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INHIBITORS OF THE SULFATION OF PROTEINS, GLYCOPROTEINS, AND PROTEOGLYCANS

Glen L. Hortin, Mary Schilling, and James P. Graham

Department of Pediatrics, Box 8116
Washington University School of Medicine, St. Louis, MO 63110

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SUMMARY: Two categories of compounds, substrates of sulfation and sulfate analogs, were tested for the ability to inhibit sulfation of macromolecules secreted by HepG2 cells. Several compounds which most effectively inhibited sulfation without toxic effects on cells were tested for their relative inhibition of sulfation of tyrosine residues (using the fourth component of complement as a model substrate), of N-linked oligosaccharides (α_2HS-glycoprotein as substrate), and of proteoglycans. Inhibitors decreased the sulfation of all three classes of substrate, but not always equally. Use of inhibitors from both categories in combination yielded synergistic effects, with more effective inhibition of sulfation and low toxicity. Such combinations of inhibitors should provide a valuable tool for probing the significance of the sulfation of macromolecules.

The sulfation of small molecules has been investigated in detail due to the recognized importance of this reaction in the excretion of drugs and the normal metabolism of many compounds (1). On the other hand, little is understood about the biological significance of the sulfation of macromolecules such as proteoglycans (2) and proteins (3-8). Sulfate comprises a significant proportion of the mass of many proteoglycans, but the contribution of sulfate groups to function are known for only a few cases such as the role of sulfates in the anticoagulant action of heparin (9). Recently, it has been noted that many proteins also are substrates of sulfation, with oligosaccharides (3, 4) or tyrosine residues (5-8) serving as acceptor sites. The function of the sulfation of proteins is not known, but it has been postulated that sulfation may direct intracellular sorting of some proteins (3, 5). In the present study, we have identified inhibitors of the sulfation of macromolecules to aid in studying the physiological role of the sulfate groups. We examined the effect of compounds specifically on the sulfation of the fourth component of complement (C4),

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α_2HS-glycoprotein, and proteoglycans, which, respectively, bear sulfate linked to tyrosine residues (7), N-linked oligosaccharides (4), and glycosaminoglycan chains (2).

MATERIALS AND METHODS

Carrier-free [^{35}S]sulfate and [^{3}H]leucine (40-80 Ci/mmol) were from ICN. Reagents were purchased from Sigma Chemical, except the following: $NaClO_3$ and $Na_2MoO_4 \cdot H_2O$ from EM Science, $NaBrO_3$, $NaCrO_4 \cdot 4H_2O$, CH_3PO_3H, and $NaClO_2$ from Aldrich Chemical. Antiserum to C4 and α_2HS-glycoprotein were obtained from Miles Laboratories and Atlantic Antibodies.

HepG2 cells were grown in Earle's minimal essential medium with 10% fetal calf serum. Incubations with labeled sulfate or leucine employed serum-free medium deficient in sulfate and leucine. Potential inhibitors of sulfation were prepared as neutral aqueous solutions (sodium salts of ions) except for pentachlorophenol, harmol, and β-naphthol which were prepared as concentrated solutions in alcohol. Incubations were for 5 h with 30 μCi [^{35}]sulfate or 20 μCi [^{3}H]leucine in 5 ml added to 25 cm^2 flasks. For immunoprecipitation studies, the amount of label was increased to 300 μCi sulfate or 200 μCi leucine in 10 ml added to 75 cm^2 flasks, and incubation time was increased to 15 h. Total incorporation of label into macromolecules was assessed by precipitation with cold 10% trichloroacetic acid after adding bovine albumin as carrier. The precipitate was collected on Metricel membrane filters (Gelman Sciences). Filters were dissolved and counted in 10 ml 3a70 (Research Products International). Nonspecific binding of label (zero time of incubation) was 5-10% of the total. Each experiment had a control incubation without inhibitors; incorporation per flask was determined as the percent of control. The variance of incorporation between flasks was about 12% (standard deviation). In multiple triplicate incubations, the standard deviation ranged from 2-24%. Immunoprecipitation and sodium dodecylsulfate-polyacrylamide gel electrophoresis were performed as previously described (7).

RESULTS AND DISCUSSION

Two classes of compounds-- substrates of sulfation and sulfate analogs-- were tested for inhibition of [^{35}S]sulfate incorporation into macromolecules (measured as total acid-precipitable label) secreted by HepG2 cells (Table 1). This assay of sulfate incorporation reflects predominantly sulfation of proteoglycans; about 90% of the incorporated label goes into this class of molecules (4). Parallel incubations with the compounds monitored the incorporation of [^{3}H]leucine into secreted products. This served as a sensitive indicator of toxic effects on cells and inhibition of secretion. A number of compounds among both classes inhibited sulfate incorporation without affecting leucine incorporation. Some compounds that were tested are not included in Table 1, because they were toxic to the cells at concentrations inhibiting sulfation. Among these were a few well-known inhibitors and substrates of sulfation, such as pentachlorophenol, para-nitrophenol, β-naphthol, and

Table 1. Effect of compounds on sulfate and leucine incorporation into macromolecules secreted by HepG2 cells

COMPOUND	CONCENTRATION	INHIBITION OF INCORPORATION[a] [^{35}S]SULFATE	[^{3}H]LEUCINE
Potential Substrates of Sulfation:			
Acetaminophen	2 mM	10%	-2%
Ascorbic acid	2 mM	56%	0%
Benzyl alcohol	2 mM	42%	-5%
Catechol	2 mM	73%	15%
Guaiacol	2 mM	40%	-13%
Homovanillyl alcohol	2 mM	75%	7%
Procainamide	2 mM	36%	-4%
Salicylamide	2 mM	63%	-9%
Salicylic acid	1 mM	3%	5%
Saligenin	2 mM	45%	1%
Tyramine	1 mM	49%	-2%
Tyrosine amide	2 mM	11% (7%)[b]	7%
Tyrosine methyl ester	2 mM	51% (34%)	25%
Tyrosylglutamic acid	2 mM	7% (-24%)	14%
Prolyltyrosine	2 mM	7% (-26%)	7%
Glycyltyrosine amide	2 mM	18% (-21%)	-1%
Sulfate Analogs:			
Bromate (BrO_3^-)	0.5 mM	47%	33%
Chlorate (ClO_3^-)	0.5 mM	77%	0%
Chlorite (ClO_2^-)	0.5 mM	69%	69%
Perchlorate (ClO_4^-)	0.5 mM	70%	11%
Iodate (IO_3^-)	0.5 mM	42%	18%
Methylphosphonate	2.5 mM	30%	-9%
Molybdate (MoO_4^{-2})	1.0 mM	64%	4%
Nitrate (NO_3^-)	2.5 mM	75%	5%
Tungstate (WO_4^{-2})	1.0 mM	61%	3%

[a] Incorporation of leucine and sulfate were determined as label precipitated by trichloroacetic acid. Values are expressed as percent inhibition relative to a control incubation. Negative values represent stimulation.
[b] Values in parentheses reflect incorporation of label into C4 rather than into total secretory product.

harmol (1) and some sulfate analogs such as selenate, selenite, and chromate. In several experiments, intracellular as well as secreted products were assayed, and results were very similar (not shown).

Several tyrosine derivatives and tyrosine-containing peptides were tested for selective inhibition of the sulfation of C4, which is sulfated on tyrosine residues (7). None of these compounds selectively inhibited sulfation of C4. Results are shown in parentheses in Table 1. Recent studies of the sulfation of tyrosine residues suggests why these compounds were

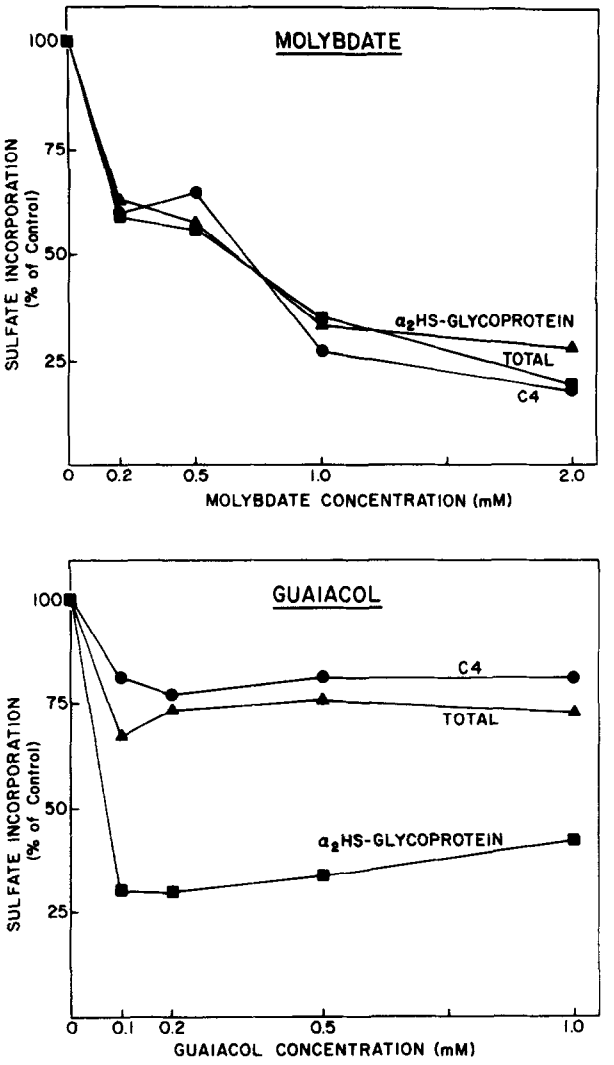

Figure 1. Inhibition of sulfation by molybdate (top panel) and guaiacol (bottom). Total incorporation of [^{35}S]sulfate into macromolecules secreted by HepG2 cells was measured as acid-precipitable product. Label in C4 and α_2HS-glycoprotein were assessed by specific immunoprecipitation from aliquots of the same culture medium.

not competitive inhibitors. Substrates of this sulfotransferase appear to require acidic amino acid residues preceding the tyrosine residue (8).

The effect of several of the more effective sulfation inhibitors on the sulfation of C4, α_2HS-glycoprotein, and proteoglycans (total incorporation) was examined. Figure 1 shows inhibition of sulfation of three types of macromolecular substrate at different concentrations of molybdate (top panel) and guaiacol (bottom panel). Both compounds inhibited sulfation of all three types of substrate, and molybdate yielded three parallel dose response curves.

Table 2. Effect of inhibitors alone and in combination on sulfation of macromolecular substrates

INHIBITOR	SUBSTRATE OF SULFATION	INCORPORATION (% OF CONTROL) [^{35}S]SULFATE	[^{3}H]LEUCINE
0.5 mM Chlorate	Total	66%	101%
	C4	74%	101%
	α_2HS-Glycoprotein	49%	102%
0.5 mM Guaiacol	Total	65%	91%
	C4	93%	104%
	α_2HS-Glycoprotein	23%	123%
0.5 mM Chlorate + 0.5 mM Guaiacol	Total	31%	96%
	C4	34%	98%
	α_2HS-Glycoprotein	10%	115%

Total incorporation of labeled sulfate and leucine were determined by precipitation of macromolecules with trichloroacetic acid. Incorporation into C4 and α_2HS-glycoprotein were determined by specific immunoprecipitation.

However, guaiacol inhibited sulfation of α_2HS-glycoprotein to a greater extent and at lower concentrations than sulfation of the other substrates. Chlorate and salicylamide (not shown) yielded results similar to those with molybdate and guaiacol, respectively. The different inhibitor profile of the two classes of inhibitor suggests action via different mechanisms. This was expected based on results of others. Small substrates of sulfation should inhibit sulfation of other molecules by depleting the intracellular pool of the sulfate donor, 3'-phosphoadenosyl-5'-phosphosulfate (1), while sulfate analogs interfere with formation of the sulfate donor (11).

The different mechanisms of the two classes of inhibitor suggested that these inhibitors could be synergistic. Combinations of the two types of inhibitor proved to be more effective at blocking sulfation and did not have toxic effects on the cells. Effects of guaiacol and chlorate alone and in combination are quantified in Table 2. Products from this experiment (Figure 2) and others have been analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Patterns of the total sulfate-labeled products secreted in the absence (lane 1) and presence of inhibitors (lanes 2-4) suggests some selectivity in the inhibition of sulfation. The intensity of some bands decreased more than others.

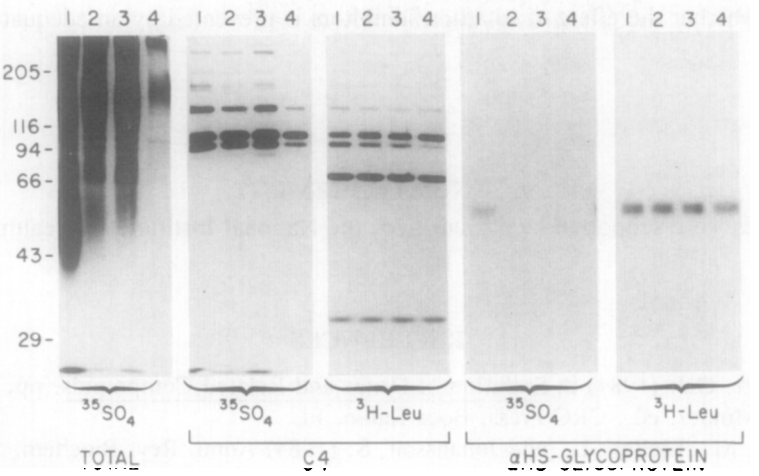

Figure 2. Effects of inhibitors of sulfation assessed by SDS-polyacrylamide gel electrophoresis. Five groups of samples are shown: Total sulfate labeled products, C4 labeled with sulfate or leucine, α_2HS-glycoprotein labeled with sulfate or leucine. Lane 1 of each group was a control incubation, Lane 2 with 0.5 mM chlorate, Lane 3 with 0.5 mM guaiacol, Lane 4 with 0.5 mM chlorate plus 0.5 mM guaiacol. The mass of molecular weight markers in kilodaltons is indicated.

Analysis of immunoprecipitates specific for C4 and α_2HS-glycoprotein showed decreased incorporation of [^{35}S]sulfate but not of [^{3}H]leucine, indicating that there was no decrease in the amount of these proteins secreted. The multiple labeled bands obtained for C4 correspond to the α-chain (2 forms about 95 kd), β-chain (75 kd), γ-chain (33 kd), pro-C4 (200 kd), and several processing intermediates (7, 11-13). Sulfate is linked only to 3 tyrosine residues in a short segment of the α-chain and of precursor forms containing the α-chain (7, 13). Huttner has suggested that sulfation of tyrosine residues may act as a secretory signal for some proteins (5). However, the present study found no evidence that secretion of C4 was affected when it was not sulfated.

Use of a synergistic combination of sulfation inhibitors in sulfate-deficient medium as described here should provide a valuable approach for examining the sulfation of macromolecules. The present study has used a single model system, a hepatocyte-derived cell line, but this approach should be generally applicable to cells in culture due to the wide distribution of phenolsulfotransferases among different cell types (1, 14). We are

examining whether the effect of sulfation inhibitors is prevented by an adequate supply of sulfate.

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