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TOXICITY OF URICOSURIC DIURETICS IN RAT HEPATOCYTE CULTURE

KEITH G. TOLMAN,* PHILLIP D. GRAY, SOUHEIR EL MASRY, ROBERT R. LUTHER and ROBERT S. JANICKI

Division of Gastroenterology and Clinical Pharmacology, Department of Internal Medicine, University of Utah School of Medicine, Salt Lake City, UT; and Abbott Laboratories, Abbott Park, IL, U.S.A.

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Abstract—Several aryloxyacetic acid diuretics have shown hepatotoxicity in humans, yet there continues to be interest in developing these compounds because of the uricosuric properties of some of them. This study was designed to test the utility of the hepatocyte monolayer culture as a model for studying these compounds. In addition, an attempt was made to define the structural components that are common to hepatotoxicity. Ticrynafen, indacrinone, ethacrynic acid and A-49816, an investigational compound, were found to be toxic in hepatocyte cultures; thus, with the exception of indacrinone, paralleling the experience in humans. The toxic compounds share a ketodichlorophenoxyacetic acid chemical structure. A-56234, an investigational uricosuric, was also found to be toxic in cultures but has not been demonstrated to be hepatotoxic in humans in limited clinical experience. It does not possess the ketodichlorophenoxyacetic acid structure proper but may be metabolized to a closely related structure. Furosemide, which does not have the ketodichlorophenoxyacetic acid structure, was not toxic in hepatocyte cultures and has not been hepatotoxic in humans. Thus, the structure common to the toxic compounds is ketodichlorophenoxyacetic acid or a closely related compound. The hepatocyte monolayer system appears to be a good model for demonstrating toxicity and, perhaps, for predicting toxicity of new compounds under development.

Since the discovery of ethacrynic acid more than 20 years ago, several other aryloxyacetic acid derivatives have been synthesized and studied for diuretic activity. Ticrynafen, one of the derivatives, was marketed as a uricosuric diuretic but was withdrawn from the U.S. market because of the rare occurrence of fatal hepatotoxicity [1, 2]. Indacrinone, another aryloxyacetic acid derivative, was studied extensively for its uricosuric diuretic activity, but its development was interrupted for unclear reasons. Ethacrynic acid has also been shown, on rare occasions, to be hepatotoxic in humans [3]. All of these potentially hepatotoxic compounds have a common ketodichlorophenoxyacetic acid structure, as shown in Fig. 1.

Because of the utility of this class of compounds as potential uricosuric diuretics, they continue to be developed. Efforts are being made to predict their potential hepatotoxicity [4] and to determine the mechanism of their toxicity [5]. The hepatotoxicity of many drugs has been studied successfully in rat hepatocyte culture [6–11], but it has been stated that ticrynafen, the classic drug in this group, is not toxic in hepatocyte culture [4]. In this report, several aryloxyacetic acid derivatives and a non-aryloxyacetic acid derivative (furosemide) were examined to determine their relative toxicity in hepatocyte culture. Of the aryloxyacetic acid derivatives examined, four compounds (ethacrynic acid, indacrinone, ticrynafen and A-49816) have the ketodichlorophenoxyacetic acid structure, while one, A-56234, does not have this structure. The study was designed to examine the association between the presence of the ketodichlorophenoxyacetic acid structure and toxicity in hepatocyte cultures. The structures of the tested compounds are shown in Fig. 1 along with the ketodichlorophenoxyacetic acid structure which is common to the known hepatotoxic diuretics.

* Correspondence: Dr. Keith G. Tolman, Division of Gastroenterology, University of Utah Medical Center, Salt Lake City, UT 84132.

METHODS

Hepatocytes were isolated from healthy adult male Sprague–Dawley rats using a modification of the method of Berry and Friend [12] and Bissell *et al.* [13]. In brief, the livers were perfused *in situ* with Hanks' balanced salt solution containing 0.05% crude collagenase (Cooper Biochemical Corp., Malvern, PA). The parenchymal cells were washed and separated from nonparenchymal cells, and then suspended in Waymouth MB 752 culture medium (Sigma Chemical Co., St. Louis, MO) containing 50 mg/ml of gentamicin (Schering Corp., Kennelworth, NJ). Cell viability of greater than 90% was confirmed by the trypan blue exclusion test [14, 15]. Suspensions of 2.5×10^6 cells were then placed on collagen coated 60 × 15 mm plastic petri dishes and allowed to form a monolayer in a humidified incubator at 37° in an atmosphere of 5% CO_2 and 95% air.

Preliminary experiments demonstrated that, in general, the compounds were more toxic in hepatocytes that were only a few hours old. However, the results were highly variable and difficult to reproduce. The most reproducible results were obtained at 24 hr—presumably a time when the cells were less metabolically active but more stable. Thus,

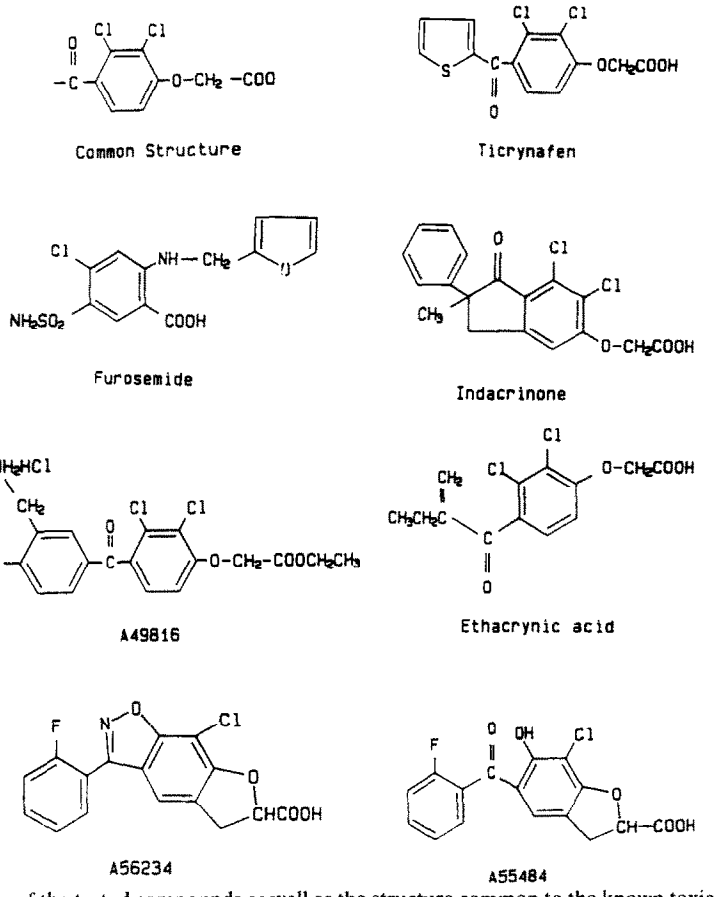

Fig. 1. Structures of the tested compounds as well as the structure common to the known toxic uricosuric drugs.

the 24-hr time was used for all subsequent studies. Following 24-hr incubation and stabilization, the supernatant fraction was aspirated and the hepatocyte cultures were treated with fresh Waymouth medium containing the test agents. Each of the test agents was added to the Waymouth solution in concentrations ranging from 0 to 1500 μg/ml. Most of the drugs had to be solubilized in either ethanol or dimethyl sulfoxide (DMSO) in concentrations known to be nontoxic in the hepatocyte cell culture system. All test drugs were provided by Abbott Laboratories.

Following a 6-hr incubation of each compound in the cell culture, the supernatant fraction was aspirated and passed through a 3 micron Millipore filter. The remaining hepatocytes in the petri dish were lysed with 2.5 ml of 1% Triton X-100, scraped from the plate, sonicated for 30 sec and then centrifuged for 10 min at 9000 g. Lactate dehydrogenase (LDH) in the filtered medium and in the cell supernatant fraction was measured using the method of Wroblewski and LaDue [16]. An LDH index (the ratio of LDH in the medium to the total LDH in the medium plus the cellular supernatant) was derived to evaluate the severity of hepatocyte injury [11, 15]. Previous experiments demonstrated that none of the test compounds affected either the LDH assay or the total amount of LDH activity in the system.

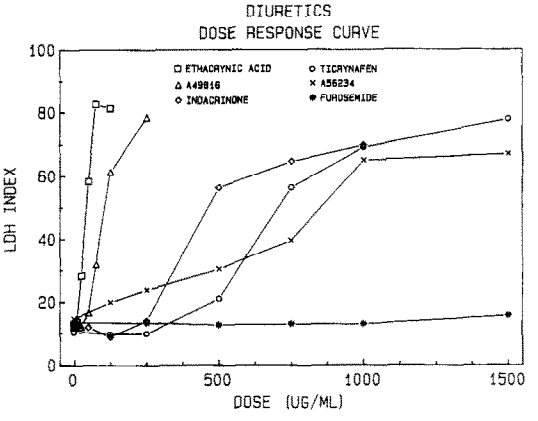

Fig. 2. Relative toxicity of the tested compounds.

RESULTS

The dose–response curves of the various compounds, as reflected by the LDH index, are shown in Fig. 2. Ethacrynic acid and A-49816 were, by far, the most toxic compounds in hepatocyte culture. Furosemide, compatible with its clinical performance, had no detectable toxicity. Ticrynafen,

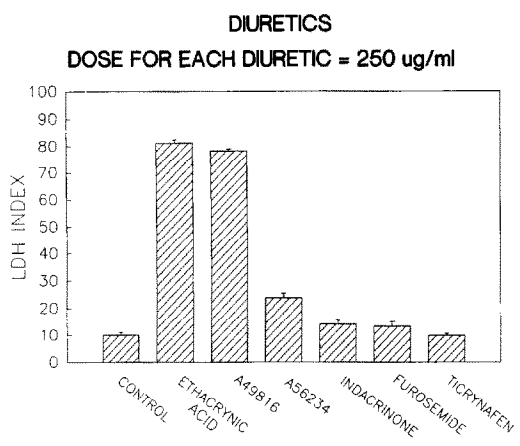

Fig. 3. Relative toxicity of each of the compounds at a concentration of 250 μg/ml. Note that the concentration of ethacrynic acid was 125 μg/ml because it demonstrated maximum toxicity at that concentration. Values are expressed as LDH index ± SEM.

indacrinone and A-56234 had comparable toxicity. The comparison of these compounds, at a concentration of 250 μg/ml, is illustrated in Fig. 3. This again demonstrated that ethacrynic acid and A-49816 were the most toxic compounds.

DISCUSSION

A previous attempt by Acosta *et al.* [4] to demonstrate ticrynafen toxicity in rat hepatocytes was unsuccessful. In the present study, ticrynafen was toxic in rat hepatocytes. The experimental conditions in the Acosta study differed from the present conditions and could have been responsible for the difference in results. Thus, in the Acosta study, neonatal rats, in which the drug-metabolizing enzyme levels may have been different, were used as the source of hepatocytes. If, as suggested earlier [17], ticrynafen hepatotoxicity is accentuated or is demonstrable only following metabolism, the neonatal cells used in the Acosta study may not have had the ability to generate the hepatotoxic species. Also, in the Acosta study, the hepatocytes were purified by using a medium which would not support fibroblast growth rather than by filtration. The effect of that medium on hepatocyte function and drug metabolism was not known. Finally, the concentrations of drug used in the Acosta study were on the low side. Previous experience with hepatocyte cell cultures suggests that relatively high doses need to be used to reproduce an injury comparable to that seen in humans. In any case, it appears that the current hepatocyte monolayer culture system is useful for studying uricosuric agents.

Furosemide, in the present study, was not toxic to the hepatocyte cultures. Unlike the other compounds which are all aryloxyacetic acid derivatives, furosemide is a furan derivative. The furan ring has been reported to be metabolically activated and, in high doses, to be hepatotoxic [18]. Mitchell *et al.* [19] have shown furosemide to cause dose-related midzonal necrosis in rats. The reason for the differences in results has yet to be determined. There have been reported cases of jaundice in humans due to furosemide [20]; however, hepatic necrosis has not been reported.

Ticrynafen, ethacrynic acid, indacrinone, A-49816 and A-56234 were all toxic in hepatocyte culture. The first two are known to be hepatotoxic in humans. A-49816 has been tested in Phase I studies in humans. One volunteer developed elevated liver enzymes which recurred upon rechallenge. The structure common to all four of these compounds is shown in Fig. 1 and is characterized by the three following features: (1) an oxyacetic acid side chain, (2) dichloro atoms, and (3) a fixed keto function opposite the constrained oxyacetic acid group. A-56234, however, is only remotely related to the other four compounds in that it does not possess the ketodichlorophenoxyacetic acid structure proper. Nevertheless, it has a chemical structure which is not dissimilar from the ketodichlorophenoxyacetic acid structure in that it has a rigid oxyacetic acid constrained in the furan ring and a monochloro substituent on the benzofuran ring. Moreover, in whole animals, A-56234 is metabolized to A-55484 (Fig. 1) unpublished observations) which has a ketomonochlorophenoxyacetic acid structure isosteric with the ketodichloro structure common to ethacrynic acid, ticrynafen, indacrinone and A-49816. If one assumes that in culture the hepatocytes metabolize A-56234 to A-55484, one may conclude that either the ketodichloro- or the closely related ketomonochlorophenoxyacetic acid structure is involved in the toxicity of hepatocytes by this class of diuretics.

Ethacrynic acid, paradoxically, appears to be the most toxic compound in the cell culture system while probably being the least toxic in humans. The specific reason for this is not known, but it is known that the cell culture system, in general, is not a very good predictor of the relative toxicity of compounds other than parent and metabolites of the same drug. The lack of predictability between compounds may be a reflection of the varying rates of loss of enzymatic functions as the cells remain in culture [21]. This also is probably the explanation for the need to use relatively high drug concentrations in this system.

The mechanism of toxicity remains unknown at this time. Ahokas *et al.* [5] have suggested that glutathione *S*-transferase inhibition may underlie the toxicity. Preliminary data from our laboratory suggest that the toxicity is more complex than can be explained by glutathione *S*-transferase inhibition alone. In any case, it appears that rat hepatocytes may be a useful model for studying the toxicity of these compounds and perhaps for predicting their hepatotoxicity in humans.

REFERENCES

1. Ticrynafen recall. *FDA Drug Bulletin* **2**: 3–4, 1980.
2. Simpson FD and Waal-Manding HJ, Total ban on tienilic acid (letter) microsomal metabolism of furosemide. Evidence for the nature of the reactive intermediate involved with covalent binding. *Lancet* **1**: 978–979, 1980.
3. Datey KK, Deshmukh SN, Dalvi CP and Purandare

NM, Hepatocellular damage with ethacrynic acid. *Br Med J* **3**: 152–153, 1967.
4. Acosta D, Mitchell DB, Santone KS, Bock A and Lewis W, Lack of cytotoxicity of ticrynafen in primary cultures of rat liver cells. *Toxicol Lett* **10**: 385–388, 1982.
5. Ahokas JT, Davies C, Ravenscroft PJ and Emmerson BT, Inhibition of soluble glutathione *S*-transferase by diuretic drugs. *Biochem Pharmacol* **33**: 1929–1932, 1984.
6. Anuforo DC, Acosta D and Smith RV, Hepatotoxicity studies with primary cultures of rat liver cells. *In Vitro* **14**: 981–988, 1978.
7. Acosta D, Anuforo DC and Smith RV, Cytotoxicity of acetaminophen and papaverine in primary cultures of rat hepatocytes. *Toxicol Appl Pharmacol* **53**: 306–314, 1980.
8. Mitchell DM and Acosta D, Evaluation of the cytotoxicity of tricyclic antidepressants in primary cultures of rat hepatocytes. *J Toxicol Environ Health* **7**: 83–92, 1981.
9. Tolman KG, Peterson P, Gray P and Hammar SP, Hepatotoxicity of salicylates in monolayer cell cultures. *Gastroenterology* **74**: 205–208, 1978.
10. Sanders SW, Dukes GE Jr, Gray P and Tolman KG, Toxicity of heparin in isolated rat hepatocytes. *Biochem Pharmacol* **33**: 2223–2226, 1984.
11. Kingsley E and Tolman KG, Hepatotoxicity of valproic acid and other anticonvulsants in rat hepatocyte culture. *Epilepsia* **21**: 699–704, 1980.
12. Berry MN and Friend DS, High-yield preparation of isolated rat liver parenchymal cells. *J Cell Biol* **43**: 506–520, 1969.
13. Bissell DM, Hammaker LE and Meyer UA, Parenchymal cells from adult rat liver in non-proliferating monolayer culture. *J Cell Biol* **59**: 722–734, 1973.
14. Tolani S, A method for viable cell count. *Tissue Culture Assoc Manual* **1**: 37–39, 1975.
15. Jaurgui HO, Hayner NT, Driscoll JL, Williams-Holland R, Lipsky MH and Galeti PM, Trypan blue dye uptake and lactate dehydrogenase in adult rat hepatocytes—Freshly isolated cells, cell suspension and primary monolayer cultures. *In Vitro* **17**: 1100–1109, 1981.
16. Wroblewski F and LaDue JS, Lactic dehydrogenase activity in blood. *Proc Soc Exp Biol Med* **90**: 210–213, 1955.
17. Zimmerman HJ, Abernathy CO, Lukacs L and Ezekiel M, Effects of ticrynafen on hepatic excretory function in the isolated perfused rat liver. *Hepatology* **2**: 255–257, 1982.
18. Wirth PJ, Betts CJ and Nelson WL, Microsomal metabolism of furosemide. Evidence for the nature of the reactive intermediate involved in covalent binding. *Mol Pharmacol* **12**: 759–768, 1976.
19. Mitchell JR, Nelson WL, Potter WZ, Sasame HA and Jollow DJ, Metabolic activation of furosemide to a chemically reactive hepatotoxic metabolite. *J Pharmacol Exp Ther* **199**: 41–52, 1976.
20. Dargie HJ and Dollery CT, Adverse reactions to diuretic drugs. In: *Meyler's Side Effects of Drugs* (Ed Dukes MNG), Vol. 8, pp. 483–591. Excerpta Medica, Amsterdam, 1975.
21. Guzelian PS, Bissell DM and Meyer UA, Drug metabolism in adult rat hepatocytes in primary monolayer culture. *Gastroenterology* **72**: 1232–1239, 1977.