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SELECTIVE AND POTENT INHIBITION OF DIFFERENT HEPATIC UDP-GLUCURONOSYLTRANSFERASE ACTIVITIES BY ω,ω,ω-TRIPHENYLALCOHOLS AND UDP DERIVATIVES

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SUMMARY. A homologous series of ω,ω,ω-triphenylalcohols and corresponding ω,ω,ω-triphenylalkyl-UDP derivatives was synthesized and tested as inhibitors of UDP-glucuronosyltransferase (UGT) activity in rat liver microsomes, with 1-naphthol, testosterone and bilirubin as substrates. Introduction of the UDP moiety in the triphenylalcohols increased their inhibition potency markedly toward the isoforms which glucuronidate 1-naphthol and testosterone, but strongly decreased that toward bilirubin. The inhibiting potency of the UDP-derivatives increased as a function of the length of the hydrocarbon chain. The best inhibitor 7,7,7-triphenylheptyl-UDP showed an I_{50} of 30 and 10 μM for 1-naphthol and testosterone glucuronidation, respectively; even a 1 mM concentration of the compound had little, if any, effect on bilirubin glucuronidation. The inhibition by 7,7,7-triphenylheptyl-UDP was mixed-type toward 1-naphthol, and non competitive toward testosterone (apparent K_i 30 μM and 1.7 μM, respectively); on the other hand, the inhibition was competitive toward the common substrate UDP-glucuronic acid (apparent K_i 1.9-1.2 μM). In addition, 7,7,7-triphenylheptyl-UDP (0.25-0.50 mM) almost inhibited glucuronidation of 1-naphthol and testosterone catalyzed by the recombinant rat liver UGT-2B1 and human liver UGT-1A1, whose cDNA has been expressed in V79 cells. In conclusion, the data indicate that 7,7,7-triphenyheptyl-UDP interacted competitively with the UDP binding site of UGT. The results also indicate that it is possible to design transition state analogue inhibitors with specificity for different UGT forms.

INTRODUCTION. UDP-glucuronosyltransferase (UGT, EC 2.4.1.17) is a multigenic family of membrane-bound enzymes involved in the detoxication of drugs and endogenous compounds such as bilirubin (1). They catalyze the binding of glucuronic acid from UDP-glucuronic acid (UDPGA) to a large variety of structurally unrelated compounds with hydroxyl-, carboxyl-, amino- and sulfhydryl groups, leading to the formation of water-soluble ß-(D)-glucuronides (2). The glucuronidation reaction involves the interaction of UDPGA and the aglycon (acceptor substrate) with a protein domain until now little explored. Since inhibitors are useful tools to probe the active site of enzymes, a series a of ω,ω,ω-triphenylalkylcarboxylic acids was recently designed which drammatically decreased bilirubin glucuronidation (3). The apparent K_i value of 7,7,7-triphenylheptanoic acid for the microsomal and the purified liver enzymes were 12.1 and 1.6 μM, respectively (4).

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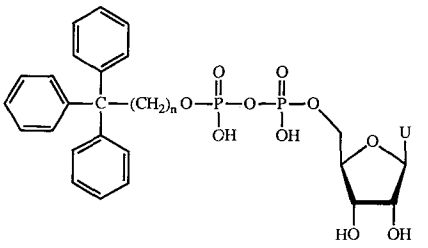

Fig. 1. General structure of ω,ω,ω-triphenylalkyl-UDP derivatives. n=1, 2, 4, 6.

In addition, possible transition state analogues, consisting of a UDP and an acceptor substrate moiety were synthesized (5) and tested as inhibitors (6), which could provide information concerning the organization of both the UDPGA and the aglycon binding sites. The best inhibitor of this series was 2,2,2-triphenylethyl-UDP which inhibited glucuronidation of phenols (harmol, 3,3',5-triiodothyronine) and *N*-hydroxy-2-acetylaminofluorene in isolated rat hepatocytes (7).
In order to improve both the selectivity and the efficiency of this type of inhibitors, a series of chemically related w,w,w-triphenylalkyl-UDP compounds was synthesized (Fig.1) and tested on the glucuronidation of 1-naphthol, testosterone and bilirubin supported by various UGT isoforms.

MATERIALS AND METHODS. **Synthesis of the inhibitors.** 2,2,2-Triphenylacetic acid and 3,3,3-triphenylpropionic acid (Aldrich Chimie, Strasburg, France) were easily reduced to their corresponding primary alcohols with borane-dimethylsulfide complex in dry tetrahydrofurane (THF), by heating at 66°C (8). The intermediate products were hydrolyzed by methanol. 2-(2,2,2-triphenylethoxy)ethanol was prepared by base catalyzed (NaH) reaction of 2,2,2-triphenylethanol with methylbromoacetate in THF at 60°C, followed by reduction ($LiAlH_4$) of methyl 2,2,2-triphenylethoxyacetate in anhydrous diethyl ether.
7,7,7-Triphenylheptanol was prepared *via* a three-step procedure: (i) The hydroxyl group of 6-chlorohexanol was protected with a tetrahydropyranyl function. (ii) LiCl/CuCl catalyzed reaction in dry THF of the thus obtained tetrahydropyranyl acetal with triphenylmethyllithium, synthesized according to Hellerman (9), gave protected 7,7,7-triphenylheptanol. Finaly acid hydrolysis gave the target compound which was purified by silica gel column chromatography. 5,5,5-Triphenylheptanol was synthesized in the same way, with 4-chlorobutanol as the starting material. The UDP-derivatives were synthesized as previously described (5).
All compounds synthesized gave satisfactory ^1H and ^{31}P NMR, infrared spectral and analytical data.
Enzyme assays. Male wistar rats (180-200 g, Iffa-Credo, St. Germain l' Abresle, France) were used. They were housed in an environmentally controlled room (12h light cycle, 22-24°C) and fed a rodent chow (UAR Alimentation, Villemoisson, France). After one week, they were killed by decapitation. Liver microsomes were prepared by differential ultracentrifugation according to Hogeboom (10). The microsomes were suspended in 1 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, and were stored until use at -20°C. The protein content was measured according to the technique of Bradford (11), with bovine serum albumin as standard. Glucuronidation of testosterone and 1-naphthol was measured on fully activated microsomes according to the method of Bock *et al.* (12), and Rao *et al.* (13), respectively. Bilirubin glucuronidation was measured according to Heirwegh *et al.* (14) on digitonin-activated microsomes.
V79 chinese hamster lung fibroblasts were transfected with a recombinant plasmid carrying the cDNA UGT_r-2, which encodes a phenobarbital-inducible rat UGT-2B1 expressed predominantly in the liver (15), and a vector including a neomycine resistance gene (16). This recombinant UGT form was chosen, as we showed that it was able to catalyze both the glucuronidation of 1-naphthol and testosterone (see results). The expression in V79 cells of the human liver cDNA encoding a UGT-1A1 which glucuronidates 1-naphthol, but not testosterone, was performed as described by Fournel-Gigleux *et al.* (16).

The inhibitors were dissolved in dimethylsulfoxide and added to rat liver microsomes or V79 cell homogenates at the final concentrations 10^{-6} to 10^{-3} M, 1 min before addition of the substrate. Controls without inhibitors were always run to represent 100% enzyme activity. I_{50} values were determined using the linear portion of the semilogarithmic plots of inhibition data. Apparent K_i were calculated from secondary plots of apparent K_m/V_{max} versus 4 to 5 concentrations of inhibitors. The best fitting lines were obtained by linear least-squares regression analysis.

RESULTS AND DISCUSSION. The compounds synthetized corresponded to the series of the triphenylalkanols with an increasing number of methylene groups. 2-(2,2,2-triphenylethoxy) ethanol was also prepared by substituting the carbon number 3 in 5,5,5 triphenylpentanol with an oxygen atom, since ethylene glycol-containing compounds were found to be effective and competitive inhibitors (17). The inhibitory values in term of I_{50} of these compounds and of their corresponding UDP-derivatives on the glucuronidation of bilirubin, 1-naphthol and testosterone, in rat liver microsomes are indicated in Table 1. The alcohols could inhibit glucuronidation of bilirubin, whatever the length of the aliphatic chain, whereas, interestingly, the UDP-derivatives failed to decrease the activity. By contrast, while the alcohols did not inhibit 1-naphthol glucuronidation, introduction of UDP led to potent inhibitory compounds. This effect markedly increased with the length of the aliphatic chain. Testosterone glucuronidation was also strongly inhibited by the UDP-derivatives, whatever the type of alcohol used. However, whereas 2,2,2-

Table 1: Comparison of the inhibitory effect of ω,ω,ω-triphenylalcohols and their corresponding UDP analogues on UDP-glucuronosyl-transferase activities

Inhibitors	I_{50} (μM)		
	1-naphthol	testosterone	bilirubin
Ph3C-CH2OH	>>1000 (75)	63	360
Ph3C-CH2-O-UDP	**446**	**141**	**>>1000 (88)**
Ph3C-CH2-CH2OH	1000	112	180
Ph3C-CH2-CH2-O-UDP	**501**	**71**	**>>1000 (117)**
Ph3C-(CH2)4-OH	>1000	>1000	190
Ph3C-(CH2)4-O-UDP	**30**	**22**	**>>1000**
Ph3C-(CH2)6-OH	>1000	>1000	360
Ph3C-(CH2)6-O-UDP	**30**	**10**	**>>1000**
Ph3C-CH2-O-CH2-CH2OH	>1000 (100)	1780	160
Ph3C-CH2-O-CH2-CH2-O-UDP	**200**	**40**	**>>1000 (62)**

The percentage of activity for the glucuronidation of 1-naphthol, testosterone and bilirubin remaining at 1 mM concentration of inhibitors is indicated between parentheses. Ph3 represents a triphenylmethyl group.

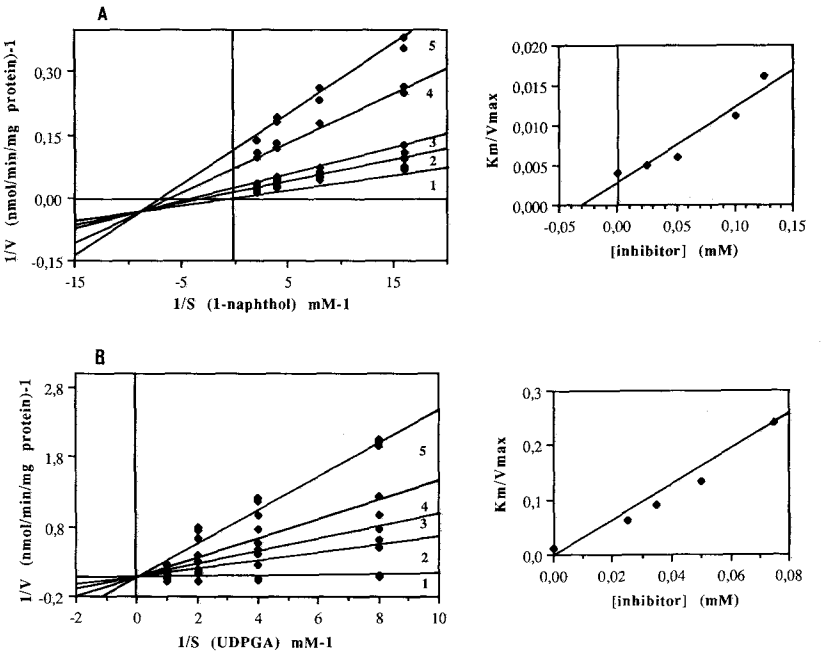

Fig. 2. Kinetics of inhibition of 1-naphthol glucuronidation by 7,7,7-triphenylalkyl-UDP. Rat liver microsomes activated with Triton X-100 were incubated in the presence of different concentrations of inhibitors. In A, the concentration of UDP-glucuronic acid was kept constant (4 mM) and the 1-naphthol concentration varied from 0 mM (graph 1) to 0.025, 0.05, 0.1, 0.125 mM (graphs 2, 3, 4, 5, respectively). In B, the 1-naphthol concentration was fixed at 0.5 mM and that of UDP-glucuronic acid varied from 0 mM (graph 1) to 0.075, 0.05, 0.035 and 0.025 mM (graphs 2, 3, 4, 5, respectively). The secondary plots K_m/V_{max} used for the calculation of apparent K_i are also indicated. The data represent the mean of triplicate measurements. The best-fitting lines were obtained by linear least-squares regression analysis.

triphenylethanol and 3,3,3-triphenylpropanol inhibited the reaction, alcohols with a longer aliphatic chain (2-(2,2,2-triphenylethoxy)ethanol, 5,5,5-triphenylbutanol and 7,7,7-triphenylhexanol) failed to decrease the activity (Table 1). The UDP-derivatives were very powerful inhibitors of the glucuronidation of 1-naphthol and testosterone; they were much more potent than those reported by Noort *et al.* (6), suggesting that the inhibitors may interact with higher affinity. Determination of the type of inhibition of the most potent inhibitor 7,7,7-triphenylheptyl-UDP was investigated by variing both the concentrations of 1-naphthol and UDPGA (Fig. 2). Mixed-type inhibition was found when 1-naphthol was the varied substrate, since both V_{max} and K_m were affected (apparent K_i 30 μM), but was competitive when the UDPGA concentration varied (K_i 1.9 μM), thus suggesting that the inhibitor competes with the UDP-sugar for the same binding site. Toward testosterone, a non competitive inhibition was found when the hormone was the varied substrate, (apparent K_i 1.7 μM), since only the V_{max} was affected (results not shown). Thus, the inhibition was much more effective than toward 1-naphthol. Like for 1-naphthol, the inhibition was competitive toward the binding of the common substrate UDPGA with a similar apparent K_i (1.2 μM).

The data clearly indicated a differential susceptibility of the isozymes considered in this study toward the inhibitors. A striking difference was observed with the bilirubin isozyme whose

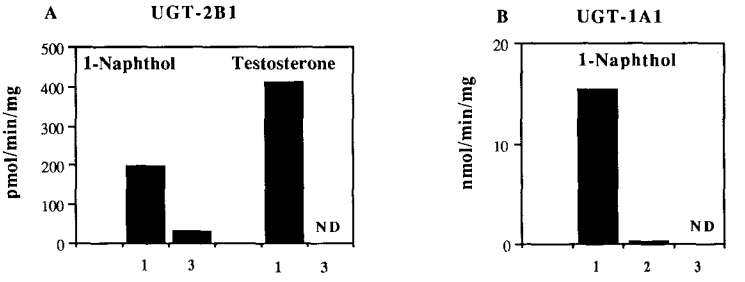

Fig. 3. Inhibitory effect of 7,7,7-triphenylheptyl-UDP on recombinant UGTs.
V79 cells lines were transfected according to (16) by a cDNA encoding a phenobarbital-inducible rat UGT-2B1 that glucuronidates 1-naphthol and testosterone (A), and by a cDNA encoding a human liver UGT-1A1 that glucuronidates phenolic compounds only (B). 1, control without inhibitor; 2, 3 with inhibitor 0.25 and 0.50 mM, respectively. ND, not detectable. The specific activities are the means of 3 to 4 determinations, the variation coefficient was less than 10%.

activity was no longer decreased upon introduction of the UDP moiety in the triphenylalcohols (Table 1). Apparently the UDPGA binding site of the UGT that converts bilirubin on one hand and 1-naphthol or testosterone on the other, possesses a different orientation toward the aglycon binding site. In this respect, it is interesting to note that triphenylalkylsulfate and -phosphate derivatives failed also to inhibit UGT bilirubin (18). This result suggests that a hydrophilic and/or negatively charged group with a very low pK_a can be less adequately accomodated in the active site of the enzyme protein than the arylalkylcarboxylic acids previously used (4) that are weaker acids or than the triphenylalkanols of this study.

Since microsomes represent a mixture of several isoforms, some of them presenting overlapping specificity, it was tempting to test 7,7,7-triphenylheptyl-UDP on the activity of recombinant UGTs. Fig. 3 (A) shows the inhibitory effect of 7,7,7-triphenyl-UDP on the glucuronidation of 1-naphthol and testosterone by the recombinant phenobarbital-inducible rat UGT-2B1. At the concentration of 0.5 mM, the activity toward 1-naphthol and testosterone was reduced to a similar extent (84 %, up to 100 %, respectively). At 0.25 mM, this inhibitor also decreased by 96 % the glucuronidation of 1-naphthol specifically catalyzed by the human liver UGT-1A1 expressed in V79 cell lines (Fig. 3 B). 7,7,7-triphenylheptyl-UDP could be a powerful tool to investigate the binding site of the UGT-1A1 which is involved in the glucuronidation of planar phenolic compounds in humans (16).

In conclusion, highly efficient inhibitors of UGT were synthesized, which appear to interact with the UDP binding site of the proteins that catalyze the glucuronidation of 1-naphthol and testosterone, but not with that involved in the glucuronidation of bilirubin. It is therefore possible to design inhibitors with specificity for different UGT forms.

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