STREPTOMYCIN: CORRELATION BETWEEN *IN VIVO* AND *IN VITRO* ACTIVITY OF TEN REPRESENTATIVE DERIVATIVES

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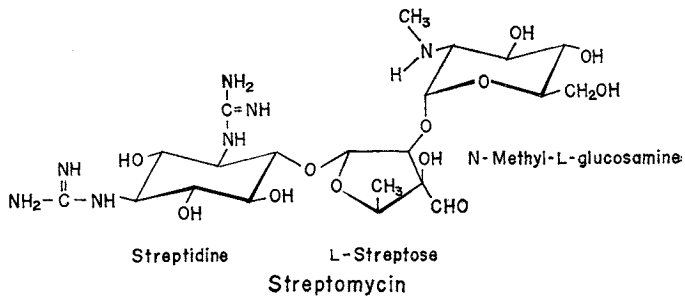
The affinities of ten streptomycin (SM) derivatives were compared to that of tritium labelled dihydro-streptomycin (T-DHS) towards "run off" 70 S ribosomal particles from a sensitive *E. coli*. The *in vitro* affinities were correlated with the *in vivo* activities (killing activities). High affinity and activity could only be demonstrated in derivatives with an intact streptidine (SD) moiety. Furthermore an interaction between the streptose branch and the amino group was necessary to obtain high affinity and activity. The highest *in vivo* activity was found when the amino group was N-mono-methyl substituted. Our results indicate that SM binds to two sites at the 70 S particles, one having the character of a cation-exchanger (RNA), the other being presumably a metal ion (Mg^{2+}). The metal ion probably forms a complex with the glucosamine and the streptose branch moieties.

Since the recognition of SM's chronic toxicity and the ability of sensitive bacteria to develop resistance a great number of derivatives has been prepared to overcome these drawbacks. The antimicrobial activities of the derivatives ranges from 0 % to 100 % as compared to SM.

SM contains, as shown in Fig. 1, a number of functional groups available for chemical modification. We decided to investigate the possible correlation between the *in vivo* activities of a series of SM derivatives and their *in vitro* activities—the target for the *in vitro* activity of SM is the ribosomal protein synthesizing system. It is well-documented that SM interferes with this system and in the last decade more than twenty papers have described different facets of this *in vitro* interaction. Only a few of these papers will be mentioned.

In a cell-free system containing poly-U as the messenger, SM causes miscoding. At higher concentrations of SM, poly-peptide synthesis is inhibited[1]. However, it is doubtful whether this miscoding and inhibition are directly related to the bactericidal

Fig. 1.



effect of SM.

In a recent study the protein composition of ribosomes from a sensitive and a resistant strain of *Escherichia coli* has been shown to be significant different. 30 S Subunits from the sensitive strain contain the genotype protein P 10 which is responsible for the binding of SM to the subunit. However, the P 10 protein itself does not bind SM[2].

According to CHANG and FLAKS[3], the function of P 10 may be that of ordering the proper configuration of other proteins and/or r-RNA responsible for the binding of the antibiotic to the ribosomes, thus disturbing protein synthesis.

When SM is added to a growing culture of a sensitive *E. coli* strain, an accumulation of 70 S particles is observed, apparently at the expense of polysomes[4].

In the preliminary study presented here, we measured the binding affinity of SM and SM-derivatives to "run off" 70 S particles and used this binding affinity as a measure of *in vitro* activity. The killing effect was estimated by tne conventional serial dilution method.

Experimental Procedures and Results

The minimal inhibitory concentration values (MIC) of the SM-derivatives were estimated by the conventional serial dilution method in Mycin assay broth (Difco) at pH 8 towards the sensitive *E. coli* strain Novo 01 (see Table 1).

The affinities of the derivatives towards 70 S particles were measured in the following manner: T-DHS (0.25 μg) was added to increasing amounts of unlabelled DHS, SM and derivatives dissolved in a constant volume (35 μl) of Tris buffer (0.01 M) containing magnesium acetate (0.014 M) and potassium chloride (0.06 M) at a pH of 7.8 and a temperature of 37°C. To this mixture was added a suspension of 70 S particles in the Tris buffer (25 μl). The resulting mixtures were incubated at 37°C for 30 minutes. Longer incubation times did not change the binding of the antibiotics to the 70 S particles.

The ribosome-bound T-DHS was isolated by Millipore filtration (0.45 μ). The filters were washed with Tris bufier (3× 1 ml) and the antibiotics were liberated from the ribosomes by treatment with 0.01 N HCl. The amount of bound T-DHS was counted in a liquid scintillation counter.

With a 15 percent binding of 0.25 μg T-DHS, a typical isotope dilution curve was obtained when increasing amounts of unlabelled DHS were added as shown in Fig. 2 A.

Derivatives of the streptose moiety (Fig. 2 B):

In good agreement with the literature[5],

Table 1. Minimal inhibitory concentrations (MIC) of SM-derivatives towards a sensitive *E. coli*, strain Novo 01, estimated by the conventional serial dilution method.

Dirivative	MIC (μg base/ml)
SM	0.4
DHS	0.4
Dihydrodesoxy-SM	0.4
Methyl-SM[6]	6.3
SM-acid[7]	100
N-Demethyl-SM (NDMS)[8]	3.2
Dihydro-NDMS (DH-NDMS)[8]	3.2
Mannosido-SM	3.2
N-Dimethyl-DHS ($N(CH_3)_2$DHS)*	25
SD[9]	100
Didiamidino-DHS[10]	100

* E. ANDRUP: unpublished, 1959

Fig. 2. Isotope dilution curves.
T-DHS was incubated with increasing amounts of SM-derivatives in the presence of 70 S particles. The amount of bound T-DHS was measured.

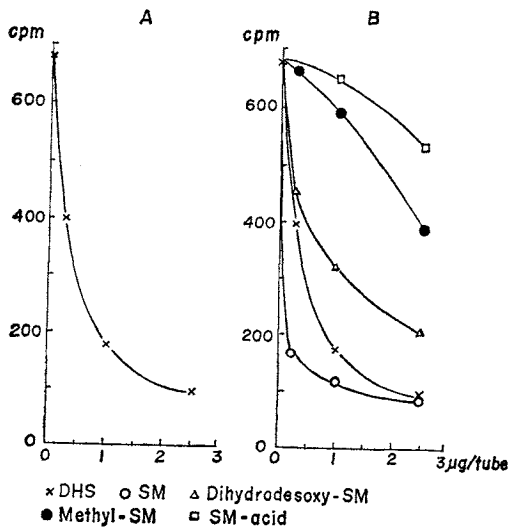
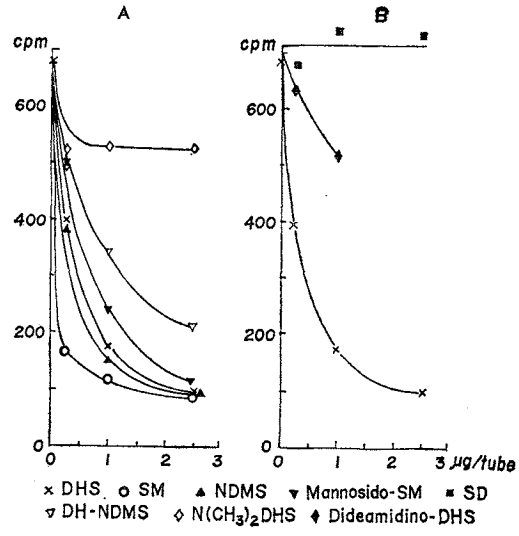


Fig. 3. Isotope dilution curves.
T-DHS was incubated with increasing amounts of SM-derivatives in the presence of 70 S particles. The amount of bound T-DHS was measured.



SM showed a higher affinity towards the 70 S particles than DHS. The difference in affinity towards the 70 S particles is not reflected in the antibacterial activities of the two antibiotics. Dihydrodesoxy-SM, having approximately the same antibacterial activity as DHS, showed in the isotope dilution assay a slightly lower affinity to the 70 S particles than DHS. Methyl-SM which is sixteen times less active than DHS (Table 1) had a very low affinity to the ribosomes. In a concentration ten times higher than that of T-DHS, it effected only a 25 % decrease in the binding of T-DHS. A similar addition of DHS decreased the binding by 80 %. SM-acid, which is devoid of antimicrobial activity, decreased under the same experimental conditions the binding of the T-DHS only by 15 %.

Derivatives of the L-glucosamine moiety (Fig. 3 A):

NDMS is eight-times less active than DHS towards the test organism (Table 1), but at low concentrations, it has an affinity to the ribosomes higher than that of DHS. At highest concentration, the affinities were practically the same. However, compared to SM, the affinity of NDMS to the ribosomes is lower.

DH-NDMS, having an antibiotical activity equal to NDMS, binds less firmly to the ribosomes than NDMS, thereby following the pattern seen in the comparison of DHS and SM.

$N(CH_3)_2$-DHS has less than 2 % antibacterial activity as compared to DHS and it only competed weakly with the T-DHS for the binding sites on the ribosomes.

Mannosido-SM has eight-times lower activity than DHS in the *in vivo* system used, and the affinitity to the ribosomes was significantly lower than that of DHS. Experessed on a molar basis, however, mannosido-SM has almost the same affinity as DHS.

Derivatives of the streptidine moiety (Fig. 3 B):

Only two derivatives were prepared and tested, dideamidino-DHS and SD. The antimicrobial activity of both is negligible. In the highest concentration tested in the isotope dilution assay the dideamidino-DHS lowered the binding of T-DHS to the ribosomes by about 35 %. SD did not interfere with the binding of T-DHS at all.

Discussion

Fig. 2 A shows a typical isotope dilution curve. The shape of the curve indicates a lack of simple stochiometry in the reaction of DHS with the 70 S particles.

The results presented in Fig. 2 B clearly demonstrate that the nature of the L-streptose branch is very important for the binding of the antibiotics to the ribosomes. The order of the compounds with respect to binding affinity (*in vitro* system) is:

SM>DHS>dihydrodesoxy-SM>methyl-SM>SM--acid

In vivo the order is:

SM=DHS=dihydrodesoxy-SM>methyl-SM>SM-acid

It appears that there is a close correlation between binding affinity and antimicrobial activity.

Changes of the amino group in the glucosamine moiety do not lead to such a simple correlation as shown in Fig. 3 A. The order of the compounds with respect to binding is:

SM($-NHCH_3$)>NDMS($-NH_2$)>DHS($-NHCH_3$)>DH-NDMS($-NH_2$)>N$(CH_3)_2$-DHS

The order of effectiveness as antimicrobial agents is:

SM($-NHCH_3$)=DHS($-NHCH_3$)>NDMS($-NH_2$)=DH-NDMS($-NH_2$)>N$(CH_3)_2$-DHS

From these data, it might be concluded that an interaction between the streptose branch and the amino group in the glucosamine moiety is of fundamental importance for the binding affinity and the activity. The two groups may interact directly with each other or with a third group, being part of the ribosome. In SM and NDMS the branch is a potential carbonyl group. NMR data indicates that it may be masked as a *gem*-diol[11]. An interaction between this group and the secondary amino group in SM and the primary amino group in NDMS is likely to take place[12]. Another possibility is, however, that the streptose branch and the amino groups may serve as ligands in a metal complex, the metal ion probably being magnesium. The slightly higher binding affinity of SM as compared to NDMS may be the result of the inductive effect of the methyl group which may increase the alkalinity of the amino group. NDMS binds more firmly to the 70 S particles than DHS, but the antimicrobial activity is almost ten-times lower. The N$(CH_3)_2$-DHS analogue has, if any, a very low affinity to the particles, and only residual antibacterial activity. The tertiary amino group will neither have a high affinity to the streptose branch nor will it be a likely ligand in a Mg^{2+}-complex. Consequently this compound has no affinity to the 70S particles. Parallel conclusions hold for methyl-SM.

High binding affinity is thus not sufficient to ensure a high antimicrobial activity. This is obtained only when the affinity is high and the amino group is mono-methyl substituted, *i.e.* SM and DHS. The relative low antimicrobial activity of mannosido-SM, which as mentioned has a high affinity, may be attributed to a decreased ability to penetrate the cell wall of the test organism. SM-acid has a very low affinity to the ribosomes and has no antibacterial activity.

The derivatives discussed above all have an intact SD-group in common. Only two derivatives of this moiety have been tested and compared to DHS, dideamidino-DHS and SD. Fig. 3 B shows that dideamidino-DHS does bind to the ribosomes, but the affinity is low. The antibacterial activity of this derivative is also very low. SD in all concentrations tested does not interfere with the binding of T-DHS and the compound has no antibacterial activity.

The conclusion of our study is that SM and antimicrobially active SM-derivatives

bind to "run off" 70 S ribosomal particles at two sites. The one site has the character of a cation-exchanger which at physiological pH values is negatively charged and in the buffer system used probably saturated with Mg^{2+}. The streptidine moiety competes successfully with this ion and a labile attachment of the antibiotic to the ribosome is obtained. Once attached the streptose branch glucosamie system interacts with another site of the ribosome. Taking the nature of the branch and the amino group into account for those derivatives that bind and have bactericidal activity, it is more likely to assume that this second site is a metal ion, probably Mg^{2+}, than one of the ribosomal proteins. The function of this magnesium ion may well be that of ordering these proteins to secure transcription of the genetic code. Disturbance of a protein-magnesium complex with an agent as streptomycin may lead to incorrect transcription (miscoding) or inhibition of peptide synthesis.

Acknowledgements

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