Reactivity and toxicity of atracurium and its metabolites *in vitro*

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Cytotoxicity of atracurium and of its metabolites was tested in vitro. *Exposure of isolated rat hepatocytes to atracurium produced cellular damage evidenced by extrusion of an intracellular enzyme, lactate dehydrogenase (LDH), into the incubation medium. Leakage of LDH was directly related to the concentration of atracurium in the medium (250 to 800 μM). If the spontaneous degradation of atracurium (presumably via Hofmann elimination) was first carried out* in vitro *and the degradation products subsequently added to the isolated hepatocytes, the leakage of LDH was also dose-dependent but larger than that observed after the addition of the parent drug. When l-cysteine was admixed to the products of the spontaneous degradation of atracurium prior to their addition to the liver cells, no leakage of LDH was observed. The results are compatible with the working hypothesis that atracurium itself and, even more so, acrylates formed in Hofmann elimination of atracurium, are reactive toward nucleophiles and damage the cells by alkylating nucleophiles present in cellular membranes. Antecedent covalent binding of acrylates to the nucleophile cysteine, i.e., the formation of acrylate-cysteine adducts, saturated the reactive capacity of acrylates for nucleophiles and thus prevented the reactive metabolites from alkylating the endogenous nucleophiles. Possible clinical consequences resulting from* in vivo *generation of reactive metabolites are not clear at the present time but are projected to be related to (a) the dose of atracurium administered, (b) the amount of acrylates generated, (c) the functional importance of the endogenous nucleophiles alkylated, and (d) the pathway and the speed of detoxification of atracurium and its metabolites.*

Key words
BIOTRANSFORMATION (DRUG): atracurium; NEUROMUSCULAR RELAXANTS: atracurium; TOXICITY: atracurium, acrylates.

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Metabolic degradation was not a prominent pharmacologic feature of the older, non-depolarizing muscle relaxants. An older drug, pancuronium, and the more recently introduced agents atracurium and vecuronium are, however, subject to metabolic degradation. This characteristic is most prominent in atracurium since the drug was deliberately synthesized to undergo spontaneous degradation *in vivo*.[1] Chemical instability of atracurium was achieved by incorporation of two reverse ester groups into an old and metabolically stable relaxant, laudexium[1-3], (Figure). In addition to being susceptible to hydrolytic degradation, the reverse ester groups destabilize the bond between the quaternary nitrogen and the vicinal carbon atom in the aliphatic chain. Thus, both the originally conceived Hofmann elimination[1] and the more recently proposed nucleophilic substitution reaction[4] may occur *in vivo* (Figure). Both pathways are interesting from a toxicologic point of view. When either reaction occurs *in vivo*, covalent bonds with endogenous nucleophiles may be formed either directly in a reaction with atracurium, i.e., in the nucleophilic substitution reaction, or indirectly in a reaction with its metabolites, i.e., by conjugation with acrylates formed in Hofmann elimination of atracurium (Figure). Since the alkylation of nucleophiles can lead to their functional inactivation, atracurium or its metabolic products may lead to impaired cellular function.

Previous experiments have indicated that the outlined mechanisms may be responsible for the deleterious effects of atracurium on isolated rat hepatocytes.[5,6] However, the question whether atracurium itself or a product of its spontaneous degradation was the culprit was not answered. In the present experiments we tested the hypothesis that the higher chemical reactivity of the products of Hofmann elimination would be manifested as cytotoxicity that is greater than that observed with the parent drug. In addition, we postulated that the reactive capacity of the metabolites could be satiated *in vitro* in a reaction with an exogenous nucleophile. If so, then the exposure of the hepatocytes to the preformed metabolite-nucleophile conjugates (or adducts) would produce only minimal or no cytotoxicity. Cysteine was selected as the test nucleophile.

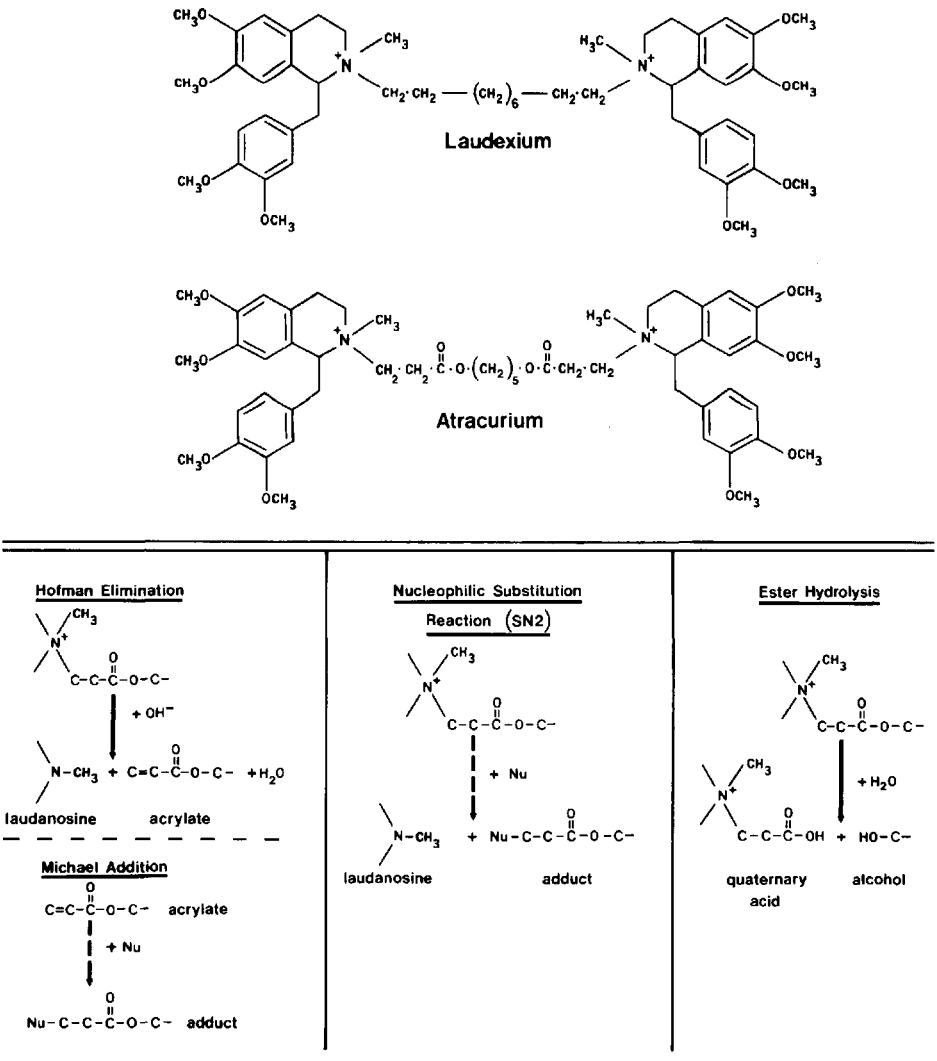

FIGURE Atracurium and the postulated degradation and reaction pathways. The upper half of the figure presents the structures of atracurium and laudexium. The three panels in the lower half present the previously suggested (solid arrows) and the presently postulated (dashed arrows) degradation pathways. Only the relevant part of the atracurium molecule is presented with bonds to hydrogen atoms omitted. Incomplete bonds connect this part of the atracurium molecule to carbon atoms of atracurium or its metabolites. In the left panel, acrylate ester – formed by Hofmann elimination – is postulated to alkylate a nucleophile (Nu) and to form an adduct with it. In the middle panel, a direct formation of the adduct is postulated to occur via a substitution of a nucleophile for the laudanosine moiety (SN2 reaction). In the right panel, the destruction of the ester group is illustrated to occur via ester hydrolysis.

Methods

Animal experiments were performed in compliance with all state and federal guidance, ordinances and laws in our animal research facility.

Isolated hepatocytes were harvested from six male Wistar rats (body weight 87 ± 7g (mean ±SE)) and incubated as previously described[5,6] using the method of Klaunig *et al.*[7] In brief, the liver and the portal vein were surgically exposed in anaesthetized rats (pentobarbitone, 60 mg · kg^{-1} IP). Perfusion of the liver via the portal vein was carried out in two stages; first, with a calcium-free solution and, subsequently, with L-15 medium containing collagenase (0.15 Uml^{-1}). One million hepatocytes, suspended in 3 ml of the L-15 medium (Gibco Laboratories) containing fetal bovine serum (0.1 ml ml^{-1} of medium), were plated to a dish. Viability of the cells, as determined by the trypan blue exclusion test, was 90 ± 1 per cent. The initial incubation of the cells was carried out for either two or five hours to allow the cells to attach to the floor of the culture dishes. Different durations of the attachment period were necessitated by the experimental protocol (*vide infra*). Following attachment the medium was replaced with fresh L-15 medium (5 ml per dish) containing no fetal bovine serum but containing either atracurium or its metabolites. Separate dishes containing no additives were included in each experiment. The dishes were incubated (at 36.5° C and pH 7.4) for four hours. At the end of incubation the cell-free medium was collected for the determination of lactate dehydrogenase (LDH). In each experiment the maximal amount of LDH releasable by the destruction of all the cells in a dish (cell lysis accomplished with Triton X-100, 50 μl per dish) was measured in three separate dishes containing no additives.

Table I presents the details on the incubation of atracurium that was carried out prior to the addition of the solutions to hepatocytes. The desired amounts of atracurium from fresh, refrigerated ampoules commercially available for clinical use (Tracrium, Burroughs Wellcome Co., 10 mg ml^{-1}) were diluted with L-15 medium (pH 7.4 37° C). The medium did not contain fetal bovine serum. Five diluted solutions of atracurium (0 to 800 μM) were thus prepared. Samples of each solution were immediately added to dishes containing hepatocytes attached for two hours. Each solution was added to three dishes. All dishes exposed to different concentrations of atracurium immediately after the addition of atracurium to the incubation medium are denoted experimental group A in Table I. The remainders of each of the five diluted solutions of atracurium were incubated for 120 minutes at 37° C and pH 7.4 (measured with a glass electrode). Afterwards, each diluted solution of atracurium was divided into two portions to which either saline (denoted experimental group B in Table I) or saline and *l*-cysteine (experimental group C) were added. The added volumes were small (1 per cent of the original volume). The final concentration of cysteine in group C was 3.0 mM. After an additional 60 minutes of incubation (37° C, pH 7.4), samples from the ten solutions (2 sets each comprised of five dilutions of atracurium) were added to the dishes containing hepatocytes attached for five hours. Five milliliters of each dilution were added to each of three dishes. Subsequently, the experiments were carried out in a manner identical to that described above.

TABLE I Schematic representation of the incubation of atracurium before the solutions were added to hepatocytes. The sequence was repeated with each of 5 solutions containing various concentrations of atracurium. See Methods for additional details

Experimental group	*Incubation of atracurium: L-15 medium; pH 7.4, 37° C; duration: 120 min*	*Continued incubation: L-15 medium; pH 7.4, 37° C; duration: 60 min*
A	No	No
B	Yes	Yes, no cysteine present
C	Yes	Yes, cysteine concentration 3 mM

The amount of LDH released from the hepatocytes into the medium by the end of incubation was taken as the quantitative expression of cytotoxicity of the compounds present in the medium.[8,9] The release was expressed as percent of the total amount of LDH released from hepatocytes lysed with Triton X-100. Since the variance for the total releasable LDH within each experiment was significantly smaller than the variance among the experiments, each experiment was evaluated using its estimate for the total releasable LDH. In addition, since the Bartlett's test[10] indicated inhomogeneity of variances among the groups exposed to various initial concentrations of atracurium, we have performed logarithmic transformation of all the percentage values. The transformation established homogeneity of variances among the treatment groups. The influence of incubation of atracurium on the release of LDH was examined in a two-way analysis of variance using the data from groups A and B. Incubation of atracurium prior to the addition to hepatocytes (performed or not) and the initial concentration of atracurium were the two independent variables. Percentages of LDH released from hepatocytes exposed to the products of atracurium degradation (groups B and C) were analyzed in an analogous way except that the concentration of atracurium and the presence of cysteine prior to the addition to hepatocytes (present or not) were the two independent variables. The results in Table II represent the reconverted values (antilogarithms) of the means for

TABLE II Leakage of LDH expressed in percent of the total amount of LDH present intracellularly. Indicated are mean values and the corresponding 95% confidence intervals in parentheses. Each concentration of atracurium in each experimental group was tested in 3 dishes prepared from each of 6 rats (n = 18). See Table I and Methods for experimental details.

Experimental group	*Initial concentration of atracurium (μM)*				
	0	*253*	*435*	*617*	*800*
A	12.4 (11.4–13.6)	18.1 (16.5–19.8)	28.1 (25.7–30.7)	34.7 (31.7–37.9)	42.3 (38.7–46.3)
B	7.99 (7.07–9.03)	27.1 (24.0–30.6)	45.8 (40.5–51.7)	60.4 (53.5–68.3)	75.4 (66.8–85.2)
C	7.73 (6.84–8.73)	9.15 (8.10–10.3)	8.89 (7.87–10.0)	9.26 (8.19–10.5)	8.42 (7.45–9.51)

the percent of LDH released and their 95 per cent confidence intervals.

Results

The amount of LDH extruded from isolated hepatocytes (Table II) exposed neither to atracurium nor to its metabolites was slightly lower when the medium was incubated for 180 minutes (groups B and C) than when the medium was not incubated (group A). The presence of cysteine (3.0 mM) did not influence the leakage of LDH from the hepatocytes (comparison of groups B and C in the absence of atracurium).

When atracurium was added to the medium and the medium immediately added to hepatocytes (group A), the leakage of LDH increased as the concentration of atracurium increased ($P < 0.001$ for the comparison with cells not exposed to atracurium). When the medium containing atracurium was incubated for 180 minutes prior to addition to hepatocytes (group B), the leakage of LDH from hepatocytes was even higher ($P < 0.001$ for the comparison of LDH leakage in groups B and A). When cysteine was added during the third hour of incubation of atracurium in the L-15 medium, i.e., prior to the addition of the medium to the hepatocytes (group C), LDH leakage produced by atracurium metabolites was completely abolished ($P < 0.001$ for the comparison of LDH leakage between groups B and C). Leakage of LDH from hepatocytes exposed to solutions containing atracurium metabolites and cysteine did not differ among the dishes exposed to different concentrations of atracurium nor did the leakage in these dishes differ from the leakage observed in dishes not exposed to atracurium.

Discussion

Assuming (a) that the leakage of LDH from isolated hepatocytes signifies the destruction of the isolated cells, and (b) that atracurium degrades spontaneously via Hofmann elimination at 37° C and pH 7.4, our results provide support for the working hypothesis that (1) the products of the spontaneous degradation of atracurium are more damaging to the isolated cells than the parent compound, and (2) the conjugation of the metabolites with a nucleophile abolishes the cytotoxicity of the metabolites.

In the present experiments fetal bovine serum was deliberately omitted from the final incubation medium for two reasons: first, the nucleophiles present in the serum might have acted as scavengers for the reactive metabolites and, second, the hydrolases present in the serum might have diverted the degradation of atracurium to pathways other than Hofmann elimination. Due to the short incubation time of the hepatocytes (four hours), the omission of the fetal bovine serum from the incubation medium did not reduce the survival of the isolated hepatocytes. It must be stressed, however, that the L-15 medium itself contains many amino acids, including cysteine (1 mM). The composition of this medium has proved optimal for cell survival and therefore we could not totally exclude cysteine or other nucleophiles from the final incubation medium.

In the absence of atracurium, the difference in LDH release between the experimental group A (no incubation of the medium) and the groups B and C (incubation of the medium carried out without or with cysteine, respectively) was small but statistically significant ($P < 0.05$). The difference was probably due to different durations of the attachment periods, two hours in group A and five hours in groups B and C.

We selected the conditions for incubation of atracurium in L-15 medium so as to mimic the conditions *in vivo* that were postulated to be conducive to spontaneous degradation of atracurium.[1] This degradation pathway (Figure, Hofmann elimination) leads to formation of (a) laudanosine, a chemically inert product, and (b) acrylates, which are electrophilic and hence highly reactive. Chemical reactivity of acrylates generated from atracurium was previously demonstrated *in vitro* as a rapid consumption of two sulfhydryl groups of cysteine for one molecule of

atracurium.[11] Laudanosine did not react with the mercapto groups of cysteine. To explain the observed cytotoxicity of the metabolites of atracurium we propose that atracurium was spontaneously degraded during incubation and, when added to hepatocytes, the metabolites alkylated the nucleophiles present in the membranes of the hepatocytes (Figure, Michael addition). The alkylation is postulated to have produced functional inactivation of the nucleophiles and, as a consequence, rupture of cell membranes and extrusion of the intracellular contents. Since laudanosine did not show reactivity toward nucleophiles,[11] we propose that the reactive products are various acrylate species. The thesis that the reactivity was the principal chemical characteristic of the metabolites responsible for the cellular damage is supported by the results of the experiments in which cysteine was added to the incubation solutions of atracurium (experimental group C). We propose that the addition of cysteine to the metabolites of atracurium resulted in formation of acrylate-cysteine adducts (conjugates) during the third hour of incubation (Figure, Michael addition, Nu = the thiol group of cysteine). These thioaether adducts are non-reactive and, therefore, their addition to hepatocytes did not exert a cytotoxic effect. Since the supply of cysteine was adequate, conjugation with cysteine satisfied the chemical avidity of acrylates for nucleophiles and so protected the nucleophiles on, or in, the membrane of the hepatocytes.

When atracurium was diluted in the incubation medium and the medium immediately added to the hepatocytes (group A), the cells were presumably exposed to atracurium as well as to its degradation products formed during the incubation. The results of this test demonstrated that the cytotoxicity was similar to that observed in previous experiments.[5,6] The toxicity, however, was not as extensive as that observed when the preformed products of the spontaneous degradation of atracurium were added to hepatocytes (group B). The higher cytotoxicity of the metabolites in comparison with the cytotoxicity of the parent drug indicates (1) that the metabolites are more reactive than the parent drug, and/or (2) that the amount of the reactive metabolites generated from atracurium was decreased in the presence of hepatocytes. It is plausible to postulate that hydrolases, extruded into the medium from the damaged hepatocytes, degraded atracurium to products other than the reactive acrylates. High activity of one of these enzymes was previously reported in the rat.[12] Since this enzyme (carboxylesterase) hydrolyzes atracurium[13,14] as well as acrylates[15], enzyme-catalyzed hydrolysis exerted a protective effect. This explanation is in agreement with previously reported results that the inhibition of the enzyme potentiates the cytotoxic effect of atracurium on isolated rat hepatocytes.[6]

A comparison of cytotoxicity of atracurium and its metabolites with that of the known hepatotoxins previously evaluated by other investigators using isolated hepatocytes is difficult. Since even a minor methodologic detail might conceivably alter the quantitative expression of toxicity of a xenobiotic, only a direct comparison would yield truly comparable data. Even so, it is remarkable that the known experimental hepatotoxin, bromobenzene,[16] produces cell death and the release of LDH under conditions (rat hepatocytes incubated for four hours in 0.75 mM bromobenzene) which are similar to our results with 0.8 mM atracurium. A comparison of our data with those obtained with acetaminophen is tenuous. Although the results with isolated hepatocyes (e.g., LDH release) are cited in support of the proposal that the hepatotoxicity from an overdose of acetaminophen occurs via a reactive metabolite, a higher concentration of acetaminophen and a longer incubation are required[17,18] to produce LDH release comparable to that observed in our experiments with atracurium and its metabolites. At the end of 60 min exposure, halothane (0.4 mM) only doubles the release of LDH from the isolated rat hepatocytes.[19] The increase of LDH is smaller than the release of LDH by atracurium metabolites that we have observed at the end of four hours of incubation with a similar concentration of atracurium. We would like to reserve the final conclusion regarding the hepatotoxic effects of atracurium and its metabolites relative to those of the known hepatotoxins for the appropriately conducted experiments.

The high initial concentrations of atracurium used in our experiments were deliberately selected since one of the purposes of the study was to test for the protective effect of a nucleophile. These high concentrations of atracurium are never encountered in clinical practice. Our results can, however, be directly translated to clinical situations since the outlined reactions may occur *in vivo* as well as *in vitro*. If the degradation of atracurium in patients proceeds via Hofmann elimination – and there are many reports supporting this notion – acrylates will be formed. Their fate *in vivo* and the consequences of their presence in the organism will depend on the amount of acrylates formed, the site of formation, and on the pathways and the rapidity of detoxification. The amount of acrylates formed will depend not only on the dose of atracurium but also on the pathway of degradation, since only Hofmann elimination leads to their formation (Figure). The site of formation of the reactive metabolites will determine whether the conjugation will occur with a mobile scavenger, e.g., glutathione, or with a structurally and/or functionally important nucleophile. Whereas the former reaction may be considered the initial step in the detoxification process via the mercapturic acid pathway,[20] the latter reaction might possibly lead to undesired

consequences. Finally, detoxification of acrylates may also proceed via enzyme-catalyzed hydrolysis to non-reactive acrylic acid.[15]

We believe that the consequences of *in vivo* formation of electrophilic acrylates from small clinical doses of atracurium are not likely to lead to clinically manifest changes of physiologic variables like heart rate or blood pressure. Rather, the changes will be subtle and more likely documented in biochemical tests. Since enzyme-catalyzed hydrolysis of atracurium is not pronounced in humans,[14,21] degradation via Hofmann elimination assumes a prominent role. Detoxification of acrylates formed in this process may be interfered with if (1) anticholinesterase agents, used in the reversal of muscle paralysis produced by atracurium, also inhibit the hydrolase responsible for hydrolysis of atracurium and acrylates, or if (2) drugs administered concomitantly with atracurium depend on, and consume, glutathione in the course of their detoxification. There are at present no clinical reports available to confirm or to reject these theoretical concerns. Future clinical research needs to be directed specifically toward answering these questions.

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Résumé

La cytotoxicité de l'atracurium et de ses métabolites a été étudiée in vitro. *L'exposition d'hépatocytes de rat à l'atracurium a produit des dommages cellulaires tel qu'exprimé par la libération de déhydrogénase lactique (LDH), dans le médium d'incubation. La quantité de LDH libérée était directement reliée à la concentration de l'atracurium dans le médium (250 à 800 μM). Après dégradation de l'atracurium* in vitro *et addition des produits de la dégradation aux hépatocytes, la libération du LDH était aussi dépendante de la dose mais bien supérieure à celle observée après l'addition de l'atracurium lui-même. Quand la l-cystéïne a été additionnée au produit de dégradation de l'atracurium avant de les présenter aux hépatocytes, aucune libération de LDH ne fut observée. Ces résultats sont compatibles avec l'hypothèse que l'atracurium elle-même ainsi que ses métabolites sont réactives sur les nucléophiles et peuvent endommager les cellules. L'addition de L-cystéïne a atténué l'affinité des acrylates pour les nucléophiles et a prévenu ainsi le dommage cellulaire. Les conséquences cliniques possibles de cette génération* in vivo *de métabolites réactifs ne sont pas encore élucidées mais peuvent être la conséquence de (a) la dose d'atracurium administrée, (b) la quantité d'acrylates générée, (c) l'importance fonctionnelle des nucléophyles endogènes alkylés, et (d) la voie et la vitesse de détoxification de l'atracurium et de ses métabolites.*