyclic feature of the pure substance is duplicated to give a substance of symmetrical structure.

In the work leading to " 528," the relatively complex configuration of stage (i) was the phenanthridine ring-system (formula I) ; and the mono- and bis-compounds of stages (ii) and (iii) were those of formulae III and IV (though we have not yet reached the stage of isolating and characterizing the actual impurity). In the work leading to synthetic sex hormones by Dodds *et al.* (1939) and Campbell *et al.* (1938, 1939), the complex structure of (i) was the naturally occurring *cyclo*pentenophenanthrene configuration ; and the mono- and bis-compounds of stages (ii) and (iii) were those shown in formulae V and VI.

The steps leading to " 528," an early outline of which was already given in our preliminary note (Keneford *et al.*, 1948), also found some analogy with those leading to antrycide, later described by Curd and Davey (1950). Here also trypanocidal activity was found in a crude but not in a pure precursor of the eventual trypanocidal compound. However, the precursor was not a mono-analogue of the eventual bis-substance, but a bis-compound already conforming to the antrycide structure, but with only one of the N-atoms quaternized. The active impurity was thought to be a substance similar to

the compound for which the synthesis was intended but with a nitrogen atom quaternized in both the ring systems, and this clue led directly to the antrycide molecule.

BIS-CINNOLINIUM AND BIS-QUINOLINIUM COMPOUNDS

The first bis-3-methylcinnolinium compounds of type IV to be synthesized and examined were those with guanidine, urea, and thiourea linkages. These proved to be quite inactive in maximum tolerated subcutaneous dosages of about 0.25, 4, and 8 mg./20 g., respectively. This was discouraging, but was obviously not enough to justify abandoning the symmetrical type of compound. One reason why our faith in compounds of that type was not seriously shaken was that we had not yet synthesized and examined the azo-substance, which we had originally suspected of being the active impurity in the crude preparation of III. Unexpected difficulties arose in synthesizing this substance, and, pending solution of these difficulties, attention was concentrated on a series of mono- and bis-compounds unmethylated in the 3- position. Here the surprising development arose among the mono-cinnolinium substances that, while pure specimens of 4 : 6-diaminocinnolinium iodide were quite inactive, unequivocally pure specimens of its 6-nitro precursor were curative at the maximum tolerated dose of about 0.75 mg./20 g. subcutaneously. This seemed to reopen the possibility of finding useful substances among those of the simpler mono-structure, and examination of these is proceeding, but our main energies continued to be directed towards the bis-substances.

The bis-compounds of type IV, unmethylated in position 3-, which then engaged our main attention might be described as N_6-linked bis-4:6-diaminocinnolinium substances and their corresponding bis-quinolinium analogues. The quinolinium salts synthesized and examined were iodides or chlorides with azo, urea, thiourea, and guanidine linkages. The maximum tolerated subcutaneous doses of the azo compound in mice was about 0.125 mg./20 g. body weight, and about half this was capable of temporarily clearing trypanosomes from the blood. The urea compound was tolerated in doses of about 1.0 mg./20 g., but it was only at this relatively high dosage-level that the blood was temporarily cleared in a few of the mice thus treated. The maximum tolerated dose of each of the other two quinolinium substances, like that of the azo compound, was about 0.125 mg., but they showed no trypanocidal activity at that dosage.

These few bis-quinolinium substances were therefore relatively toxic and ineffective as a group. The azo compound was more active than the other derivatives, and this vindicated in some measure our belief that the active impurity in the crude preparation which first directed our attention to these bis-substances might have been an azo-linked compound. However, the azo-bis-cinnolinium derivative in the present series, as in the 3-methyl series above, also proved unexpectedly difficult to synthesize, and chemotherapeutic observations on this substance must await a later communication. The urea compound was found to clear the blood temporarily but not permanently in maximum tolerated doses of about 1.0 mg./20 g. body weight; and the thiourea was inactive at maximum doses of about 0.5 mg./20 g. But with the guanidine compound the activity observed was of a sufficiently high order to demand precise definition. This is the substance which, for convenience, we designate as "528."

N^1 : N^3-BIS(4′-AMINOCINNOLYL-6′)-GUANIDINE DIMETHIODIDE (" 528 ")

The appropriate chemical designation of 528 appears at the head of this paragraph, and its formula is VII. It is a yellow crystalline substance, fairly soluble in water, giving solutions which are also yellow in colour.

In a series of experiments the toxic and therapeutic actions of 528 in mice were compared with those of antrycide methylsulphate. Each drug was given subcutaneously, in aqueous solution, in a single dose per mouse and in a volume of 0.4 ml. per 20 g. body weight. For the toxicity tests uninfected mice were used. For therapeutic tests the trypanosome strain used was the Busimbi *T. congolense* of Curd and Davey (1949, 1950), kindly sent to us by Dr. Davey. This produced acute fatal infections similar to those caused by the strain on which our earlier observations had been made. Mice were inoculated intraperitoneally so that 1 to 20 trypanosomes per microscope field could be seen in thin blood films two or three days later, at which stage they were treated.

TABLE

RESULTS OF TESTS WITH " 528 " AND ANTRYCIDE METHYLSULPHATE. TOXICITY IN UNINFECTED MICE AND THERAPEUTIC ACTIVITY IN MICE INFECTED WITH *Trypanosoma congolense*

Compound	Dose (mg./20 g.)	No. treated	Result	Log dose probit regression slope (*b*)	Lethal doses (mg./20 g.)		Curative doses (mg./20 g.)		Therapeutic indices (with 95% fiducial limits)	
					LD50	LD10	CD50	CD90	LD50/CD50	LD10/CD90
"528"			% killed	5.42	1.73	1.03			22.9 (20.5–25.5)	11.6 (9.6–13.9)
	2.20	40	67							
	1.69	48	52							
	1.30	43	23							
	1.00	23	9							
			% cured	18.02			0.076	0.089		
	0.123	21	100							
	0.095	22	95							
	0.073	21	43							
	0.062	26	4							
Antrycide			% killed	6.07	0.82	0.49			33.4 (30.0–37.3)	12.5 (10.1–15.6)
	1.10	45	78							
	0.84	83	55							
	0.75	60	37							
	0.65	52	29							
			% cured	6.40			0.024	0.039		
	0.042	20	100							
	0.032	20	75							
	0.031	36	72							
	0.025	22	41							
	0.019	22	36							
	0.016	36	11							

Toxicity was measured by percentage mortalities at different doses. The deaths occurred within about six hours of injection, usually within the first hour, both after antrycide and after 528. The two drugs were also alike in producing similar signs of toxic action before death, as briefly described elsewhere (Lourie and Walker, 1951). In the therapeutic tests, mice in which the peripheral blood remained free of trypanosomes for 30 days after treatment were regarded as cured.

The results of these tests for toxicity and therapeutic action are set out in the Table. The subcutaneous LD50 for antrycide was found to be considerably higher than those reported by Curd and Davey (1949, 1950) and by Goodwin and Walls (1950), our figure being 0.82 mg./20 g., while Curd and Davey quote 0.4 to 0.5 and Goodwin and Walls 0.46 mg./20 g. body weight, administered in volumes of unspecified size. It is notorious that small differences of technique may lead to wide differences in the results of tests for toxicity or for therapeutic action. We have examined one particular example of this in respect of antrycide methylsulphate, without being in a position to say whether it has contributed to the discrepancies between our own toxicity figures and those of the other workers. This is that the lethal effect of a subcutaneous dose of antrycide methylsulphate (and probably of any other drug which kills quickly) is largely dependent on the volume of the dose injected. All our own injections were given in a volume of 0.4 ml. per 20 g. body weight, and it seems specially advisable to quote the dose-volume (or drug concentration) whenever precise figures are given for the lethal action of any drug that kills rapidly. This point is elaborated in a separate communication (Lourie and Walker, 1951).

The Table shows that both the lethal and the curative doses of 528 are higher than those of antrycide, and in very roughly the same proportions for the curative as for the lethal doses. The effect is that the differences between the therapeutic indices of the two drugs are slight. When the indices compared are those given by LD50/CD50 they are 22.9 for 528 and 33.4 for antrycide, i.e., giving a ratio of 1.46 in favour of antrycide. Calculation of fiducial limits to these particular indices shows that this slight advantage in favour of antrycide is statistically significant.

The 50 per cent end-points are rightly much favoured for the statistical definition of biological properties, because their standard errors are less than those of any other end-points that might be chosen (Trevan, 1927). They suffer from the disadvantage, however, that for practical chemotherapy the doses which kill 50 per cent of subjects and which cure only 50 per cent are very far from the ideal. Doses required in practice are those which kill practically none and cure practically all; but the figures representing doses (such as the LD1 and the CD99) which approach those ideal requirements have such large standard errors that very little reliance can be placed on them. A compromise is needed between the accuracy coupled with therapeutic undesirability of the 50 per cent end-points, on the one hand, and the inaccuracy coupled with therapeutic virtues of the LD1 and CD99 on the other. This compromise may be struck by the LD10 and CD90, and the Table shows that in the therapeutic indices derived from those doses (i.e., the indices given by LD10/CD90) the slight superiority of antrycide over 528 is lost and the two compounds emerge as approximately equal in value, their indices now being 11.6 and 12.5. (It is the very steep slope of *b* for cures with 528 which redresses for that drug the less favourable relationship to antrycide which it assumed when the therapeutic indices were derived from 50 per cent end-points.)

The close agreement between the therapeutic indices for 528 and for antrycide methylsulphate in mice clearly justifies a trial of this new compound for *T. congolense* infections in cattle. As for its potentialities against other trypanosome species, we have made a few provisional tests with an old laboratory strain of *T. rhodesiense*. The tests have shown that mice infected with this strain may be cured by the same doses of 528 as are curative for *T. congolense* infections. In the present studies we have, however, concentrated on *T. congolense* rather than on *T. rhodesiense* because, for many reasons, field workers in Africa still regard the control of cattle trypanosomiasis as a more pressing and difficult problem than that of human trypanosomiasis.

SUMMARY

1. Trypanocidal activity was sought in representatives of what might be regarded as a simplification of the phenanthridinium type, namely, quaternary quinoline compounds, and in members of the closely related quaternary cinnolines and quinazolines.

2. The trypanocidal activity detected in crude preparations of one particular cinnolinium salt was lost when pure specimens of the same substance were examined. It was therefore inferred that the trypanocidal activity found in the crude preparations must have been a property of one of the impurities present in those preparations.

3. On both chemical and biological grounds it was thought likely that the active impurity might be a substance with two cinnolinium residues joined by a simple linkage. A series of such substances was therefore synthesized and examined, and some of them were in fact found to be trypanocidally active. The most active was the compound $N^1 : N^3$-bis(4′-aminocinnolyl-6′)-guanidine dimethiodide which, for convenience, we designate as " 528."

4. When tested against *T. congolense* infections in mice, the therapeutic index, LD10/CD90, of 528 was found not to differ significantly from that of antrycide methylsulphate.

We have greatly benefited by consultation with Dr. D. J. Finney, Lecturer in the Design and Analysis of Scientific Experiment in the University of Oxford, and we are indebted to him also for calculating the statistical values shown on the Table.

REFERENCES

Browning, C. H., and Calver, K. M. (1943). *J. Path. Bact.*, **55**, 393.
Browning, C. H., Cohen, J. B., Ellingworth, S., and Gulbransen, R. (1926). *J. Path. Bact.*, **29**, 317.
Browning, C. H., Morgan, G. T., Robb, J. V. M., and Walls, L. P. (1938). *J. Path. Bact.*, **46**, 203.
Brownlee, G., Goss, M. D., Goodwin, L. G., Woodbine, M., and Walls, L. P. (1950). *Brit. J Pharmacol.*, **5**, 261.
Campbell, N. R., Dodds, E. C., and Lawson, W. (1938). *Nature, Lond.*, **142**, 1121.
Campbell, N. R., Dodds, E. C., and Lawson, W. (1939). *Proc. Roy. Soc.*, B, **128**, 253.
Carmichael, J., and Bell, F. R. (1944). *Vet. Record*, **56**, 495.
Curd, F. H. S., and Davey, D. G. (1949). *Nature, Lond.*, **163**, 89.
Curd, F. H. S., and Davey, D. G. (1950). *Brit. J. Pharmacol.*, **5**, 25.
Dodds, E. C., Golberg, L., Lawson, W., and Robinson, R. (1939). *Proc. Roy. Soc.*, B, **127**, 140.
Goodwin, L. G., and Walls L. P. (1950). *Trans. Roy. Soc. Trop. Med. Hyg.*, **44**, 233.
Iensch, H. (1937). *Ang. Chem.*, **50**, 891.
Keneford, J. R., Lourie, E. M., Morley, J. S., Simpson, J. C. E., Williamson, J., and Wright, P. H. (1948). *Nature, Lond.*, **161**, 603, 758.
Keneford, J. R., and Simpson, J. C. E. (1947). *J. chem. Soc.*, 917.
Lourie, E. M., and Walker, J. M. (1951). *Brit. J. Pharmacol.*, **6**, 630.
Trevan, J. W. (1927). *Proc. Roy. Soc.*, B, **101**, 483.