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SHORT COMMUNICATIONS

Structural specificity of the NaK-ATPase inhibition by sanguinarine, an isoquinoline benzophenanthridine alkaloid*

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Sanguinarine, an isoquinoline benzophenanthridine alkaloid, was recently shown to be a potent inhibitor of guinea pig brain NaK-ATPase [1]. Since sanguinarine also has a cardiotonic effect [2] on isolated guinea pig atria, it resembles the clinically important class of cardioactive agents, the cardiac glycosides, whose pharmacologic receptor has been discussed [3, 4] in terms of the cardiac NaK-ATPase enzyme system. Furthermore, benzophenanthridine derivatives exhibit antitumor activity [5, 6], another action which has been related to NaK-ATPase inhibition in the cases of strophanthidin derivatives [7] and diketocoriolin B [8]. The structure of sanguinarine [9] is unique among NaK-ATPase inhibitors [10], combining hydrophilic and hydrophobic properties by virtue of its iminium cation being contained within an aromatic polycyclic ring system. Hetero-, polycyclic NaK-ATPase inhibitors containing a single-ring nitrogen atom include two quinoline compounds, quinidine [11] and primaquine [12], and the isoquinoline compound, quindonium bromide [13]. To determine whether other quinoline and isoquinoline compounds were inhibitors of NaK-ATPase, we tested a variety of quinolines and isoquinolines, some of which shared several structural features with sanguinarine. When none of these compounds showed significant NaK-ATPase inhibition, we tested some benzophenanthridine compounds to determine the structural specificity of sanguinarine NaK-ATPase inhibition. To further characterize the nature of sanguinarine inhibition, several other experiments were performed, including time course, dose–response, reversibility, and sulfur nucleophile studies.

NaK-ATPase was prepared from guinea pig brain using the method of Akera and Brody [14]. The enzyme was then treated with sodium thiocyanate using the method of Skou [15] to increase the ouabain sensitive portion to more than 95 per cent of the total activity. The specific activity averaged 300 μmoles P_i/hr/mg of protein, as determined by the method of Lowry *et al.* [16], and ranged from 42 to 75 μg/ml. The assay for NaK-ATPase activity in the presence of a potential inhibitor was carried out by placing the enzyme in the presence of optimum concentrations of its cofactors (6 mM Mg, 100 mM Na, 20 mM K and 132 mM Cl) and of a 0.1 mM inhibitor concentration. The pH was maintained at 7.4 with imidazole buffer (50 mM), and the reaction was initiated by making the solution 3 mM in ATP and placing the reaction tubes in a shaking water bath at 37°. The order of the reagent addition was kept fixed (cofactors, buffer, inhibitor, enzyme, distilled water), the cool solution of ATP being added last to the cooled mixture of cofactors, buffer, inhibitor and enzyme. The final volume for each sample was 1 ml. The reaction was terminated with 0.1 cm^3 cold 10% trichloroacetic acid (TCA) after a 10-min incubation period. In the case of the benzophenanthridines, a 30-min incubation period was used. The released inorganic phosphate was analyzed by the method of Fiske and Subbarow as described by Leloir and Cardini [17]. The method described was generally precise to within ± 5 per cent. Mg-ATPase activity was determined by including 2.5 mM ouabain in the incubation mixture. NaK-ATPase activity was then calculated by subtracting the Mg-ATPase activity from the total ATPase activity. Each inhibitor test was performed in triplicate. Each compound was tested at least four times, with most compounds being tested six times. Per cent inhibitions and standard deviations were calculated for each compound. The average standard deviation of per cent inhibition was 4. However, some compounds, notably acridine, acriflavine, laudanosine and nitidine, had larger standard deviations (8, 7, 6 and 6 respectively). In most cases these larger deviations were probably due to solubility problems discussed below. The reason for the large standard deviation with acriflavine is unknown.

Acridine, acriflavine chloride, protopine, capaurine, corycavine oxalate, coralyne chloride, corydaline, ethidium bromide, and *d*-tetrahydropalmatine were purchased from Aldrich Chemical Co. Berberine hydrochloride, boldine, chelidonine, laudanosine and hydrastine were purchased from Pfaltz & Bauer, while emetine hydrochloride came from Lily. Nitidine was a gift of Dr. J. L. Hartwell, of the National Cancer Institute, Bethesda, MD. These compounds were used as supplied. Synthesis of the sanguinarine analogs used in this study has been previously reported [5]. All of these compounds that were tested for NaK-ATPase modifying ability were made up to 1 mM in aqueous solution. However, several of the compounds were not soluble in distilled water. Acridine, boldine, corydaline, capaurine, protopine and hydrastine were soluble in aqueous solution acidified (about pH 3) with several drops of 0.1 N HCl. Chelidonine and laudanosine required ethanol (40 and 25%, respectively) for dissolution in addition to the acidification. Nitidine required more strenuous conditions. By alternately heating a suspension in acidified water and spinning on a vortex mixer, a pale yellow solution was obtained. Solubilizing solutions were added to the control tubes in the same amounts as those in the inhibitor-containing tube.

The reversibility of compound 8q inhibition was determined by exhaustive dialysis. Equal volumes of stock enzyme solution and of 1 mM inhibitor solutions were preincubated at 37° for 5 min. Controls were preincubated with enzyme and water at 37° for 5 min. After preincubation half of each of the solutions was incubated for 10 min in the presence of buffer and ATP. The other half was dialyzed at 6° against 100 vol. of a buffer solution (0.125 M sucrose, 2.5 mM histidine and 0.5 mM EDTA, pH 7.1). The dialysis was carried out for 24 hr with three changes of buffer. The dialyzed enzyme was then tested for activity as before.

The time course of inhibition was studied by incubating cofactors, buffer, inhibitor, ATP and enzyme together at

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37°. One-ml aliquots were removed at intervals during the first hour of reaction. The reaction was stopped with 10% TCA. The control was incubated without inhibitor and treated as above. Dose–response data for fagaronine, compound 8q, and sanquinarine were obtained by varying the concentration of inhibitor added to the reaction vessel before incubation. Values for pI_{50} were taken from plots of concentration vs per cent of inhibition.

In the preincubation experiments on protection with sulfur nucleophiles (mercaptoethanol, sodium bisulfite, dithioerythritol and cysteine), the enzyme was placed on a shaking water bath for 10 min at 37° in the presence of its cofactors (ATP absent), sanguinarine (0.01 mM) and one of the sulfur nucleophiles. The total volume of each tube was brought to 0.9 ml. At the end of this preincubation, the tubes were returned to the ice bath, and 0.1 ml of 30 mM ATP was added. The tubes were then incubated for 10 min as previously described. The reversal experiments were carried out similarly, with a 10-min preincubation without nucleophile and ATP, followed by a 10-min preincubation with nucleophile, then finally a 10-min incubation with both nucleophile and ATP. All experiments with sulfur nucleophiles were carried out in duplicate four times.

Table 1 shows the degree of inhibition on NaK-ATPase activity produced by a group of non-benzophenanthridine compounds which included both imine and iminium quinolines, a phenanthridinium isoquinoline, and six subclasses of isoquinoline alkaloids [9]. From the low inhibitory activities exhibited by these compounds, it is apparent that substituted quinoline and isoquinoline skeletons of the types represented are not potent modifiers of guinea pig brain NaK-ATPase. In particular, the aromatic, rigid ring systems of the iminium cations ethidium bromide, coralyne and berberine, which share several structural features with sanguinarine, are inactive.

From the inhibitory activities of the various benzophenanthridines shown in Table 2, it can be concluded that there is a high degree of structural specificity for sanguinarine inhibition of guinea pig brain NaK-ATPase. This conclusion is derived from the fact that small deviations in the ring substituent pattern of sanguinarine result in large differences in inhibition. When the 7,8-methylenedioxy ring is opened by conversion to the 7,8-dimethoxy derivative (chelerythrine), there is a definite loss of inhibitory potential of 16 per cent. Thus, the presence of the less bulky, less hydrophobic methylenedioxy group enhances inhibition but is not an absolute requirement. However, inhibition of NaK-ATPase is reduced by more than 50 per cent with the remaining compounds. Nitidine, a ring A structural isomer of chelerythrine, gives a greater than 50 per cent reduction in inhibition. A similar loss in inhibition also occurs in the remaining compounds, each structurally modified from sanguinarine in both the A and D rings. One of the important modifications is the loss of the methylenedioxy rings. Hence, the tentative conclusion can be drawn that the methylenedioxy ring in the A and/or the D ring is important for inhibition of the NaK-ATPase. The conformation and charge distribution needed for maximal guinea pig brain NaK-ATPase inhibition thus appears to favor planar, fully aromatic benzo[c]phenanthridinium compounds with substituents that are less bulky and hydrophobic than methoxy groups.

Results from fagaronine, chelidonine, and the tri- and tetra-methoxy compounds substantiate this view of the essential features of the sanguinarine structure and also indicate a possible basis for the structural specificity of sanguinarine. Chelidonine, which has no iminium group, is inactive. The iminium compounds vary in activity from no inhibition to 97 per cent inhibition. In addition, the iminium compounds vary widely in their physicochemical properties. For example, nitidine is only sparingly water soluble in contrast to sanguinarine, which is both water and lipid soluble. Thus, the iminium function is likely to be a necessary but not sufficient factor in sanguinarine inhibition of NaK-ATPase, while physicochemical properties (e.g. solubility and partition coefficient) also play an important role.

Table 1. Inhibition of NaK-ATPase by quinolines and isoquinolines*

Compound	% NaK-ATPase inhibition	N
Acridine	8 ± 8	9
Acriflavine	13 ± 7	6
Ethidium bromide	11 ± 3	8
Berberine	2 ± 3	5
Coralyne	5 ± 4	6
Capaurine	1 ± 4	5
Corydaline	4 ± 3	3
d-Tetrahydropalmatine	4 ± 4	5
Protopine	5 ± 4	6
Corycavine oxalate	4 ± 4	5
Papaverine	1 ± 1	4
Laudanosine	−2 ± 6	6
Hydrastine	0 ± 5	6
Boldine	4 ± 1	5
Emetine	6 ± 1	4

* The reaction mixture contained 20 mM KCl, 6 mM $MgCl_2$, 100 mM NaCl, 50 mM imidazole (pH 7.4), 65 μg enzyme protein (average value), and an inhibitor concentration of 0.1 mM in a final volume of 1 ml. The reaction was initiated by making the solution 3 mM in ATP and was terminated by the addition of 10% TCA after a 10-min incubation period at 37°. NaK-ATPase activity, averaging 300 μmoles P_i formed/hr/mg of protein, was determined spectrophotometrically, as indicated in the text. Values given are mean per cent inhibition ± S.D. N = number of experiments.

The time course of inhibition for a moderate inhibitor, compound 8q, and sanguinarine shows that after 10–15 min of reaction the inhibition reaches a maximum (37 per cent for compound 8q; 97 per cent for sanguinarine). The level of inhibition does not change after this initial 10 to 15-min period for either inhibitor. Thus, the values reported here for per cent of inhibition were taken after the system had reached equilibrium. Values obtained as the pI_{50} of fagaronine, compound 8q and sanguinarine were 1.5, 3.8 and 5 respectively. This distribution reflects the degree of inhibition found for the inhibitors at 0.1 mM. Twenty-four hr of dialysis did not change the value obtained for the per cent of inhibition for compound 8q and sanguinarine. This indicates that inhibition of NaK-ATPase by benzophenanthridines is not readily reversible, regardless of the degree of inhibition.

Experiments with sulfur nucleophiles suggest a role for the iminium function and the nature of the interaction between sanguinarine and the NaK-ATPase enzyme system. The results shown in Table 3 indicate that the enzyme activity can be protected against sanguinarine inhibition by some sulfur nucleophiles, presumably by attacking the iminium carbon. Table 4 indicates that only sodium bisulfite can effect partial reversal of sanguinarine inhibition. This indicates a high degree of irreversibility for the reaction between sanguinarine and NaK-ATPase. It was first suggested by Sarkar [18] that sanguinarine inhibition of –SH enzymes may involve a combination of sanguinarine with essential sulfhydryl groups. Howell *et al.* [19] related the detoxification of sanguinarine by fungi to production of the reduced dihydro derivative. Structure–activity studies by Stermitz *et al.* [20] and by Zee-Cheng and Cheng [6] on cytotoxic effects of sanguinarine analogs

Table 2. Inhibition of NaK-ATPase by benzophenanthridines*

Compound	% NaK-ATPase inhibition	N
Sanguinarine chloride $R_1 + R_2 = -CH_2-$ $R_3 + R_4 = -OCH_2-$ $R_5 = -H$	97 ± 1	5
Chelerythrine chloride $R_1 + R_2 = -CH_2-$ $R_3 = R_4 = -OCH_3$ $R_5 = -H$	81 ± 7	6
8q $R_1 = R_2 = -CH_3$ $R_3 = -H$ $R_4 = -OCH_3$ $R_5 = -OH$	37 ± 4	7
2,3,8-Trimethoxy-5-methylbenzophenanthridinium chloride $R_1 = R_2 = -CH_3$ $R_4 = -OCH_3$ $R_3 = R_5 = -H$	33 ± 5	5
8o $R_1 = -CH_3$ $R_2 = -H$ $R_3 = -H$ $R_4 = R_5 = -OCH_3$	30 ± 3	6
Nitidine chloride $R_1 + R_2 = -CH_2-$ $R_4 = R_5 = -OCH_3$ $R_3 = -H$	28 ± 6	9
2,3,7,9-Tetramethoxy-5-methylbenzophenanthridinium chloride $R_1 = R_2 = -CH_3$ $R_3 = R_5 = -OCH_3$ $R_4 = -H$	19 ± 3	4
2,3,9-Trimethoxy-5-methylbenzophenanthridinium chloride $R_1 = R_2 = -CH_3$ $R_5 = -OCH_3$ $R_3 = R_4 = -H$	9 ± 4	5
Fagaronine chloride $R_1 = -H$; $R_2 = -CH_3$ $R_4 = R_5 = -OCH_3$ $R_3 = -H$	6 ± 4	5
Cheldonine	5 ± 2	6
8M $R_1 = R_2 = -CH_3$ $R_3 = -H$ $R_4 = -OCH(CH_3)$ $R_5 = -OCH_3$	5 ± 4	4
8H $R_1 = R_2 = -CH_3$ $R_3 = -H$ $R_4 = -OCH_3$ $R_5 = -OCH_2CH_3$	0 ± 2	5

* Experiments were carried out using a test inhibitor concentration of 0.1 mM, as described in the text and Table 1, and a 30-min incubation period replacing the 10-min period desribed in the previous table. Values are the mean per cent inhibition ± S.D. N = number of separate experiments.

also emphasize the possible importance of nucleophilic attack at the iminium carbon. Furthermore, other inhibitors of NaK-ATPase, such as *N*-ethylmaleimide [21] and ethacrynic acid [22], have strong electrophilic centers and are thought to alkylate essential sulfhydryl groups on NaK-ATPase. Thus, sanguinarine may belong to the sulfhydryl reagent class of NaK-ATPase inhibitors by virtue of the electrophilic nature of its iminium function.

If the reactivity of the iminium group of sanguinarine is the dominant feature in NaK-ATPase inhibition, inhibitory activity would be expected to correlate with enhancement of the electrophilicity of the iminium carbon from ring substituent groups. The order of activity in Table 2 provides no such unifying hypothesis. This serves as another indication of the possible importance of other factors, such as the physicochemical properties of the

Table 3. Protection by sulfur nucleophiles against sanguinarine inhibition*

Nucleophile	% Inhibition with 10^{-5} sanguinarine	N
None	80 ± 9	4
Mercaptoethanol (10 mM)	32 ± 10	5
Sodium bisulfite (10 mM)	8 ± 6	4
DTE (10 mM)	20 ± 5	4
Cysteine (10 mM)	28 ± 3	4

* Preincubation was carried out in the presence of enzyme (23.6 μg protein/ml), 50 mM imidazole (pH 7.4), 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 0.01 mM sanguinarine, and nucleophile as specified. Background was measured by incubating nucleophiles without sanguinarine. All preincubations were for 10 min at 37°. Incubations were for 10 min at 37° in the presence of 3 mM ATP in addition to cofactors, buffer, inhibitor and nucleophile. Final total volume of each tube was 1.0 ml. Values given are the mean per cent inhibition ± S.D. N = number of separate experiments. DTE = dithioerythritol.

Table 4. Reversal of sanguinarine inhibition by sulfur nucleophiles*

Nucleophiles	% Inhibition with 10^{-5} sanguinarine	N
None	87 ± 3	4
Mercaptoethanol (10 mM)	80 ± 9	4
Sodium bisulfite (10 mM)	52 ± 8	4
DTE (10 mM)	76 ± 14	4
Cysteine (10 mM)	80 ± 4	4

* Enzyme was preincubated for 10 min at 37° in the presence of 50 mM imidazole (pH 7.4), 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$ and 0.01 mM sanguinarine. Then, 0.1 ml of the specified nucleophile was added and preincubation continued for another 10 min. Finally, 0.1 ml of 30 mM ATP was added and incubation carried out for 10 min. Values given are mean per cent inhibition ± S.D. N = number of separate experiments. DTE = dithioerythritol.

compounds examined. Furthermore, comparison of the benzophenanthridinium structure–activity ordering for antileukemic activity [5] with that found in Table 2 shows no correlation. Although there is evidence in the cases of strophanthidin derivatives [7] and diketocoriolin B [8] that the biochemical mechanism of some antitumor agents may be NaK-ATPase inhibition, no evidence is provided here for such a mechanism in the case of benzophenanthridines. However, results in Table 2 do show some correlation with the antimicrobial activity of the benzophenanthridinium chlorides [5]. This observation suggests again that the relative importance of the physicochemical properties of sanguinarine derivatives must be considered when attempting to account for differences in structure–activity correlations. For example, a partition coefficient favorable for a molecule reaching its target site in one system may be unfavorable in another system due to differences in the composition and structure of the systems.

Since it is likely that sanguinarine is capable of alkylating a biologically significant nucleophile, such as the sulfhydryl group, and that an optimal balance of chemical reactivity and physicochemical properties can be achieved such that the alkylation can be both highly selective and potent, these findings encourage the design and synthesis of new benzophenanthridinium salts and their testing in a variety of biological and biochemical systems.

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