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European Journal of Pharmacology 483 (2004) 127–132
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Antitumor antibiotic streptonigrin and its derivatives as inhibitors of nitric oxide-dependent activation of soluble guanylyl cyclase

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Received 6 March 2003; received in revised form 9 October 2003; accepted 14 October 2003

Abstract

The influence of streptonigrin on the activity of human platelet guanylyl cyclase was investigated. Streptonigrin (0.1–5 μM) had no effect on the basal activity of the enzyme, but inhibited in a concentration-dependent manner the sodium nitroprusside-induced activation of human platelet soluble guanylyl cyclase with an IC_{50} value of 4.16 μM. Streptonigrin (10 μM) also inhibited (by 28%) the activation of the enzyme by the direct nitric oxide (NO) donor–spermine–NONO (100 μM), but had no influence on the stimulation of soluble guanylyl cyclase by protoporphyrin IX. The absence of a correlation between the inhibition of NO-stimulated guanylyl cyclase activity by streptonigrin (**I**) and its derivatives (streptonigrone (**IV**), streptonigrone-2′-imine (**V**), amide of 1 and 2′-deoxy-2′-amino-D-glucose (**VI**), amide of 1 and 2′-deoxy-2′-amino-2′-D-galactose (**VII**), amide of 1 and 1-*O*-methyl-6-deoxy-6-amino-D-glucose (**VIII**), diphenylmethyl ester of **I** (**IX**), conjugate of **I** and daunorubicin (**X**)), and the level of cytotoxic effects of these compounds excludes the involvement of guanylyl cyclase in the mechanism of antitumor action of streptonigrin. Inhibition of guanylyl cyclase activation by NO donors but not by protoporphyrin IX represents a new biochemical effect of streptonigrin, which should be taken into account in addition to its antitumor action.

Keywords: Guanylyl cyclase; Nitric oxide (NO); Streptonigrin

1. Introduction

Intracellular signaling pathways, such as the nitric oxide (NO)-soluble guanylyl cyclase-cyclic-3′,5′-guanosine monophosphate (cGMP) pathway, play an important role in the regulation of various physiological processes (Hobbs, 1997). The NO-cGMP system is involved in vasorelaxation, platelet aggregation, central nervous system function and septic shock (Ignarro, 1999; Adrie, 1996; Paakkari and Lindsberg, 1995; Vasilev et al., 1996). NO may have opposite effects on apoptosis in different cell lines, and may induce apoptosis through either cGMP-dependent or cGMP-independent mechanisms (Shimojo et al., 1999; Ward et al., 2000). However, cGMP may protect against cell death provoked by NO production (Thippeswamy and Morris, 1997). The effect of NO on tumor growth is also contradictory and is determined by a number of factors: tumor type, development stage, NO level and use of antitumor therapy (Wink et al., 1998). It has been shown that in vitro NO production increases the effectiveness of some chemotherapeutic drugs (Cook et al., 1997; Wink et al., 1997), whereas inhibition of NO biosynthesis by some medical drugs (for example, 5-fluorouracyl) decreases the proliferation of some tumor cell types (Jin et al., 1996).

Recently, it was shown that the intracellular signaling pathway NO-soluble guanylyl cyclase-cGMP is involved in the survival of proliferating L1210 leukemia cells. 1*H*-[1,2,4]oxadiazole[4,3-α]quinoxalin-one (ODQ), a soluble guanylyl cyclase inhibitor, was found to induce a marked increase in caspase activity, which was associated with a loss of cell viability and a reduction in cGMP content (Flamigni et al., 2001). Furthermore, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl-indazole (YC-1, an allosteric activator of soluble guanylyl cyclase) and 8-Br-cGMP (a cell-permeable analogue of cGMP) exerted some protection against various

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doi:10.1016/j.ejphar.2003.10.018

apoptotic stimuli (Flamigni et al., 2001). Methylene blue (a well-known inhibitor of nitric oxide-dependent activation of soluble guanylyl cyclase) also can inhibit the growth of L1210, P388 leukemia cells and Ehrlich ascitic tumor in mice. The average life span of the treated animals was clearly longer than that of the control animals (Lai et al., 1989). Moreover, the plasma cGMP level of untreated patients with cancer is noticeably high and normalizes during remission (Chawla et al., 1980 ; Peracchi et al., 1983, 1987). Thus, inhibitors of NO-dependent guanylyl cyclase activation (ODQ, methylene blue) may inhibit the growth of some tumor cells.

Earlier, it was demonstrated that the antitumor antibiotic streptonigrin (bruneomycin) inhibits L1210 leukemia cell growth (Shorin and Bazhanov, 1974; Boger et al., 1987). The biochemical mechanism of its pharmacological action was studied in detail only with regard to its effect on nucleic acids. Until now, it was considered that streptonigrin inhibits topoisomerase II activity, provokes the destruction of DNA and RNA and blocks their biosynthesis (Bolzan and

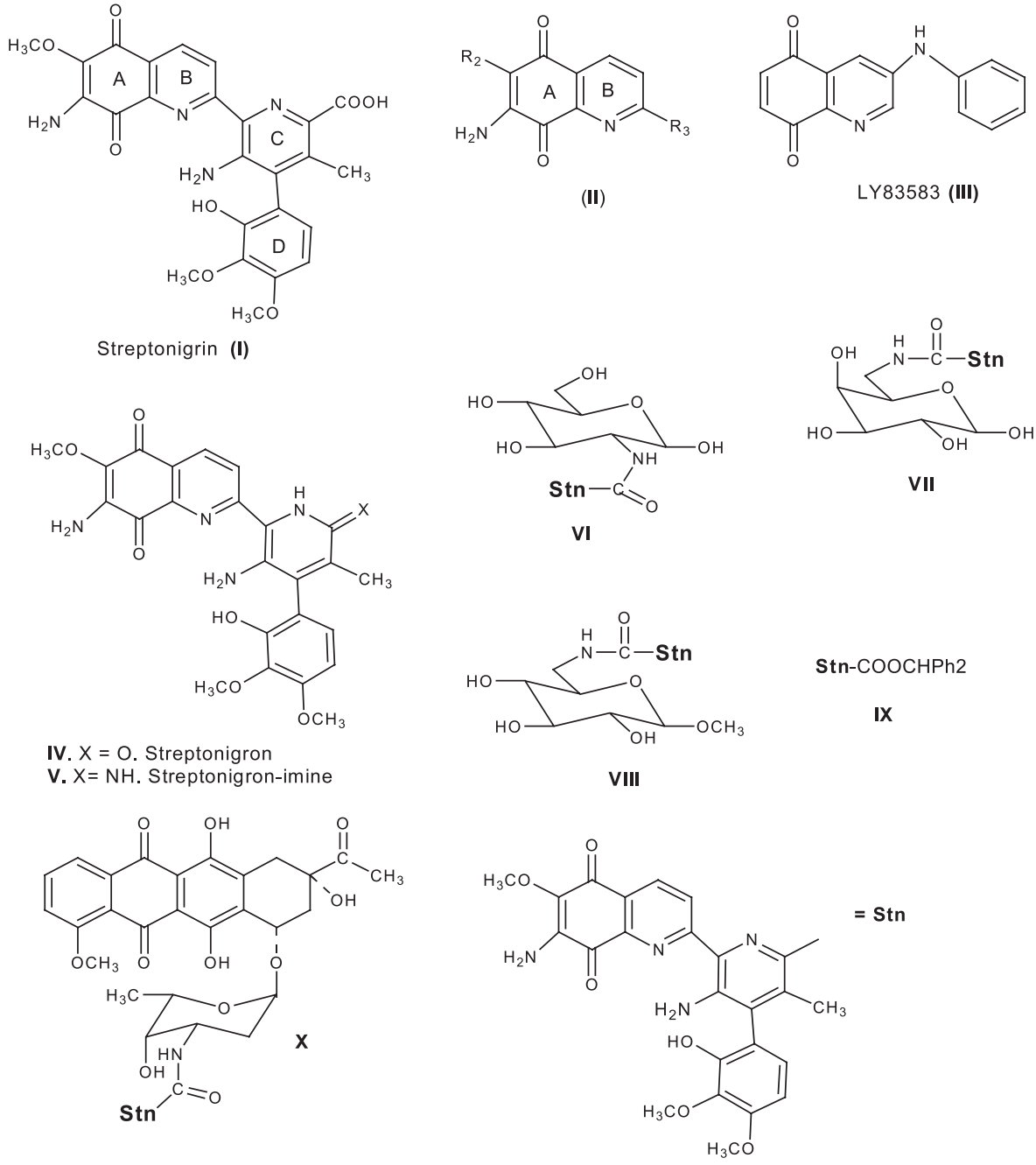

Fig. 1. Structures of streptonigrin (**I**), its pharmacophoric quinoline-5,8-quinoline fragment (**II**), LY 83583 (**III**), streptonigrone (**IV**), streptonigrone-2′-imine (**V**). Amide of **I** and 2′-deoxy-2′-amino-D-glucose (**VI**), amide of **I** and 2′-deoxy-2′-amino-2′-D-galactose (**VII**), amide of **I** and 1-*O*-methyl-6-deoxy-6-amino-D-glucose (**VIII**), diphenylmethyl ester of **I** (**IX**), conjugate of **I** and daunorubicin (**X**).

Bianchi, 2001). The main pharmacophoric fragment of the streptonigrin molecule is the substituted quinolinequinoic fragment (II, Fig. 1) (rings A and B, Fig. 1), and the presence of rings C and D in the streptonigrin molecule (Fig. 1) is less important for the cytotoxic activity of this drug (Boger et al., 1987). The same quinolinequinone fragment (II, Fig. 1) is also present in the structure of LY83583 (III, Fig. 1), an inhibitor of NO-dependent activation of soluble guanylyl cyclase (Mulsch et al., 1988).

This paper, therefore, investigates the influence of streptonigrin and some of its derivatives (Fig. 1) on human platelet soluble guanylyl cyclase and its effect on the activation of the enzyme by NO donors (sodium nitroprusside and spermine–NONO).

2. Materials and methods

In this study, human platelets were used as a source of soluble guanylyl cyclase. Platelets were isolated from the blood of healthy donors as described earlier (Chirkov et al., 1987). A suspension of washed platelets in 50 mM Tris–HCl buffer (pH 7.6) containing 0.2 mM dithiothreitol was sonicated in an MSE 5–78 ultrasonic sonicator (UK) for 20 s at 2 °C and centrifuged at 105 000 × *g*. The supernatant obtained from 40 ml of blood from one donor was used as human platelet soluble guanylyl cyclase in one experiment.

Guanylyl cyclase activity was measured as described by Garbers and Murad (1979). Briefly, the samples (final volume 150 μl) contained 50 mM Tris–HCl buffer (pH 7.6), 1 mM guanosine-5′ -triphosphate, 4 mM $MgCl_2$, 4 mM creatine phosphate, 20 μg (120–160 units) creatine phosphokinase, 10 mM theophylline, 20 μg of human platelet supernatant and other additives including streptonigrin and its derivatives if necessary. The effect of streptonigrin was studied in the concentration range 0.1–10 μM. To compare the effect of streptonigrin with that of its derivatives, 10 μM concentrations of the compounds were used. Compounds were first preincubated (10 min at 2 °C) with guanylyl cyclase before the addition of NO donors. Because of the poor solubility of streptonigrin and its derivatives in the buffer solution, they were initially dissolved in dimethyl sulfoxide (DMSO) with subsequent dilution in 50 mM Tris–HCl buffer (pH 7.6) to the required concentration. Control samples contained the same amount of DMSO.

The amount of cGMP formed (15 min, 37 °C) was estimated by an enzyme-linked immuno-sorbent assay (ELISA) method using Bioimmunogen kits (Russia). The influence of streptonigrin on NO release from sodium nitroprusside was measured as NO_2 formation by the Griess method (Schmidt and Kelm, 1996) with minor modifications. Protein was determined by the method of Bradford (1976). The following reagents were used: guanosine triphosphate sodium salt (Fluka, Switzerland), superoxide dismutase and other reagents were from Sigma. Derivatives of streptonigrin were synthesized according to Tolstikov et al. (1992a,b), Preobrazhenskaya et al. (1992) and Tolstikov et al. (1989).

Statistical differences were evaluated using Student's *t*-test.

3. Results

Streptonigrin in the concentration range 0.1–5 μM had no influence on the basal activity of human platelet soluble guanylyl cyclase. In these experiments, the basal enzyme activity was 58 ± 7 pmol cGMP mg^{-1} min^{-1} without streptonigrin and 62 ± 6, 64 ± 8 and 66 ± 7 pmol cGMP mg^{-1} min^{-1} in the presence of 0.1, 1 and 5 μM streptonigrin, respectively. At 10 μM, streptonigrin slightly enhanced the basal enzyme activity up to 99 ± 8 pmol cGMP mg^{-1} min^{-1} (about 1.7-fold). Fig. 2 shows that streptonigrin in a concentration-dependent manner inhibited sodium nitroprusside-stimulated guanylyl cyclase activity with an IC_{50} value of 4.16 μM. NO release from sodium nitroprusside (100 μM) in the presence of 100 μM dithiothreitol without and with streptonigrin (10 μM) was 2.61 ± 0.09 and 2.83 ± 0.11 μM NO_2^- ($P > 0.05$), respectively. Thus, streptonigrin did not alter the production of NO. Streptonigrin (10 μM) also inhibited the activation of the enzyme by the direct NO donor-spermine NONO. Guanylyl cyclase activity in the presence of 100 μM spermine NONO without and with streptonigrin (10 μM) was 1096 ± 109 and 790 ± 71 pmol cGMP mg^{-1} min^{-1}, respectively ($P < 0.05$).

The molecular mechanism of DNA degradation by streptonigrin involves the generation of superoxide-anion

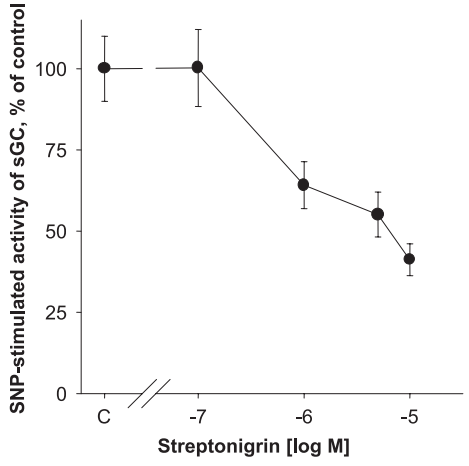

Fig. 2. Effect of streptonigrin on sodium nitroprusside-stimulated soluble guanylyl cyclase activity in human platelets. Soluble guanylyl cyclase (sGC) was determined in the presence of 100 μM sodium nitroprusside (SNP) and in the absence (con) of streptonigrin (0.1–10 μM). Abscissa: streptonigrin concentration in the sample (log M). Ordinate: sodium nitroprusside-stimulated activity in the absence of streptonigrin (con) was taken as 100%. Basal guanylyl cyclase activity was 58 ± pmol cGMP mg^{-1} min^{-1}. Guanylyl cyclase activity in the presence of 100 μM sodium nitroprusside was 840 ± 84 pmol cGMP mg^{-1} min^{-1}. Data represent means ± S.D. of four independent experiments with <10% error.

radicals, and this process was completely blocked by superoxide dismutase (Cone et al., 1976). Superoxide-anion radicals may interact with NO to form peroxinitrite and/or may directly oxidize Fe^{2+} heme to Fe^{3+} heme, leading to a decrease or prevention of heme-dependent activation of guanylyl cyclase by NO donors. To elucidate whether oxygen-derived radicals are involved in the inhibitory effect of streptonigrin, we studied the influence of superoxide dismutase on the inhibition by streptonigrin of sodium nitroprusside-induced guanylyl cyclase activation. Sodium nitroprusside (100 μM)-stimulated guanylyl cyclase activity without streptonigrin and superoxide dismutase was 1998 ± 161 pmol cGMP mg^{-1} min^{-1}. In the presence of streptonigrin (10 μM) and without and with superoxide dismutase (167 units ml^{-1}), guanylyl cyclase activity was 795 ± 58 and 854 ± 41 pmol cGMP mg^{-1} min^{-1}, respectively. Thus, the presence of superoxide dismutase in the sample did not influence the inhibition of guanylyl cyclase activation by streptonigrin.

Inhibition of NO-dependent guanylyl cyclase activation by streptonigrin was also confirmed in a series of independent experiments with protoporphyrin IX-induced soluble guanylyl cyclase activation. Protoporphyrin IX, an immediate heme precursor, is an endogenous stimulator of guanylyl cyclase activity (Ignarro et al., 1982). However, in contrast to NO, which requires the guanylyl cyclase heme for activation, the latter is not involved in the protoporphyrin IX-induced stimulation of the enzyme. The addition of 10 μM streptonigrin (final concentration) did not influence human platelet guanylyl cyclase activation by protoporphyrin IX (5 μM) (guanylyl cyclase activity with and without streptonigrin was 388 ± 23 and 400 ± 20 pmol cGMP mg^{-1} min^{-1}, respectively; the basal guanylyl cyclase activity was 58 ± 7 pmol cGMP mg^{-1} min^{-1}).

Table 1
Effect of streptonigrin (**I**) (10 μM) and its derivatives (**IV**–**X**) (10 μM) on the sodium nitroprusside (SNP, 100 μM)-stimulated activity of human platelet guanylyl cyclase (sGC)

Compounds and additives	SNP-stimulated activity of sGC (pmol cGMP mg^{-1} min^{-1})	Inhibition of sGC activation (%)
SNP	2484 ± 189	0
SNP + **I**	1094 ± 81	56
SNP + streptonigrone (**IV**)	1173 ± 90	53
SNP + streptonigrone-imine (**V**)	885 ± 65	64
SNP + amide (**VI**)	806 ± 69	68
SNP + amide (**VII**)	1094 ± 78	56
SNP ± amide (**VIII**)	701 ± 49	72
SNP + ester (**IX**)	1829 ± 150	26
SNP + conjugate of **I** and daunorubicin (**X**)	360 ± 22	85

Data represent means ± S.D. of four independent experiments with < 10% error.

Table 2
Effect of streptonigrin (**I**), daunorubicin (DNR) and streptonigrin–daunorubicin–conjugate (**X**) on sodium nitroprusside (SNP, 100 μM)-stimulated activity of human platelet soluble guanylyl cyclase (sGC)

Compounds and additives	SNP-stimulated activity of sGC (pmol cGMP mg^{-1} min^{-1})	Inhibition of sGC activations (%)
SNP	1771 ± 131	
SNP + **I** (10 μM)	950 ± 72	53
SNP + DNR (10 μM)	1251 ± 81	36
SNP + **I** (10 μM) + DNR (10 μM)	632 ± 49	71
SNP + conjugate **X** (10 μM)	230 ± 17	87

Data represent means ± S.D. of three independent experiments with < 10% error.

Investigation of the influence of several streptonigrin derivatives containing different substituents in the 2′ position of the central pyridine fragment (Fig. 1, Table 1) on sodium nitroprusside-stimulated guanylyl cyclase activity demonstrated that the inhibitory effects varied from 26% to 85% depending on the structure of the compounds used (Table 1). Taking into consideration the sharply increased inhibitory effect of the streptonigrin derivative containing in its molecule the residue of an antitumor antracycline antibiotic-daunorubomicin (conjugate **X**, 85%, Table 1), we studied the influence of daunorubicin alone and in the presence of streptonigrin (**I**) on the sodium nitroprusside-induced activation of guanylyl cyclase in a series of independent experiments. Our earlier unpublished data demonstrated that sodium nitroprusside (100 μM)-stimulated human platelet guanylyl cyclase activity without and with 1 and 10 μM daunorubicin was 547 ± 38, 350 ± 25 and 351 ± 22 pmol cGMP mg^{-1} min^{-1}, respectively; basal guanylyl cyclase activity was 48 ± 6 pmol cGMP mg^{-1} min^{-1}. Thus, 10 μM daunorubicin caused maximal inhibition of NO-stimulated guanylyl cyclase activity. Table 2 shows that the inhibitory effect of daunorubicin (10 μM) and streptonigrin (10 μM) incubated together (71%) was very close to the arithmetic sum (89%) of the inhibitory effects of 10 μM streptonigrin (56%) and 10 μM daunorubicin (36%) alone and also to the inhibition produced by 10 μM conjugate (**X**) (87%) (Table 2).

4. Discussion

The antitumor antibiotic streptonigrin inhibits the growth of L1210 leukemia cells (Shorin and Bazhanov, 1974; Boger et al., 1987). According to current concepts, the molecular mechanism of the antitumor effect of streptonigrin involves the inhibition of topoisomerase **II** activity. The latter results in the destruction of DNA and RNA and blockade of their biosynthesis (Bolzan and Bianchi, 2001). The data presented here demonstrate for the first time that

this antibiotic is a rather potent inhibitor of the NO-dependent activation of soluble guanylyl cyclase by sodium nitroprusside, but has no influence on protoporphyrin IX-induced enzyme activation. The ability of streptonigrin to inhibit the activation of guanylyl cyclase by NO donors but not by protoporphyrin IX suggests the involvement of the guanylyl cyclase heme in this process. Unfortunately, the presence in the 105 000 g supernatant used as the source of guanylyl cyclase of other heme-containing proteins excluded the possibility of spectrophotometric determination of the streptonigrin-guanylyl cyclase heme interaction. Therefore, we have studied the interaction of streptonigrin with the heme group of oxyhemoglobin. There were no changes in oxyhemoglobin UV/visible light absorption spectra in the presence of streptonigrin. The maximal changes after a 30-min incubation of 10 μM oxyhemoglobin with 10 μM streptonigrin was $\leq 1\%$ and there was no shift in oxyhemoglobin absorption maxima (data not shown). This finding does not exclude that streptonigrin may interact with the guanylyl cyclase heme, but in order to prove the involvement of heme in the strepronigrin-induced inhibition further investigations are necessary.

It is known that LY83583 (**III**) (Fig. 1) inhibits the sodium nitroprusside-induced activation of guanylyl cyclase (Mulsch et al., 1988) and its structure (like streptonigrin) contains the quinolinequinone fragment (Fig. 1) (Mulsch et al., 1989). This inhibitory effect was sharply decreased in the presence of superoxide dismutase. This suggests that the inhibitory effect is due to the generation of the superoxide-anion radical (Cone et al., 1976). Streptonigrin is also able to generate this radical under aerobic conditions. However, the lack of effect of superoxide dismutase on the inhibitory action of streptonigrin excluded this possibility.

Results obtained with the use of the streptonigrin derivatives (Table 1) demonstrate that substitution of the carboxyl group of the streptonigrin molecule with hydrophilic carbohydrate fragments (**VI**–**VIII**)or a carbonyl or imino group (**IV**–**V**) practically did not influence the intensity of the inhibitory effect, which changed from 53% to 73% (Table 1). The increase in hydrophobic properties of streptonigrin derivative (**IX**) sharply decreased the inhibitory effect (up to 26%, Table 1). The most interesting results were obtained with the conjugate (**I**-DNR, **X**, Tables 1 and 2). The inhibitory effect of this compound (10 μM) (87%) was equal to the arithmetic sum (89%) of the inhibitory effects of 10 μM streptonigrin (53%) and 10 μM daunorubicin (36%) alone (Table 2). The inhibition of the stimulated enzyme activity (by 71%) in the presence of streptonigrin (10 μM) and daunorubicin (10 μM) incubated with the enzyme together was equal to 80% of the sum of the inhibitory effects of these antibiotics (Table 2). The additivity of the inhibitory effects of daunorubicin (10 μM, maximal concentration) and streptonigrin (10 μM) on the enzyme suggests that both these drugs act independently at two different sites on the enzyme in the immediate proximity of the heme prosthetic group. Whether these drugs cause inhibition by the same or by different mechanisms need further investigation.

It should be mentioned that the inhibition of stimulated guanylyl cyclase activity by streptonigrin (**I**) and its derivatives (**IV**–**X**) did not correlate with the level of cytotoxic effects of these compounds. Streptonigrin (**I**) is a highly cytotoxic compound (IC_{50} for L1210 is 0.044 ± 0.009 μM), whereas the investigated streptonigrin derivatives are several orders less toxic (for L1210 cells $IC_{50} = 2.57 \pm 0.28$ (**IV**), 2.46 ± 0.24 (**V**), >100 (**VI**), 34 ± 5.1 (**VII**), 2.3 ± 0.08 μM (**VIII**)) (Preobrazhenskaya et al., 1992; Tolstikov et al., 1992a). The conjugate of streptonigrin and daunorubicin (**X**), which was the most potent inhibitor of stimulated guanylyl cyclase activity, was only slightly cytotoxic to murine C-127 cell culture (IC_{50} for **X**: 200 μg/ml; IC_{50} for **I**: 0.02 μg/ml) and was not toxic to mice in the dose of 20 mg/kg (Tolstikov et al., 1989). The absence of a correlation between the inhibitory effects of streptonigrin (**I**) and its derivatives (**IV**–**X**) and the level of the cytotoxic effects of these compounds suggests that guanylyl cyclase is not involved in the mechanism of antitumor action of this drug. The high cytotoxicity of streptonigrin does not allow this drug to be used as an inhibitor of NO-dependent guanylyl cyclase activation. However, the low cytotoxicity of the conjugate of streptonigrin and daunorubicin (**X**) and its high inhibitory properties demonstrate that this compound (or appropriate derivatives) may be used as a potent inhibitor of NO-dependent activation of soluble guanylyl cyclase especially in cases of excessive NO formation.

Thus, the data for streptonigrin-induced inhibition presented here demonstrate for the first time a new biochemical effect of streptonigrin. This finding should be taken into account in addition to the antitumor action of this drug.

Acknowledgements

These studies were supported by the Russian Foundation for Basic Research (No. 02-04-48319).

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