Hellebrigenin 3-Haloacetates: Potent Site-Directed Alkylators of Transport Adenosinetriphosphatase

Abstract. *Hellebrigenin, which differs from strophanthidin only in its lactone ring, has 30 times the affinity of strophanthidin for the brain (Na + K)-activated adenosinetriphosphatase. Hellebrigenin 3-acetate and hellebrigenin 3,5-diacetate are about three times more potent toward this enzyme than hellebrigenin is. The 3-iodoacetate and 3-bromoacetate of hellebrigenin were synthesized and were highly potent irreversible inhibitors of the enzyme. The iodoacetate was 20 times more potent than the bromoacetate, as expected from the superior alkylating power of iodoacetate as compared to bromoacetate. The irreversible inhibition of the enzyme by hellebrigenin 3-bromoacetate and by strophanthidin 3-bromoacetate paralleled the affinities of the nonesterified steroids for reversible inhibition; this is further strong evidence for the site-directed alkylation of the (Na + K)-activated adenosinetriphosphatase by the haloacetate derivatives of the cardiotonic steroids.*

The membrane-bound (Na + K)-activated adenosinetriphosphatase (Na-K ATPase) has received considerable attention because of its probable role in the coupled transports of Na and K (*1*). This enzyme is rather specifically inhibited by cardiotonic steroids (Fig. 1), which also inhibit the coupled transports of Na and K (*2*). Attempts at extensive purification of the native enzyme have so far been unsuccessful, although recent efforts in this direction show promise (*3*). One approach to molecular characterization of this enzyme is alkylation of the protein with a radioactive site-directed alkylating agent and purification of the labeled protein. Strophanthidin 3-iodoacetate (SIA) and strophanthidin 3-bromoacetate (SBA) alkylate the cardiotonic steroid site of the Na-K ATPase (*4*). Strophanthidin 3-[1-^{14}C]-bromoacetate of high specific activity has been synthesized, and, at concentrations which are required to alkylate a fair percentage of the enzyme ($10^{-4}M$), much more membrane protein was labeled than could be accounted for by specific labeling of the enzyme (*5*). A haloacetate derivative of a cardiotonic steroid with a much higher affinity for the cardiotonic steroid site of the enzyme appeared desirable. We now report on such compounds (Fig. 1).

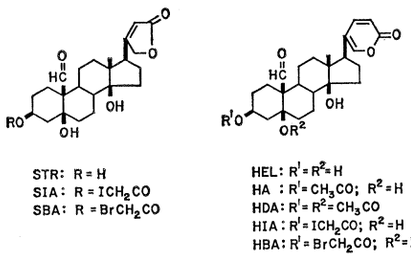
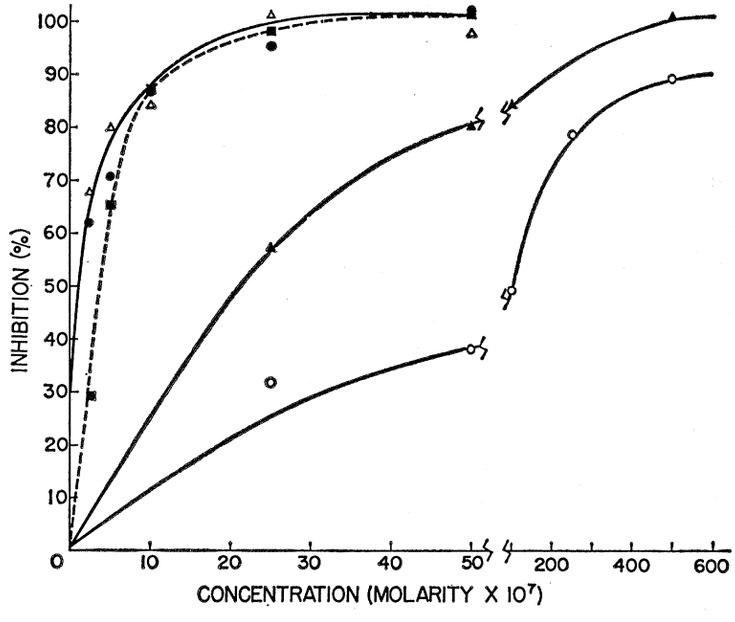


Fig. 1 (left). Structures of the cardiotonic steroid derivatives.

In a search for tumor inhibitors of plant origin, Kupchan *et al.* (*6*) found that hellebrigenin 3-acetate (HA) and hellebrigenin 3,5-diacetate (HDA) were highly cytotoxic against human nasopharyngeal carcinoma cells grown in tissue culture. In view of the correlation between cytotoxic activity and inhibition of the Na-K ATPase (*7*), we tested the activity of the hellebrigenin derivatives toward the enzyme. In Fig. 2 the inhibitory potencies of derivatives of hellebrigenin and two cardenolides on the membrane-bound Na-K ATPase of guinea pig brain (*8*) are compared.

The concentrations of the various



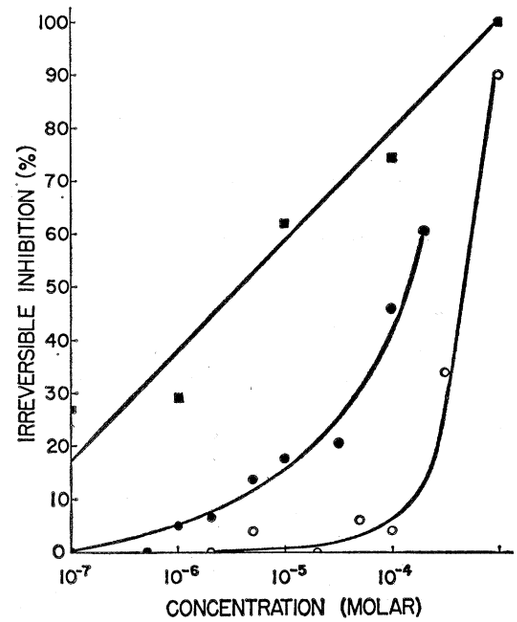


Fig. 2 (left). Reversible inhibition of the (Na + K)-activated adenosinetriphosphatase by bufadienolides and cardenolides. Enzyme from guinea pig brain, prepared as described (*8*), was assayed (*8*) in the presence and absence of the indicated steroid. The aglycones (final concentration is shown) were added in dimethylformamide (1 percent of the final volume). ○, STR; ▲, ouabain; ■, HEL; △, HA; ●, HDA. Dashed line, hellebrigenin. Fig. 3 (right). Irreversible inhibition of the (Na + K)-activated adenosinetriphosphatase by steroid haloacetates. NaI-treated beef-brain microsomes were prepared by the method of Nakao *et al.* (*11*). The enzyme suspensions with appropriate additions in a final volume of 1.0 ml were incubated for 2 hours at 37°C, the particulate enzyme was washed seven times, and the enzyme and protein were assayed on the final residue as described (*4*). The steroids were added in 10 μl of dimethylformamide. ○, SBA; ●, HBA; ■, HIA.

steroids for half-maximum inhibition (I_{50}) of the enzyme are: HA and HDA, $1.3 \times 10^{-7}M$; hellebrigenin (HEL), $3.6 \times 10^{-7}M$; ouabain, $1.4 \times 10^{-6}M$; strophanthidin (STR), $10^{-5}M$. It is of interest to compare the affinities of hellebrigenin and strophanthidin, since their structures differ only in their lactone rings; the affinity of hellebrigenin was 25 to 30 times that of strophanthidin. Our results are similar to those of Glynn (*9*), who found that the bufadienolide, scillaren A, was seven times more potent as an inhibitor of the active transport of K in erythrocytes than the potent cardenolide, digoxin.

When the cardiotonic activities of HA and strophanthidin acetate on the guinea pig atrium (*7*) were compared, very little difference was found. This is an interesting departure from the close correlation between cardiotonic activity and Na-K ATPase inhibitory activity previously found among the derivatives of strophanthidin (*7*) and supports the view, advanced earlier (*2*), that the cardiotonic effect is not brought about by inhibition of the enzyme. It also indicates that variations in the structure of the lactone ring have less effect on cardiotonic activity than on Na-K ATPase inhibitory activity.

In view of the high affinity of the hellebrigenin derivatives for the Na-K ATPase, we prepared hellebrigenin 3-haloacetates in order to study their irreversible inhibition of the enzyme. Hellebrigenin 3-bromoacetate (HBA) was prepared by treatment of a solution of hellebrigenin (300 mg) in dioxane (25 ml, distilled from lithium aluminum hydride) with bromoacetyl chloride (700 mg) at room temperature for 16 hours. The solution was diluted with water (125 ml) and filtered, and the precipitate was dried and crystallized from methanol to yield HBA: 272 mg; $C_{26}H_{33}BrO_7 \cdot H_2O$; melting point, 180° to 182°C; $[\alpha]_D^{25}$, + 26 deg (chloroform solution). The absorption maximum in methanol solution at 298 nm showed a molar extinction coefficient (ϵ) of 5800. (Additional product was obtained by extraction of the filtrate with chloroform and chromatography of the extracted product on silicic acid.) Hellebrigenin 3-iodoacetate (HIA) was prepared in an analogous manner from hellebrigenin (300 mg) and iodoacetyl chloride (750 mg). The dried, pale yellow precipitate (320 mg) was crystallized from methanol, with prior charcoal treatment, to yield colorless prisms: $C_{26}H_{33}IO_7$; melting point, 144° to 145°C; $[\alpha]_D^{26}$, + 26 deg (chloroform solution). The absorption maximum in methanol at 298 nm showed a molar extinction coefficient of 5000 (*10*).

The concentrations of HIA, HBA, and SBA for half-maximum irreversible inhibition of the Na-K ATPase in NaI-treated beef-brain microsomes (*11*) were, respectively, $5 \times 10^{-6}M$, $10^{-4}M$, and $6.6 \times 10^{-4}M$ (Fig. 3). Thus, HIA is about 100 times more potent as an irreversible inhibitor of the enzyme than SBA is and 20 times more potent than HBA. The considerably greater potency of HIA as compared to HBA is consonant with the view that the alkylation reaction is the rate-limiting step and that the superior leaving ability of iodine as compared to bromine plays an important role. It should be noted that the microsomes were washed seven times to remove any unreacted steroid haloacetates. Six washings were required to fully restore the enzyme after incubation with the reversible inhibitor, HA. For very short incubation periods (up to 20 minutes), concentrations of HIA between $10^{-7}M$ and $10^{-6}M$ produced very little irreversible inhibition of the enzyme. It was thus possible to determine the reversible I_{50} for HIA by assaying for short periods in the presence of the steroid, and this was found to be $4.2 \times 10^{-7}M$. This value agrees well with the I_{50} for HA and HDA and indicates that the halogen does not appreciably influence the affinity of the steroid for the site on the enzyme.

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10. Hellebrigenin, HBA, and HIA showed significant cytotoxicity (ED_{50}) against cell cultures of human carcinoma of the nasopharynx (KB) at 10^{-3}, 10^{-6}, and 10^{-6} μg/ml, respectively [see H. Eagle and G. E. Foley, *Amer. J. Med.* **21**, 739 (1956); *Cancer Res.* **18**, 1017 (1958)]. The compounds failed to show significant inhibitory activity against Walker carcinosarcoma 256 in rats at doses up to 100, 25, and 50 mg per kilogram of body weight, respectively. Assays were run, under the auspices of the Cancer Chemotherapy National Service Center, NCI, by the procedures described in *Cancer Chemotherap. Rep. 25* (1962), p. 1.
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Two-Chain Immunoglobulin A Molecules: Abnormal or Normal Intermediates in Synthesis

Abstract. *Immunoglobulin A (γA) myeloma proteins secreted by plasma-cell tumors of mice are of two types, a common four-chain molecule and a rare two-chain (3.9S) molecule. The close similarity between two-chain γA molecules and four-chain γA molecules and their polymers is demonstrated in tryptic peptide maps of isolated polypeptide chains and by precipitin reactions with rabbit antiserums to γA immunoglobulins. However, a difference between these two types is distinguishable with homologous antiserums. Homologous antiserums to two-chain γA immunoglobulins are specific and do not cross-react with four-chain γA immunoglobulins.*

Plasma-cell tumors are readily induced in the inbred BALB/c strain of mice by Lucite shavings, by Freund's adjuvant, and by mineral oil (*1*). Over 50 percent of these tumors synthesize and secrete γA immunoglobulins (*2*). Most γA myeloma proteins are polydisperse in ultracentrifugal analysis, manifesting characteristic peaks with sedimentation coefficients of 7, 9, 11, and 13*S*, and are presumed to consist of four-chain (7*S*) units and polymers (n = 2, 3, and 4) thereof (*3*).

Immunoglobulin production in plasma-cell tumors is often disorderly, and abnormal forms of immunoglobulin molecules are frequently present. The most common types of abnormality observed are either the production of an excess of light chains concomitant with production of the usual four-chain immunoglobulins or the absence of any synthesis of heavy chains. Another type of disorder, so far reported only in mice, is the production