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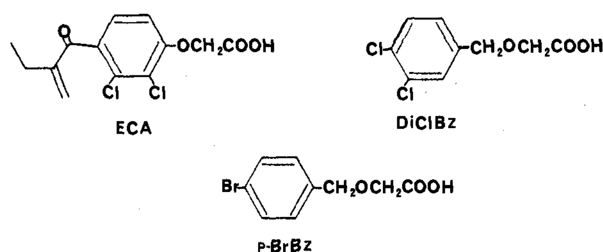
Communications to the Editor

Design, Synthesis, and Testing of Potential Antisickling Agents. 3. Ethacrynic Acid[†]

Sir:

Recently we reported the antigelling properties of several halogenated aromatic acids.[1-3] Other workers have also reported the ability of halogenated aromatic compounds to inhibit HbS polymerization.[4-6] Using the chemical information obtained from our X-ray binding studies and testing program, we searched the drug literature for approved drugs that have the necessary structural parameters for activity. Specifically, we have discovered that an aromatic ring containing two halogens attached to a benzyloxy or phenoxyacetic acid imparts strong antigelling properties to a molecule. It was evident from our search of the drug literature that the potent diuretic ethacrynic acid (ECA) contains these features. Although it seemed unlikely that ECA would be of use as a therapeutic agent in treating sickle cell anemia (due to its strong diuretic effect, which might precipitate a sickle cell crisis), we decided to test it for the purpose of extending our knowledge of structure–activity relationships. As anticipated, we discovered that ECA is an extremely potent antigelling compound and to our great surprise found that it has other properties that may make it or an analogue a viable drug for the treatment of sickle cell anemia. The following communication details our findings.

Solubility Assay. The first assay that we use to screen potential antigelling agents involves the measurement of a compound's ability to increase the solubility of sickle hemoglobin (HbS).[7] In this assay, ECA exhibited the highest activity at low concentrations (5 mM) of any compound that we have tested to date. Table I compares ECA with two of our other halogenated (benzyloxy)acetic acid molecules.[1,2] The relative solubility is given as a ratio, obtained by dividing the solubility of HbS with drug (grams per deciliter) by the solubility of the untreated sample (grams per deciliter). The higher the ratio, the higher the activity of the compound. The potency of these agents can best be visualized by noting the percentage numbers which indicate the amount of HbS polymer dissolved by the drug compared to the control (see Table I legend for details). It should be noted that our more active agents, such as ECA, [(3,4-dichlorobenzyl)oxy]acetic



acid (DiClBz), and [(*p*-bromobenzyl)oxy]acetic acid (*p*-BrBz) begin to show saturation effects around 20 mM. We also discovered that ECA reacts with the deoxygenating agent dithionite in the solubility assay; therefore, care must be taken to let the ECA react completely with the HbS under aerobic conditions before the addition of dithionite. The chemistry of this reaction is still under investigation. When ECA is incubated overnight at 4 °C before the addition of dithionite, even higher results than reported in Table I are observed (3 mμ, 1.072, 17.1%, 5 mM, 1.143, 34.2%).

Covalent Binding. The ability of the acrylophenones, such as ECA, to undergo Michael-type additions with sulfhydryl-containing agents has been evident from their initial conception as diuretics (for a recent review, see ref 8). Therefore, we analyzed the supernatant obtained after the gelation assay described above to see if ECA had reacted with HbS. Electrophoresis on cellulose acetate strips showed that one major new band appeared at all concentrations. This band migrated to the area where fetal hemoglobin is found and short of the normal adult hemoglobin (HbA) (see Figure 1a). The HbS–ethacrynic acid adduct (HbS–ECA) was resynthesized to produce a completely reacted material by incubation of ECA (12.4 mM) with HbS (4.1 mM) overnight at 4 °C (see Figure 1b). Purification of HbS–ECA was accomplished with a DEAE-Sephacel column, which was first equilibrated with 0.05 M, pH 8.6 Tris buffer and eluted with 0.05 M, pH 8.4 Tris buffer. Electrophoresis showed that the first 200 mL of eluant consisted primarily of one band with the same position as that observed in the gelation assay. The fact that the HbS–ECA complex did not dissociate upon chromatographic separation indicates that it is bound covalently or that the physical binding is extremely tight.

Titration of HbS Sulfhydryl Groups. The next step was to see if the surface-reactive Cys-93β residues had

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Table I. Comparison of Solubility Ratios and Percent HbS Solubilized[b] for Ethacrynic Acid (ECA), [(3,4-Dichlorobenzyl)oxy]acetic Acid (DiClBz), and [(p-Bromobenzyl)oxy]acetic Acid (p-BrBz)

compd[a]	5 mM acid	10 mM acid	20 mM acid	40 mM acid	no. of runs
ECA	1.106 (0.013) 25.3% (3.4)	1.166 (0.028) 39.5% (4.1)	1.317 (0.010) 76.2% (8.1)	1.375 (0.032) 89.4% (3.1)	4[c]
DiClBz	1.087 (0.020) 22.1% (4.2)	1.166 (0.019) 42.6% (3.5)	1.321 (0.016) 82.3% (5.4)	1.345 (0.049) 88.0% (9.8)	5[d]
p-BrBz	1.059 (0.023) 13.0% (4.8)	1.118 (0.036) 25.8% (7.3)	1.233 (0.045) 51.0% (9.1)	1.398 (0.034) 87.7% (4.8)	4[e]

[a] All compounds were dissolved in 0.15 M phosphate buffer, pH 7.4, with 1 equiv of sodium bicarbonate to make the sodium salt, at a concentration of 0.18 M. Appropriate aliquots of this solution (10, 20, 40, and 80 μL) were mixed with buffer to equal 90 μL. The 90-μL solutions were added to 250 μL of HbS (0.15 M phosphate), usually around 35 g %, and then 20 μL of dithionite (1.06 M) was added before sealing of the EPR tubes. Final concentrations of drug were 5, 10, 20, and 40 mM in four separate tubes. A set of six tubes was spun on each run, which included the four drug concentrations, one dHbS (d = deoxy) control (90 μL of buffer, no acid), and a 40 mM phenylalanine control. The above dilution procedure produces identical HbS initial concentrations for all six tubes. The initial HbS concentrations (in grams per deciliter) for each run after addition of the acids and dithionite and the respective solubility of deoxyhemoglobin S (in grams per deciliter) for each control run (no acid) follow for each compound studied. The ratios are calculated as follows: sol of HbS (drug) (g/dL)/sol of HbS (control) (g/dL). Standard deviations are shown in parentheses. [b] Calculated from the following equation: % increase in sol = [[sol of HbS (drug) − sol of HbS (control)]/[sol of HbS (initial) − sol of HbS (control)]] × 100. Standard deviations are shown in parentheses. [c] Initial HbS concentrations, in grams per deciliter, for each run: 24.81, 23.22, 24.47, 24.47. Respective solubility of dHbS controls for each run at 35 °C, in grams per deciliter: 17.29, 17.29, 17.00, 16.70. [d] Initial HbS concentrations, in grams per deciliter, for each run: 24.02, 23.82, 23.83, 23.97, 23.14. Respective solubility of dHbS controls run at 35 °C, in grams per deciliter: 17.07, 16.93*, 17.00, 17.58, 16.85. *This control value assumed from other runs as control was erroneously high. These values update those reported in ref 1, which were for three runs. [e] Initial HbS concentrations, in grams per deciliter, for each run: 22.85, 24.68, 24.99, 24.84. Respective solubility of dHbS controls for each run at 35 °C, in grams per deciliter: 15.93, 17.00, 16.70, 17.14.

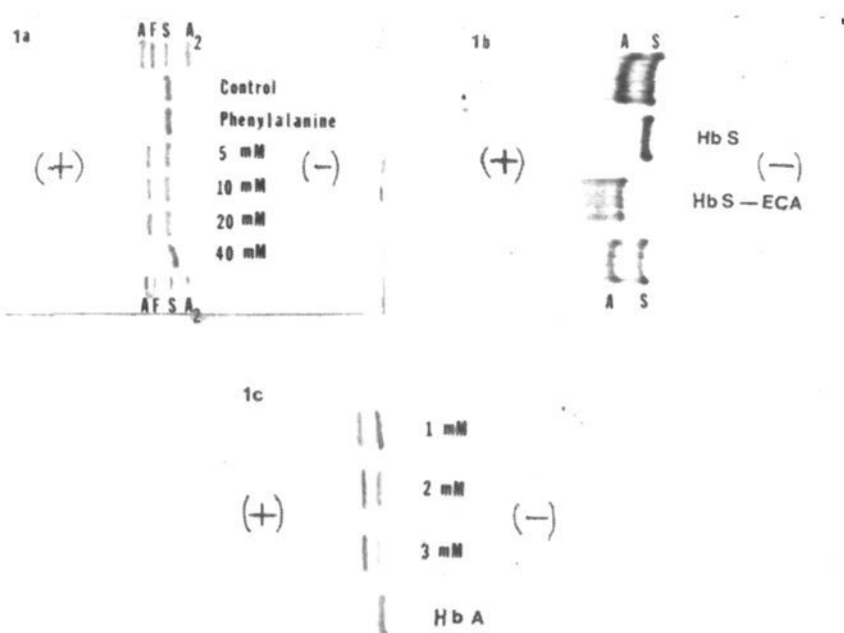

Figure 1. Electrophoresis experiments with ECA. (a) The results of the solubility assay are shown. The new HbS–ECA band is stronger as the concentration of ECA is increased, except at 40 mM. Several runs at 40 mM all produced the same results. Perhaps at this high ratio of drug to hemoglobin (ca. 10.5:1 ECA/HbS), the HbS is split into dimers or denatured and the binding of the drug is altered. (b) This demonstrates that a 3 M to 1 M ratio of ECA to HbS is sufficient to produce primarily a single product (HbS–ECA) at 4 °C. (c) This electrophoresis pattern shows the ability of ECA to cross the erythrocyte membrane in the presence of plasma proteins to form the HbA–ECA product at 1, 2, and 3 mM when incubated at 37 °C for 2 h. The increase in HbA–ECA product with increasing concentration of ECA is clear in this experiment.

reacted with ethacrynic acid. These Cys sulfhydryl groups have been shown to react covalently via a Michael-type addition with maleimide derivatives.[9,10] Titration of the free sulfhydryl residues on HbS–ECA was performed with the reagent p-mercuribenzoate.[11] The drug-free HbS (control) titrated for 2.2 free sulfhydryl groups (as expected) and the HbS–ECA solutions from the gelation assay showed 2.5 (5 mM), 2.4 (10 mM), 2.6 (20 mM), and 2.6 (40 mM) free sulfhydryl groups. Titration of purified HbS–ECA showed 2.0 free sulfhydryl groups vs. 2.1 for the control. These results suggest that ECA is not reacting with the free surface sulfhydryl groups. Recently, we have reported the selective reaction of the buried sulfhydryl groups at positions 104α and 112β with a hydrophobic heavy atom derivatizing agent, methylphenylmercury.[12] This type of internal reaction of ECA with a buried HbS sulfhydryl group in a hydrophobic pocket is a possibility for HbS–ECA. The Trp-14α pocket reported earlier[2] as a binding site for halogenated aromatic compounds does have a Thr residue (108α) located in a position that would be in proximity to effect a Michael addition on ECA. However, to our knowledge, no protein reactions with Thr as a nucleophile have ever been observed. The exact location of the binding of ECA with HbA must await crystallographic analysis, which we hope to report on soon.

Transport Across Erythrocyte Membranes. Freshly washed normal erythrocytes (0.46 mL) were incubated for 2 h at 37 °C with ECA (9.54 mL) at 1.0, 2.0, and 3.0 mM in buffer (0.1 M NaCl and 0.05 M phosphate, pH 7.4). The cells were washed 4 times with isotonic saline and lysed. Electrophoresis showed the presence of the new band at all concentrations. In order to see the effect of plasma on the transport of ECA, we incubated whole blood (1 mL, hematocrit 0.46) with 9 mL of ECA at 1.0, 2.0, and 3.0 mM under the same conditions, and the same band appeared (see Figure 1c). The plasma was checked to see whether the erythrocytes were lysed to any degree. The results (cyan-met determination) showed that the amount of lysing was minimal and only slightly above the control value of 0.037 g % HbA (1 mM = 0.066; 2 mM = 0.073; and 3 mM = 0.073 g %]. These findings establish the ability of ECA to cross the red blood cell membrane and react with hemoglobin.

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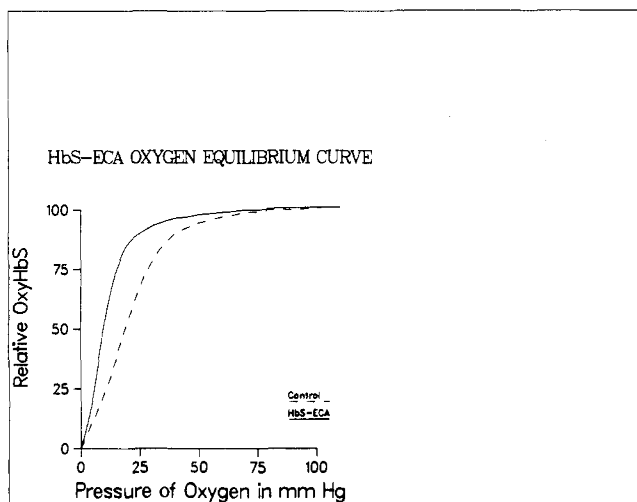

Figure 2. The oxygen equilibrium curve of the HbS–ECA (4 g %) product. This oxygen equilibrium curve was obtained on an Aminco Hem-O-Scan under conditions stated in the text of the paper. The P-50 value was essentially the same for a sample of 12–14 g % HbS–ECA (9.5 vs. 19 mmHg for the control sample).

Oxygen Equilibrium. Finally, we measured the effect of ECA on the oxygen equilibrium curves using an Aminco Hem-O-Scan with 0.5, 1.0, and 2.0 mM ECA (HbA, 2.4 g %; 0.15 M phosphate, pH 7.4). The results demonstrated that ECA raises the oxygen affinity of HbA dramatically. The control had a P-50 value of 18 mmHg, while the ECA solutions showed left-shifted P-50 values of 14 (0.5 mM) and 11 mmHg (1.0 and 2.0 mM). The left-shifted curve is the first we have observed with our benzyloxy or phenoxy compounds, which to date, all right shift the oxygen equilibrium curve (decrease the oxygen affinity of HbA).[2,13] A left-shifted curve (see Figure 2) was also obtained when we ran the oxygen equilibrium curve with the purified HbS–ECA product (0.15 M phosphate, 4 g % HbS–ECA, pH 7.4, P-50 = 7.5 vs. 17 mmHg for control).

Conclusions. ECA has the following desirable features that would be useful in a therapeutic agent: (1) ECA crosses the erythrocyte membrane and reacts covalently with HbS. Covalent or strongly bound noncovalent inhibitors have a possible advantage since they can be administered at lower doses, minimizing unwanted side effects or other therapeutic indications of the drug. (2) The primary mechanism of ECA's antigelling activity is a chemical or stereospecific interaction on HbS, which destabilizes the polymer. This is known from the solubility assay, which is run under completely deoxygenated conditions, therefore eliminating an allosteric (increased oxygen affinity) explanation for its activity. Stereochemical alteration of Hbs has been acknowledged as "an exciting alternative strategy" to finding a therapeutic agent.[3,14] (3) ECA shifts the oxygen equilibrium to the left. Such allosteric effectors act by a separate mechanism of action.[14]

If the diuretic properties of ECA preclude its use as a therapeutic agent, we have been assured[8,15] that derivatives containing the necessary binding moieties (aromatic halogens and vinyl group) and devoid of diuretic effects do exist. It will certainly be worth looking at the antigelling properties of any of these agents.

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