Vol. 148, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1987 Pages 493-499

EFFECT OF HYDROXYORGANOBORANES ON SYNTHESIS, TRANSPORT AND N-LINKED GLYCOSYLATION OF PLASMA PROTEINS

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Received September 3, 1987

SUMMARY. Utilizing a recently developed method (Boradeption) for transfering water-insoluble hydroxyorganoborane compounds into the cells, we observed inhibition of protein synthesis by three of these compounds and inhibition of secretion of plasma proteins by four of them in human hepatoma HepG2 cells. These effects were specific in that the cell viability was not affected and an increase in protein catabolism was not observed. Three compounds caused a compound-specific alterations in the electrophoretic mobility of secreted glycoproteins due to underlying changes in the N-linked carbohydrate moieties. Results presented suggest a potential new source of cellular probes.

Recent development of a method for solubilizing and transfering across plasma cell membrane water-insoluble derivatives of boronic or borinic acids, has made available a new array of potential cellular probes (1,2). This method Boradeption (boronic acid-dependant-phase-transfer), consists of solubilization of a water-insoluble hydroxyorganoborane (HOB) in a physiological buffer which contains a receptor group for formation of a reversible boronate-adduct (1). In the presence of a lipoidal cell membrane, the equilibrium for adduct formation is presumed to shift, and free HOB (but not the buffer carrier) enter the cells. Once inside, the distribution of many HOBs is probably determined by the lipoidal traffic flow, and the solubility and chemical properties of the agent. The latter determine the scope and extent of reversible and competitive interactions with hydroxyl groups and other electron-donating nitrogen-containing groups which are enhanced in compartments of low water concentration.

Boradeption has so far facilitated the development of a number of fluorescent vital stains, Fluoroboras, which showed distinct vital cellular staining patterns. Since some of the Fluoroboras localize to the

0006-291X/87 $1.50

area of the endoplasmic reticulum (a site of synthesis and processing of secreted proteins) and Golgi complex (a site for their processing), we tested the usefulness of HOBs as probes for various aspects of protein production. We analyzed the effects of five hydroxyorganoboranes (Fig. 1) on the synthesis, transport and processing of plasma proteins by human hepatoma cell line, HepG2. The latter was chosen because its plasma protein biosynthesic profile is well characterized (3-5).

METHODS AND MATERIALS

D, F and T compounds (Fig. 1) were obtained respectively from Aldrich, (Milwaukee, MN), Polyscience, Inc., (Warrington, PA), and Lancaster Synthesis, Ltd., (Windham, NH). P^+ was synthesized from 2,4,6-triphenylpyrylium tetrafluoroborate (Alfa Products, Danvers, MA) and 3-aminophenylboronic acid (Aldrich) according to Katritsky et al (6); P_H was prepared from P^+ by sodium borohydride reduction in ethanol. TAPSO (3-[N-tris(hydroxymethyl)-methylamino]-2-hydroxypropanesulfonic acid), sodium salt, which forms reversible and soluble adducts by interaction of its hydroxyls and secondary amino group with boronates, was obtained from Sigma, St. Louis, MO. Four mg of each HOB compound were dissolved in 100 µl of DMSO and then diluted in 1 ml of 250 mM TAPSO buffer pH 7.4 and 8.9 ml of Dulbecco's minimal essential medium (DMEM, M.A. Bioproducts, Walkersville MD).

Replicate human hepatoma HepG2 cultures were set in 16 mm wells (Costar, Cambridge, MA) and radiolabeled or pulse-chased and lysed as described before (5) and as indicated in the figure legends. DMEM containing 1% DMSO and 25 mM TAPSO buffer pH 7.4 (medium A) with (+) or without (-) methionine was used. Metabolic labeling was done with [^{35}S]methionine (sp. act. >1000 Ci/mmol; NEN, Boston MA) at 250 µCi/ml of DMEM lacking methionine (medium A-).

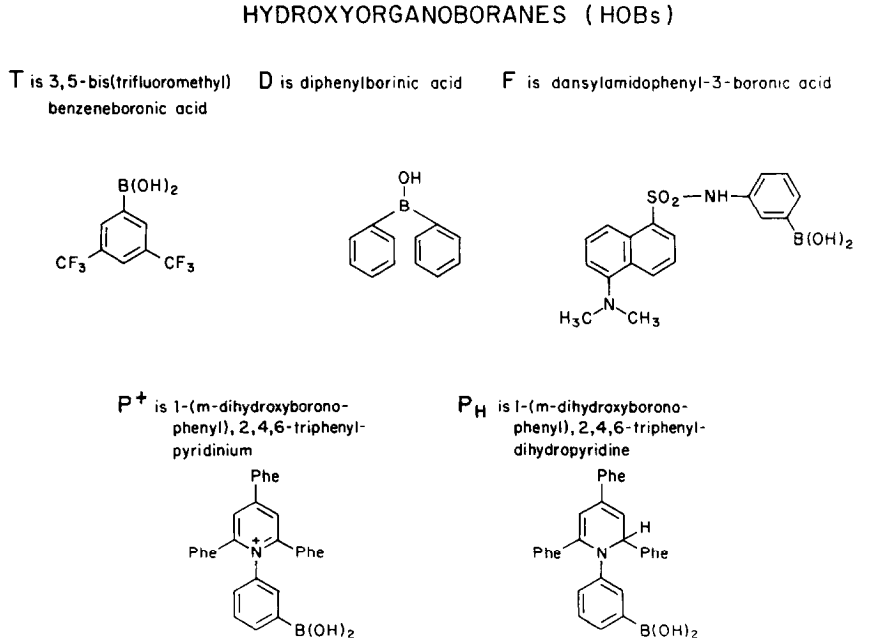


Fig. 1. Structures, formulas and codes of the HOBs used.

The immunoprecipitation was done with goat antihuman sera (Atlantic Antibodies Inc. Westbrook, MA) and IgG-Sorb (The Enzyme Center, Cambridge, MA) as described before (5). The analysis of radiolabeled proteins was done by TCA precipitation (7) or SDS/PAGE under reducing conditions according to Laemmli (8) as before (5).

Protein deglycosylation by N-glycanase treatment was done following the recommendation of the manufacturer (Genzyme, Boston, MA). One µl of 5% SDS/7% 2-mercaptoethanol was added to 9 µl of the extracellular protein in the culture media, boiled for 3 min., diluted with 0.55 M sodium phosphate buffer, pH 8.6, 0.1 M 1,10 phenanthroline in methanol and 7.5% NP-40 to a final concentration of 0.2 M, 0.01 M and 1.25% respectively. The mixtures were then incubated with or without 10 units/ml of the enzyme overnight at 37°C (9).

Protein desialidation was done as follows: One µl of the culture media was diluted 8-fold with PBS and incubated with 1 µl of 0.1 M EDTA or 1 µl of neuraminidase (100 units/ml in 0.1 M EDTA) (obtained from Sigma) overnight on ice (10).

RESULTS AND DISCUSSION

The effect of HOBs on protein synthesis was assessed by comparing the incorporation of [^{35}S]methionine into the intracellular proteins in the presence and absence of these compounds. This was done by both TCA precipitation and SDS/PAGE. A dose-dependant inhibition of protein synthesis was observed for D, F, and T (Fig. 2), but not P^+ or P_H (not shown). The onset of this inhibition was rapid since it occured within 5 minutes of incubation

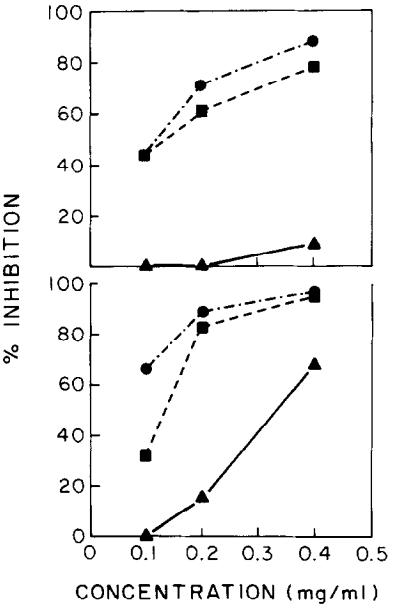
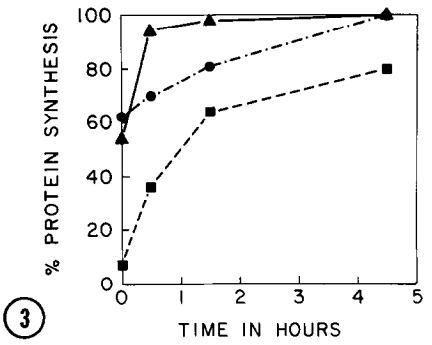


Fig. 2. Dose-dependant inhibition of protein synthesis by HOB derivatives D, F, and T. Replicate HepG2 monolayers were pulse-labeled with 200 µl of medium A- containing [^{35}S]methionine for 5 min. (upper panel) and 1 hr. (lower panel) in the presence of three doses of D (▲), F (■) and T (●). Control cultures were radiolabeled in absence of compounds. At the end of labeling the cells were rinsed and lysed in 200 ul of Dulbecco's PBS containing 1% Triton X-100 and 0.5% Deoxycholate. Protein synthesis was assessed by the incorporation of the label into TCA precipitable counts (4) and the results are expressed as percent inhibition compared to the control cultures.



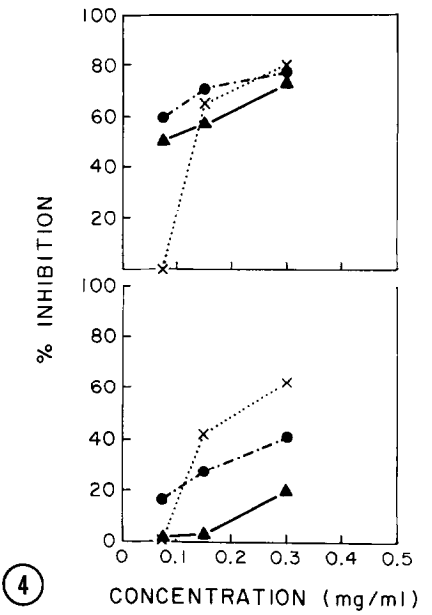


Fig. 3. Reversibility of protein synthesis inhibition by HOBs. Replicate HepG2 cultures were incubated for 1 hr with 200 µl of medium A+ in the absence or presence of 0.4 mg/ml of D (▲), F (■), or T (●) (see legend to Fig. 2). The cells were washed, incubated with medium A+ for 0, 0.5, 1.5 and 4.5 hrs. At the end of each period the cells were pulse-labeled with DMEM containing [^{35}S]methionine for 15 min. The cells were processed and the protein synthesis assessed as described in the legend to Fig. 2.

Fig. 4. Dose-dependent effect of HOBs on protein secretion. Replicate cultures of HepG2 line were pulse-labeled for 10 min. with [^{35}S]methionine in DMEM lacking methionine and then chased in medium A+ alone or with various concentrations of D (▲) and T (●). Since P_H (×) does not affect protein synthesis, its effect on protein secretion was analysed by coincubation of the compound with the medium A- containing [^{35}S]-methionine. The effect on total protein secretion at 4 (upper panel) and 8 hrs. (lower panel) was assessed by TCA precipitation. The effect on secretion of compound F was similar to that of T.

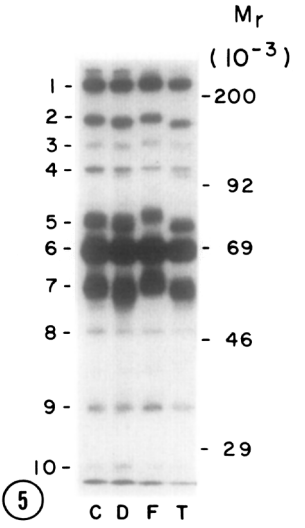
in the presence of maximal doses of T and F compounds and lasted for at least 4 hours. The inhibitory effect on the synthesis was reversible and the kinetics of recovery different for each compound (Fig. 3). This effect was also primary since viability of the cells assessed by the trypan blue exclusion was not reduced following the incubation for 4 hours with 0.4 mg/ml of each compound (data not shown).

An inhibitory effect of HOB compounds on protein secretion was observed with all the compounds except P^+. Since three of these compounds showed effect on protein synthesis (D, F and T) while two others did not (P^+ and P_H), the effect on secretion was assessed respectively by a pulse-chase experiment (for the former three) or by coincubation of HOB and the label (for the latter two). The inhibition of secretion was dose-dependant (Fig. 4) and reversible (data not shown) and observed

within one hour of incubation with the compounds. The effect on secretion was primary since the eventual recovery of the secreted proteins (after a 4 or 8 hour chase) was similar to the control (Fig. 4) and no breakdown of intracellular precursors was observed (data not shown). The inhibition of protein secretion by P^+ and not by P_H which are quite similar except that the former is charged and the latter is neutral, might be attributed to their differential distribution in the cell determined by the difference in charge.

The effect of HOBs on posttranslational processing of secreted proteins was assessed in a 10 min. pulse labeling of replicate cultures and a 4 hr. chase followed by an SDS/PAGE and autoradiography analysis. In the presence of the D, F and T (Fig. 5) but not P^+ or P_H (data not shown) alterations in the electrophoretic mobility of some secreted proteins were noted. These changes were confined to the same proteins and its direction was compound-specific. No alterations in protein mobility were observed among the proteins synthesized in the control cultures and incubated with HOBs afterwards. Among the proteins whose mobility was altered we identified transferrin and α_1-antitrypsin known to contain N-linked complex carbohydrates (11). The mobility of albumin which is unglycosylated on the other hand was not altered (12). The differences in the mobility were determined by the carbohydrate moiety of glycoproteins since a treatment with N-glycanase, a glycosidase which removes all types of N-linked carbohydrates, abrogated the differences (Fig. 6A).

There was no alteration in the mobility among proteins synthesized during a 10 min. pulse-labeling, and processed and secreted during a 20 min. chase in the presence of the compounds followed by 4 hrs. in their absence (data not shown). Also the mobilities of the intracellular precursors of transferrin synthesized during a 10 min. pulse-labeling and processed during a 1 hr. chase in the presence of these compounds were the same (data not shown). These results suggest that their effect was at the "late" steps of N-linked carbohydrate processing (12, 13). Two known inhibitors of "late" N-linked carbohydrate processing the indolizidine alkaloid swainsonine and the carboxylic ionophore monensin prevent sialidation of glycoproteins (14,15). In contrast, the effect of the D, F and T on the content of sialic acid in the glycoproteins



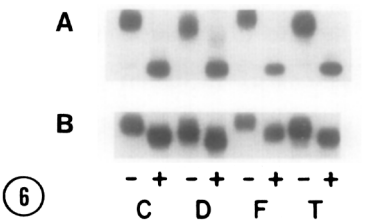


Fig. 5. Effect of HOBs on the mobility of secreted proteins. Replicate HepG2 cultures were pulse-labeled washed and chased in the absence and presence of 0.4 mg/ml of D, F and T for 4 hours (see leg. to Fig. 4). Five µl from each culture were analyzed by SDS-8% PAGE and autoradiography. The M_r of proteins corresponding to bands 1-3, 5 and 7 but not 4, 6 nor 8-10 were affected by HOBs (downwards by D and T or upwards by F). Identification of transferrin (band 5), albumin (band 6) and α_1-antitrypsin (band 7) was done by immunoprecipitation. In addition, it was determined by 2D SDS-PAGE analysis that bands 5-7 represented predominantly these specific proteins (data not shown). On the right side the position of M_r standards is shown.

Fig. 6. Analysis of the effect of HOBs on the N-linked glycosylation and sialidation of α_1-antitrypsin. Aliquots of the cultures analysed in Fig. 4 were treated (+) or not (-) with N-glycanase (A) or neuraminidase (B) and analyzed by SDS/PAGE and autoradiography. The effect of these enzymes on the M_r of α_1-antitrypsin demonstrates that the difference in mobility of these glycoproteins induced by HOBs is abrogated following a removal of the N-linked carbohydrate (A) and that the content of sialic acid does not change perceptibly as a result of incubation with HOBs (B). Similar observations were made for transferrin (data not shown).

assessed by treatment with neuraminidase was minimal if at all (Fig. 6B). Moreover, while other inhibitors of glycosylation diminish the M_r of glycoproteins, incubation with F generates glycoproteins of apparently larger size (fig. 5).

Though the mechanism of action of these compounds has not been analyzed in this study, the results presented indicate HOBs as a potential new source of cellular probes. This potential is further enhanced by considering that 1) various chemical groups can be derivatized by hydroxyboron and then solubilized by Boradeption and targetted to various lipoidal compartments in the cell and 2) hydroxyorganoboranes are not found in nature suggesting that special enzyme systems for their handling might not have evolved.

ACKNOWLEDGMENTS

We are indebted to Professor Herbert C. Brown for his continued interst in this work and for his many ideas and suggestions. We thank Edward Henson for the synthesis of the P^+ and P_H compounds and Kathy Pearson for her expert technical assistance. This work was supported by USPHS grants AG 04727, GM 33293 and AM 34369

REFERENCES

1. Gallop, P.M., Paz, M.A., and Henson, E. (1982) Science 217, 166-169.
2. Gallop, P.M., Paz, M.A., Henson, E. and Latt, S.L. (1984) BioTechniques 2, 32-36.
3. Knowles, B.B. Howe, C.C., and Aden, D.P. (1980) Science 209, 497-499.
4. Morris, K.M., Aden, D.P., Knowles, B.B., and Colten, H.R. (1982) J. Clin. Invest. 70, 906-913.
5. Goldberger, G., Arnaout, M.A., Aden, D.P., Kay, R., Rits, M., and Colten, H.R. (1984) J. Biol. Chem. 259, 6492-6497.
6. Katritsky, A.R., Lloyd, J.M., and Patel, R.G. (1982) J. Chem. Soc. Perkin Trans. 1, 117-123.
7. Roberts, B.F., and Paterson, B.M. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2330-2334.
8. Laemmli, U.K. (1970) Nature (Lond.) 227, 680-685.
9. Tarentino, A.L, Gomez, C.M., and Plummer, T.H. Jr., (1985) Biochemistry 24, 465-4671.
10. Awdeh, Z.L., and Alper, C.A. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3576-3580.
11. Lodish, H.F., Kong, N., Snider, M., and Strous, G.J.A.M. (1983) Nature 304, 80-83.
12. Yeo, K.-T., Parent, J.B., Yeo, T.-K., and Olden, K. (1985) J. Biol. Chem. 260, 7896-7902.
13. Kornfeld, R., and Kornfeld, S. (1985) Ann. Rev. Biochem. 54, 631-664.
14. Yeo, T.-K., Yeo, K.-T., Parent, J.B., and Olden, K. (1985) J. Biol. Chem. 260, 2565-2569.
15. Alonso-Caplen, F.V., and Compans, R.W. (1983) J. Cell Biol. 97, 659-680.