Biochemical Pharmacology, Vol. 42, No. 5, pp. 1137–1142, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00
Pergamon Press plc

COMPARATIVE CYTOTOXIC EFFECTS OF ACETAMINOPHEN (*N*-ACETYL-*p*-AMINOPHENOL), A NON-HEPATOTOXIC REGIOISOMER ACETYL-*m*-AMINOPHENOL AND THEIR POSTULATED REACTIVE HYDROQUINONE AND QUINONE METABOLITES IN MONOLAYER CULTURES OF MOUSE HEPATOCYTES

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(*Received* 18 *December* 1990; *accepted* 4 *May* 1991)

Abstract—Toxic effects of acetaminophen (paracetamol, *N*-acetyl-*p*-aminophenol, APAP) in monolayer cultures of mouse hepatocytes developed over a period of 18 hr. *N*-Acetyl-*m*-aminophenol (AMAP) was approximately 10-fold less toxic than APAP, despite the fact that it bound covalently to a greater extent to hepatocyte macromolecules. AMAP did not deplete glutathione to as great an extent as APAP, indicating that their reactive metabolites may bind to different proteins or that oxidative damage in addition to arylation of proteins may be involved in the development of cell death. The toxicity of 3-methoxy-acetyl-*p*-aminophenol was similar to that of APAP, whereas the other hydroquinone and quinone metabolites were 8–10 times more cytotoxic than APAP. The potencies of these analogs were in the order: acetyl-*m*-aminophenol-*p*-benzoquinoneimine ≥ 2,5-dihydroxyacetanilide ≥ 3-methoxy-acetyl-*p*-benzoquinone ≥ *N*-acetyl-*p*-benzoquinone imine (NAPQI) ≥ acetyl-*m*-aminophenol-*o*-benzoquinone ≥ 3-hydroxy-acetyl-*p*-aminophenol. The relative toxic potencies of the hydroquinone and quinone metabolites of AMAP were comparable to that of NAPQI, and do not readily explain the marked difference between the cytotoxic effects of AMAP and APAP.

Acetaminophen (paracetamol, *N*-acetyl-*p*-aminophenol, APAP) is widely used as a non-prescription analgesic and antipyretic, both as a single agent and as a component of several multiple-drug formulations. It is generally recognized that high acute doses of APAP may cause hepatic necrosis in both man and laboratory animals [1–3]. Recent studies also indicate that long term exposure of man to high therapeutic doses of APAP is correlated with increased risk of chronic renal disease [4]. APAP-induced liver tumors in mice [5] and bladder carcinomas in rats [6] have been reported. Furthermore, genotoxic effects of APAP have been found *in vitro* [7–9], in laboratory animals [10] and in man [11–14]. Whereas the genotoxic effects of APAP may be due to a direct effect of APAP on ribonucleotide reductase [15], the cytotoxic effects are most probably mediated by a reactive metabolite of APAP.

Initially, the reactive metabolite of APAP was believed to result from oxygenation of the drug to either *N*-hydroxy or 3,4-epoxy-paracetamol followed by dehydration to the electrophile *N*-acetyl-*p*-benzoquinone imine (NAPQI) [16–18]. More recent studies indicate a direct two-electron oxidation of APAP to NAPQI by cytochrome P450 or, alternatively, a one-electron oxidation to *N*-acetyl-*p*-benzosemiquinone imine by peroxidase, prostaglandin H synthase or cytochrome P450 [19, 20].

NAPQI can both bind covalently to and oxidize thiols [21]. One or both of these properties may be relevant to its high cytotoxicity [21–23]. The further development of the cytotoxic effects seems to be associated with a disruption of intracellular Ca^{2+}-homeostasis caused by the interaction of NAPQI with hepatocyte thiols [24].

Acetyl-*m*-aminophenol (AMAP) is a regioisomer of APAP reported to possess analgesic and antipyretic properties in mice [25]. However, in contrast to APAP, AMAP is apparently not hepatotoxic [26] and does not cause any inhibition of replicative DNA synthesis [27].

In the present study we compared the cytotoxic effects of APAP, AMAP and their reactive hydroquinone and quinone metabolites (see Fig. 1 for structures) in monolayers of mouse hepatocytes in order to explain the difference in cytotoxic effect of the two regioisomers (APAP and AMAP).

MATERIALS AND METHODS

Chemicals. [Ring UL-^{14}C]AMAP and APAP were purchased from Pathfinder Laboratories Inc. (St Louis, MO, U.S.A.) and tested for purity (>98%) as described previously [28]. Other compounds, 3-hydroxy-acetyl-*p*-aminophenol (3-OH-APAP) [29], 2,5-dihydroxy-acetanilide (2,5-diOH-AA)[30], 3-methoxy-acetyl-*p*-aminophenol (3-MeO-APAP)

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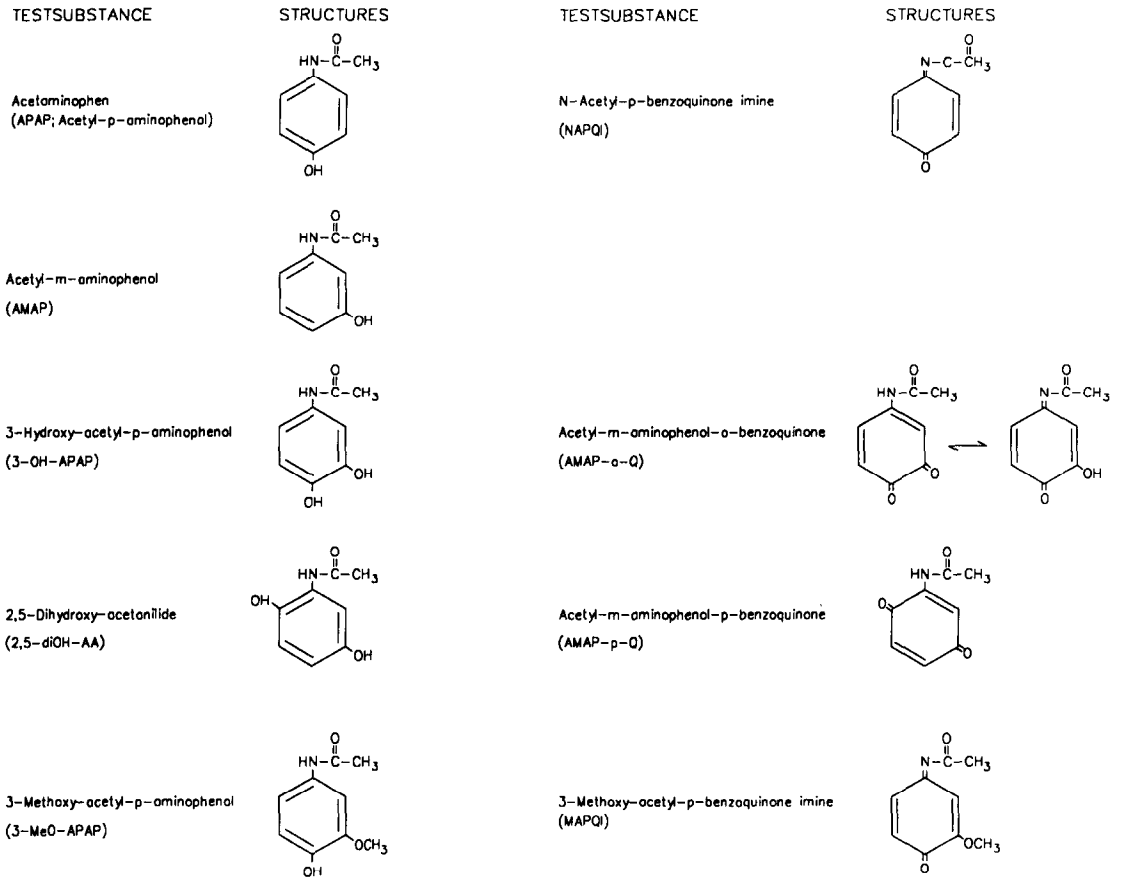

Fig. 1. Structures of acetaminophen (APAP), acetyl-*m*-aminophenol (AMAP) and several hydroquinone and quinone metabolites.

[29], *N*-acetyl-*p*-benzoquinone imine (NAPQI) [31], acetyl-*m*-aminophenol-*p*-benzoquinone (AMAP-p-Q) [32], acetyl-*m*-aminophenol-*o*-benzoquinone (AMAP-o-Q) [33] and 3-methoxy-acetyl-*p*-benzoquinone (MAPQI) [34] were synthesized as described. APAP, bovine serum albumin (BSA V), dexamethasone, insulin and trypan blue were from the Sigma Chemical Co. (St Louis, MO, U.S.A.); AMAP was obtained from the Aldrich Chemical Co., Inc. Milwaukee, WI, U.S.A.) then recrystallized from water prior to use; collagenase (CLS II) was from the Wortington Biochemical Corp. (Freehold, NJ, U.S.A.); horse serum and Dulbecco's modified Eagle medium without cysteine were from the National Institute of Public Health, Oslo, Norway; and fetal bovine serum was from Gibco (Grand Island, NY, U.S.A.). Other chemicals were commercial p.a. grade.

Animals. Male Swiss mice (CRL-CD/I(ICR)BR;20–30 g) were given standard pelleted feed from RMI(E), Special Diet Services (U.K.) and water *ad lib.*

Isolation and culture of hepatocytes. Hepatocytes were prepared by the two-step collagenase perfusion method [35], as described elsewhere [18]. The perfusion rate was 8 mL/min. The viability determined by trypan blue exclusion was always above 85% before seeding the cells. Hepatocytes were incubated as monolayer cultures (1.5×10^6 cells/dish) in 60 mm dishes containing 3 mL Dulbecco's modified Eagle medium without cysteine and supplemented with δ-aminolevulinic acid 17 μg/mL, asparagine 0.5 mg/mL, leucine 0.17 mg/mL, insulin 4×10^{-8} M, dexamethasone 2.6×10^{-7} M, penicillin 100 units/mL, streptomycin 0.1 mg/mL and mycostatin 60 units/mL. When seeding the cells, the medium contained 15% horse serum and 2.5% fetal calf serum [36]. After 2 hr, the cells were exposed to test substance (dissolved in 0.5% dimethyl sulphoxide immediately before exposure) in medium with 1% BSA.

Cytotoxicity. The incubation was terminated by placing the cells on ice and viable cells were measured as cells excluding trypan blue.

Glutathione (GSH). Intracellular levels of GSH were estimated by the method of Tietze [37], as described previously [18].

Covalent binding of APAP and AMAP to cellular macromolecules. Monolayers of mouse hepatocytes were exposed to [^{14}C]APAP or [^{14}C]AMAP (1, 2 or 3 mM, 500 cpm/nmol) in hepatocyte medium with 1% BSA for 1, 2 or 3 hr. After exposure the cells were washed in buffer without BSA, precipitated with 10% trichloroacetic acid, methanol and ethanol-ether (1:1, v/v) and dissolved in NaOH. The amount

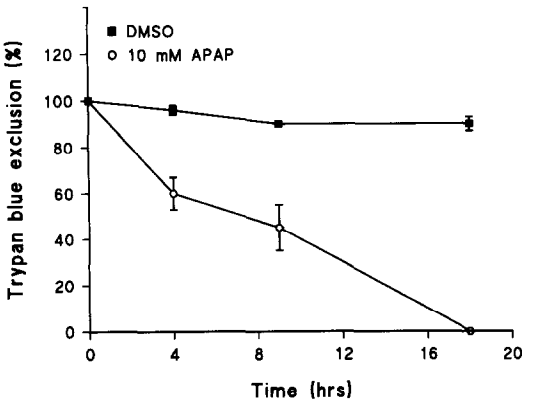

Fig. 2. Time course of APAP-induced cytotoxicity. Monolayer cultures of mouse hepatocytes were exposed to APAP or DMSO for 3, 8 and 18 hr and viable cells were measured as per cent cells excluding trypan blue. The data are means ± SD of three incubations.

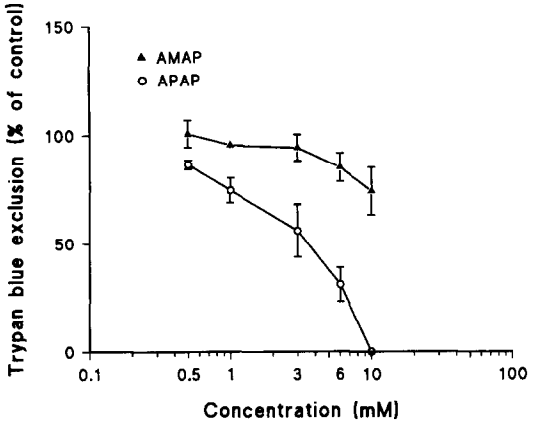

Fig. 3. Concentration-dependent cytotoxicity of APAP and AMAP. Monolayer cultures of mouse hepatocytes were exposed to various concentrations of APAP and AMAP for 18 hr. Cytotoxicity is expressed as per cent viable cells of DMSO controls. The data are means ± SD of three different experiments.

of radioactivity remaining was determined by liquid scintillation counting and protein was measured according to the method of Lowry *et al.* [38]. In all determinations zero-time values were subtracted.

RESULTS

The data in Fig. 2 show that a significant toxic effect was observed after exposing monolayer cultures of mouse hepatocytes to 10 mM APAP for 3 hr. After 18 hr exposure, all the hepatocytes were dead.

The toxicities of APAP and AMAP were compared in monolayer cultures of mouse hepatocytes after 18 hr of exposure (Fig. 3). Concentrations of APAP of 1.0 mM and higher caused cytotoxic effects, with LC_{50} about 3.0 mM. By contrast, cytotoxic effects of AMAP were first noted at a concentration of 10 mM.

To compare the extent of covalent binding of the two drugs, hepatocytes were exposed to 3 mM of either [^{14}C]APAP or [^{14}C]AMAP for 1, 2 or 3 hr; or to 1, 2 or 3 mM of the drug for 3 hr (Fig. 4). Both concentration- and time-dependent increases in covalent binding were observed. The covalent binding of AMAP was 2-fold greater than that of APAP. If the cells were exposed to 3 mM APAP for 3 hr and the drug then washed out, toxicity would develop after 18 hr. During the 3 hr exposure period significant covalent binding of both APAP and AMAP occurred. Preliminary results indicate that there was no apparent loss of binding of neither APAP nor AMAP, 15 hr after removing the drug (data not shown).

The toxicities of several of the hydroquinone and quinone metabolites of AMAP were compared with the reactive APAP metabolite NAPQI in order to explain, if possible, the difference between APAP and AMAP (Fig. 5). The cytotoxicity of 3-methoxyacetyl-*p*-aminophenol (3-MeO-APAP) was of the same order as that of APAP, whereas the other hydroquinone and quinone metabolites were 8–10 times more potent than APAP. The potencies of these analogs were in the order: AMAP-p-Q ≥ 2,5-diOH-AA ≥ MAPQI ≥ NAPQI ≥ AMAP-o-Q ≥ 3-OH-APAP.

Experiments were then carried out to determine the effects of APAP and AMAP on intracellular concentrations of GSH. As can be seen from Fig. 6, both APAP and AMAP caused a concentration-dependent decrease in GSH levels to approximately 8 and 45% of control values after 3 hr exposure to 10 mM of APAP and AMAP, respectively.

DISCUSSION

When compared to APAP, AMAP is less hepatotoxic to mice *in vivo* [17] and to mouse hepatocytes *in vitro* [Fig. 3]. Despite this decreased toxicity, the reactive metabolites of [^{14}C]AMAP bind covalently to cellular macromolecules as extensively as [^{14}C]APAP in hamsters [39, 40] and in mice [28], and even more extensively in experimental systems with microsomes [30] and mouse hepatocytes (Fig. 4). Furthermore, the covalent binding of AMAP seems to persist like that of APAP (data not shown).

In contrast to APAP, AMAP cannot be directly oxidized to a reactive quinone imine. However, AMAP is metabolized to at least three different proximate reactive metabolites, 2,5-diOH-AA, 3-OH-APAP and 3-MeO-APAP, which form the reactive metabolites, AMAP-p-Q, AMAP-o-Q and MAPQI, respectively (Fig. 1) [34]. Two of the proximate reactive metabolites of AMAP, 3-OH-APAP and 3-MeO-APAP, are also metabolites of APAP [29]. The toxicity of 3-MeO-APAP is in the same order as that of APAP, both to the mouse liver *in vivo* [29] and to mouse hepatocytes exposed *in vitro* (Fig. 5). In contrast, 3-OH-APAP has been reported to be essentially nonhepatotoxic *in vivo*, whereas in mouse hepatocytes it was as toxic as NAPQI (Fig. 5). Furthermore, the other proximate reactive metabolite of AMAP, 2,5-diOH-AA, was

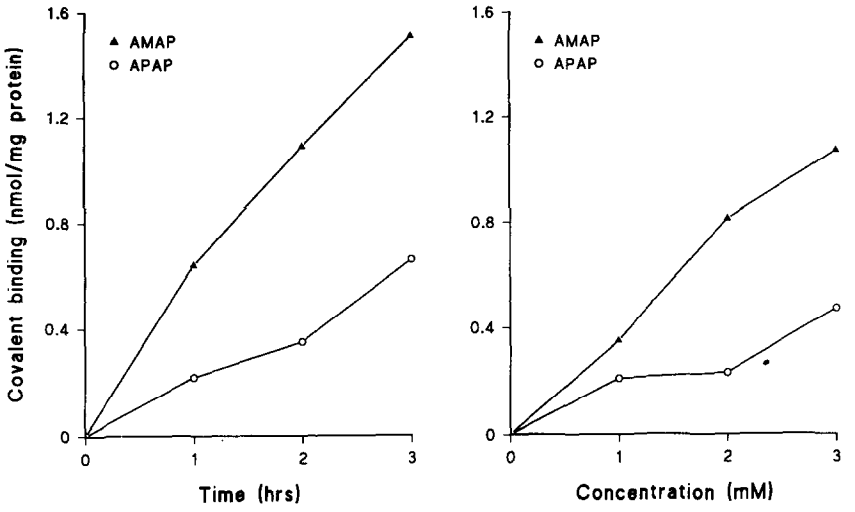

Fig. 4. Time course and concentration-dependence of [^{14}C]APAP and [^{14}C]AMAP covalent binding to macromolecules in monolayers of mouse hepatocytes. The concentration of test substance was 3 mM and incubation was for 3 hr in the time course and concentration-dependence experiments, respectively. Values represent two typical experiments. The means ± SD of [^{14}C]APAP and [^{14}C]AMAP (3 mM; 3 hr incubation) covalently bound to macromolecules in hepatocytes isolated from three different mice were 0.54 ± 0.08 and 1.24 ± 0.19 nmol/mg protein, respectively.

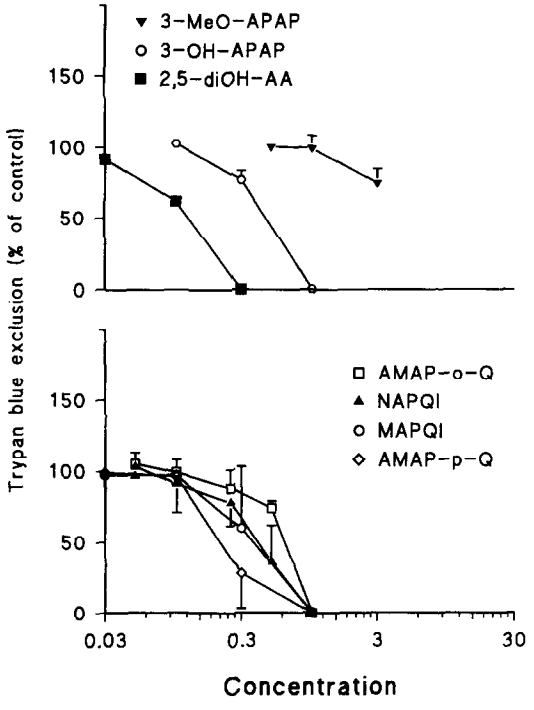

Fig. 5. Concentration-dependent cytotoxicity of hydroquinone and quinone metabolites of APAP and AMAP. Monolayers of mouse hepatocytes were exposed to various concentrations of test substance for 18 hr. Cytotoxicity is expressed as per cent viable cells of DMSO controls. The data are means ± SD of three different experiments.

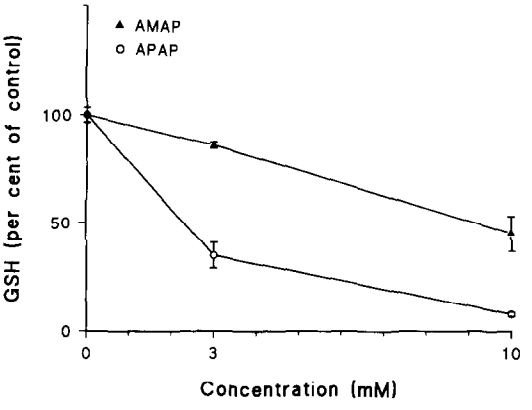

Fig. 6. Concentration-dependence of APAP- and AMAP-induced GSH depletion. Monolayers of mouse hepatocytes were exposed to 3 and 10 mM test substance for 3 hr. GSH was determined by the method of Tietze [37]. The values are means ± SD of three different incubations and represent a typical experiment.

even somewhat more potent than NAPQI (Fig. 5). The potencies of the quinone metabolites were in the same order as that of NAPQI (Fig. 6). Thus, comparing the toxicities of the hydroquinone and quinone metabolites of AMAP with that of NAPQI does not explain the difference between AMAP- and APAP-induced toxicity. This finding indicates that kinetic differences in the formation of reactive metabolites may be involved. However, it is important to be careful when interpreting results from studies where cells have been exposed to postulated reactive metabolites added to the medium. There are obvious differences in concentration and duration of exposure to the cell organelles of added reactive metabolites when compared to endogenously formed metabolites.

In general, there is a good correlation between the extent of covalent binding of APAP to

macromolecules and the degree of hepatotoxic effect [39, 41]. However, recent reports have questioned the simple relationship between covalent binding of APAP and cell death [21, 42, 43]. Dithiothreitol, GSH and *N*-acetyl-L-cysteine added after APAP exposure have been shown to decrease cytotoxicity, indicating that oxidation of protein thiols may also be an important event in the development of toxicity [21, 22, 44]. Connected to this, it is important to note that AMAP seems to have less effect, when compared to APAP, on the level of cellular GSH both *in vivo* [26, 28, 40] and in mouse hepatocytes *in vitro* (Fig. 6), and in particular on the mitochondrial GSH pool [45]. These findings support the notion that depletion of GSH is an important event in the development of APAP-toxicity. A decreased level of GSH will make the cells more susceptible to endogenously activated oxygen species which may cause oxidative stress and, in combination with the covalent binding, lead to cell death.

An alternative explanation is that the extent of covalent binding of the drug to critical target proteins or specific sites on proteins, rather than the overall amount of covalent binding, is the determinant of drug-induced cell death [46]. The fact that the reactive metabolite of AMAP does not react with GSH as extensively as APAP (Fig. 6); [40] could also support this hypothesis. In fact, APAP reactive metabolites seem to bind more extensively to mitochondrial proteins than AMAP reactive metabolites [45]. Furthermore, inhibition of mitochondrial respiration may be an important step in APAP-induced cell death [47].

The present study shows that the relative toxic potencies of the hydroquinone and quinone metabolites of AMAP were comparable to that of NAPQI, which does not readily explain the marked difference between the cytotoxic effects of AMAP and APAP. However, AMAP did not deplete GSH to as great an extent as APAP, indicating that their reactive metabolites may bind to different proteins or that oxidative damage in addition to arylation of proteins may be involved in the development of cell death.

Acknowledgements—The authors wish to thank A. L. Solbakken for artwork and S. Batalden, J. Erichsen, and K. Kihlstrøm for typing the manuscript. The work was partially supported (S.D.N.) by NIH Grant: No. 6M 25418.

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