Study of the Ability of Phenacetin, Acetaminophen, and Aspirin to Induce Cytotoxicity, Mutation, and Morphological Transformation in C3H/10T½ Clone 8 Mouse Embryo Cells

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ABSTRACT

Use of the analgesic compounds acetylsalicylic acid (aspirin), phenacetin, and acetaminophen has been correlated with increased risk of renal cancer in humans. Hence, we studied these compounds for ability to induce cytotoxicity, mutation, and morphological transformation in cultured C3H/10T½ clone 8 (10T½) mouse embryo cells. All three compounds were cytotoxic from 0.5-μg/ml to 2-μg/ml concentrations as evidenced by decreased plating efficiency. None of the compounds induced detectable base substitution mutations to ouabain resistance even at cytotoxic concentrations. Aspirin did not induce morphological transformation. Both phenacetin and acetaminophen induced low but concentration-dependent numbers of atypical, weak type II morphologically transformed foci; at equimolar concentrations, phenacetin was 1.1- to 3.0-fold more active in inducing these foci. Neither phenacetin nor acetaminophen was cotoxifying with 3-methylcholanthrene, and neither compound promoted cell transformation when added to 3-methylcholanthrene-initiated 10T½ cells. The focus-inducing potency of both compounds was increased by addition of an Arochlor-induced hamster liver S9 fraction as an exogenous metabolizing system. However, seven putative metabolites of phenacetin and acetaminophen that were tested—N-hydroxyphenacetin, p-henetidine, p-aminophenol, p-nitrosophenol, benzoquinone, acetamide, and N-acetyl-p-benzoquinonimine—were inactive in transformation assays at the concentrations reducing plating efficiency of treated cells to 50% of the plating efficiency of nontreated (control) cells. Several acetaminophen- and phenacetin-induced foci were cloned, expanded into cell lines, and characterized. These cell lines stably formed type II foci when maintained at confluence for 2 to 4 wk in reconstruction experiments with nontransformed 10T½ cells; however, they did not exhibit significantly increased saturation density compared to 10T½ cells, and they did not grow in soft agarose. These results suggest that metabolic intermediates of high concentrations of phenacetin and acetaminophen induce a low frequency of nonneoplastic morphological transformation of 10T½ mouse embryo cells.

INTRODUCTION

There is now widespread interest in tentatively identifying suspected human carcinogens through epidemiological investigations and confirming these observations by testing chemicals in studies of mutation and transformation of mammalian cells in vitro and in whole animal carcinogenesis bioassays (1). Recently, epidemiological evidence has accumulated to suggest that use of certain analgesic compounds such as aspirin, phenacetin and acetaminophen may be associated with increased risk of renal cancer in humans (reviewed in Ref. 2).

Aspirin (acetylsalicylic acid), a nonsteroidal inhibitor of prostaglandin biosynthesis, is perhaps the most well-known and commonly used nonprescription analgesic. To our knowledge there is no definitive experimental evidence linking aspirin usage to human cancer induction. Aspirin is noncarcinogenic to rodents when administered alone even at doses that induce chronic gastric irritation and ulceration (3). There is limited evidence that chronic high doses of aspirin may be cocarcinogenic with MNNG* in rat forestomach (3). This effect is probably related to the aspirin-induced erosion and hyperplasia sensitizing the gastric epithelium to MNNG. On the other hand, aspirin may inhibit FANFT-induced urinary bladder lesions, possibly by inhibiting metabolism of FANFT by prostaglandin endoperoxide synthetase (4). Aspirin also inhibits TPA promotion of mouse skin carcinogenesis and blocks the TPA-dependent induction of ornithine decarboxylase activity in mouse skin (reviewed in Ref. 5).

Phenacetin was widely used in humans as an analgesic and antipyretic drug, either alone or in combination with aspirin and caffeine (reviewed in Refs. 2 and 6), but its use has been curtailed due to reports of toxic side effects. Long-term use of phenacetin, usually attributable to substance abuse, is associated with kidney damage, renal carcinoma, and tumors of the bladder (2-8). Phenacetin was carcinogenic in long-term feeding studies in C57BL/6 × C3H F1, (hereafter called B6C3F1) mice (7) and Sprague-Dawley rats (8-10), inducing tumors of the kidney and lower urinary tract. There is also limited evidence that N-hydroxyphenacetin, a minor metabolite, is carcinogenic in rats (11). Bacterial mutagenesis assays have yielded conflicting results (2, 12), but it is generally accepted that rodent liver microsomal and S9 fractions can activate phenacetin and N-hydroxyphenacetin to metabolites that are mutagenic to Salmonella tester strain TA100 (13, 14). N-Hydroxyphenetidine and p-nitrosophenol are thought to be the direct-acting mutagenic metabolites (15-18). Hamster liver S9 activated phenacetin weakly in a forward mutation test in V79 Chinese hamster cells (13). Phenacetin itself weakly induced chromosomal aberrations in the absence and strongly induced them in the presence of rat liver S9 in Chinese hamster ovary cells (13).

Acetaminophen, a widely used over-the-counter analgesic/antipyretic, is safe at recommended dosages but can be toxic and fatal if ingested in large quantities. Overdoses of acetaminophen induce severe hepatic necrosis and renal failure (reviewed in Ref. 19). Long-term rodent carcinogenesis bioassays have yielded conflicting results, since acetaminophen was carcinogenic to liver in one study (20) but noncarcinogenic in two other studies (21, 22). Animals in all three studies had evidence of hepatic toxicity. Acetaminophen was nonmutagenic to bacteria in the absence or presence of metabolic activating systems (16) and nongenotoxic to rat primary hepatocyte cultures (23).

Much evidence implicates metabolites of phenacetin and acetaminophen as the ultimate toxic and carcinogenic species

* The abbreviations used are: MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; MCA, 3-methylcholanthrene; TPA, 12-O-tetradecanoylphorbol-13-acetate; Ouα, ouabain resistant; NABQI, N-acetyl-p-benzoquinonimine; FANFT, N-[4-(F-nitro-2-furyl)-2-thiazoyl]formamide; DMSO, dimethyl sulfoxide; LC50, 50% lethal concentration.

Received 3/11/88; revised 10/7/88; accepted 11/16/88.

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1 Supported by Grant ES-03816 from the National Institute of Environmental Health Sciences, NIH, to J. R. L.; by Grant CA41277 from the National Cancer Institute, NIH, to J. R. L.; and by Grant SIG-2 from the American Cancer Society to B. E. H., and J. R. L.

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(reviewed in Refs. 6, 19, and 24), and a schematic summary of these reviews is provided here as Fig. 1. In general, in all animal species studied, approximately 70% of ingested phenacetin is converted to acetaminophen via dealkylation by liver P450 enzymes. Furthermore, both phenacetin and acetaminophen can undergo numerous ring hydroxylation reactions which are not shown in Fig. 1 and are not addressed in this study. Several of the metabolic intermediates of phenacetin and acetaminophen can form covalent adducts with cellular macromolecules (6, 19, 25), and others can generate free radicals and superoxide (26, 27). For instance, para-phenetidine can form a homopolymer free radical, N-(4-ethoxyphenyl)benzoquinoneimine, and NABQI is a toxic intermediate common to both acetaminophen (via epoxidation or acetaminophen free radical formation) and phenacetin (via N-hydroxylation and hydrolysis of conjugation products) (24). Formation of such reactive metabolites is thought to be critical to the toxicity, mutagenicity, and potential carcinogenicity of these compounds. Therefore, the purpose of this investigation was to (a) test the hypothesis that these analogues are carcinogenic, by studying several of these compounds in assays for mutation and morphological transformation in cultured mammalian cells, and (b) to attempt to identify the potential carcinogenic intermediates by testing several metabolites of these compounds for in vitro cell transformation.

MATERIALS AND METHODS

Chemicals. MCA, acetylsalicylic acid (aspirin), 4-nitrosophenol, p-phenetidine, acetamide, benzoquinone, and 4-aminophenol were purchased from Aldrich Chemical Company, Milwaukee, WI. Ouabain octahydrate, MNNG, TPA, acetaminophen, and phenacetin were purchased from Sigma Chemical Company, St. Louis, MO. Spectrophotometric grade DMSO was purchased from the J. T. Baker Company, Phillipsburg, NJ. Spectrophotometric grade acetone was purchased from Mallinkrodt, Inc., Paris, KY. N-Hydroxyphenacetin and NABQI were generous gifts from Dr. Sidney Nelson, Department of Medicinal Chemistry, University of Washington (28).

Cell Culture and Cytotoxicity Assays. The C3H/10T1/2 clone 8 (10T1/2) mouse embryo fibroblast cell line, derived by Reznikoff et al. (29) and used in studies of chemical transformation (30, 31), was cultured as previously described (29) with recent modifications detailing serum screening and medium preparation procedures (reviewed in Ref. 32), in medium without antibiotics. Cells were routinely screened for Mycoplasma by growth on Mycoplasma agar (33) and found to be negative. Cytotoxicity assays were conducted as previously described (30) and directly paralleled mutagenesis and transformation assays. Colonies containing greater than 20 cells were scored under a microscope as viable.

Assays for Mutation to Ouabain Resistance in C3H/10T1/2 Cells. These assays, which detect stable base substitution mutations to ouabain resistance that map to mouse chromosome 3 (31, 34-36; reviewed in Ref. 37), were performed as previously described (31). Briefly, from ten to twenty 100-mm dishes were each seeded with 104 10T1/2 cells, and cells were treated 24 h later with 50 μl of a solution of MNNG in acetone, or of test compounds in DMSO, for 3 or 24 h. Following treatment, the medium was removed and replaced with medium not containing the compounds, and cells were incubated for a further 48 h, which yields the maximum expression time for MNNG and N-acetoxyacetaminofluorene-induced mutations to ouabain resistance in 10T1/2 cells (31). Cells were then trypsinized, and twenty 100-mm dishes were reseeded at an inoculum of 104 cells per 100-mm dish to assay Ouα’ mutant colonies, and five 60-mm dishes were each seeded with 200 cells and five dishes with 2000 cells each, to assay plating efficiency of replated cells as described above. Twenty-four h after reseeding, the medium on dishes containing 104 cells per 100-mm dish was removed and replaced with medium containing 3 mM ouabain. Medium was removed and replaced with fresh medium containing 3 mM ouabain again after 6 and 12 days of incubation. After 16 days of total incubation in ouabain, the Ouα’ mutant colonies were fixed with methanol and stained with Giemsa, and viable colonies containing greater than 20 cells were counted under a microscope. Mutation frequencies were calculated as follows.

\[
\text{Mutation frequency} = \frac{\text{total Oua' colonies/total no. of dishes}}{\text{plating efficiency of reseeded cells} \times 10^3}
\]

Assays for Morphological Transformation. These assays were conducted exactly as described in 10T1/2 cells (30), except that the time of treatment with compound was either 3 h in the presence of S9 fractions or 24 h without S9 (38). In some experiments cells were treated beginning on Day 5 after seeding according to the procedure of Nesnow et al. (39). In these experiments, both type II and type III transformed colonies were scored and tabulated (30; reviewed in Ref. 32). The cell inoculum treated in each assay was 2000 cells per 60-mm dish, and 20 dishes were treated for each concentration of compound studied per experiment. To biologically characterize the loci induced by various compounds, foci were ring cloned and expanded into cell lines. For analysis of growth rate and saturation density, 104 cells were seeded per 60-mm dishes culture, four dishes for each time point, and allowed to grow with medium changes every 3 days. Cells were harvested by trypsinization and counted electronically on a Model Z Coulter Counter every 2 days for up to 20 days. Cells were tested for ability to grow in soft agarose using the original methods of MacPherson (40) modified by use of agarose instead of agar as per Boreiko and Heidelberger (41).

Isolation of Hamster Liver S9. Aroclor 1254-induced hamster liver S9 was isolated from female Syrian golden hamsters (110 g) essentially as previously described for rat liver (38). Protein concentration was determined by Biorad assay to be 7 to 10 mg/ml of homogenate in 0.15 M KCl, using bovine serum albumin as the standard. Homogenate was added to the activation mixture and diluted 1:3 with serum-free medium to yield a final S9 protein concentration of 2 to 2.5 mg/ml as previously described (38). This was empirically determined to be the maximum noncytotoxic amount of S9 that cells could tolerate for 3 h. This complete activation mixture was filtered through a 0.45-μm filter and applied to cells in the absence or presence of test compound for 3 h (38).

RESULTS

Standard Assays for Cytotoxicity, Mutagenesis, and Transformation

Acetylsalicylic Acid. The results of cytotoxicity, mutagenesis, and transformation experiments when 10T1/2 cells were treated with acetylsalicylic acid are summarized in Table 1. This compound decreased the plating efficiency in a dose-dependent manner to 72, 32, and 25% of control values when 1.0, 2.0, and 3.0 mg/ml, respectively, were applied to 10T1/2 cells for 24 h. At these concentrations acetylsalicylic acid did not induce detectable mutation to ouabain resistance in experiments in which MNNG induced an increase of 152-fold in mutation frequency, to 305 × 10−6. Table 1 also shows the cumulative data from 2 transformation assays utilizing the standard treatment regimen (31). Acetylsalicylic acid induced no significant transformation in two experiments in which the positive control, 3-methylcholanthrene, induced a total of 16 type II and type III foci. Based on these negative results, together with our unpublished observations5 that aspirin actually inhibits 3-methylcholanthrene-induced transformation, we did not investigate aspirin further.

Acetaminophen. The cytotoxicity and genotoxicity of acetaminophen are also summarized in Table 1. Treatment of 10T1/2 cells with 0.5, 1.0, and 2.0 mg/ml of acetaminophen caused a dose-dependent cytotoxicity, decreasing survival from 49% to 9%, but did not induce detectable mutation to ouabain resistance.
acetaminophen and phenacetin could be enhanced by tumor promoters, 10T1/2 cells were treated on Day 1 with acetaminophen or phenacetin and then exposed to 0.125 μg/ml of TPA for the duration of the experiment as per Mondal et al. (42). Conversely, to determine whether acetaminophen and phenacetin have promoter-like activity, cells were initiated with a low dose (0.1 μg/ml) of MCA for 24 h on Day 1 after seeding and then exposed to acetaminophen or phenacetin for the duration of the experiment beginning on Day 5 after seeding. Table 2 shows that continuous exposure of cells initiated with 0.1 μg/ml of MCA to 0.125 μg/ml of TPA increased focus formation 3-fold. However, TPA did not significantly increase the frequency or type of foci induced in cells initiated with 1 mg/ml of acetaminophen or phenacetin. Table 2 also shows that con-

### Table 1 Summary of the activities of aspirin, phenacetin, and acetaminophen in standard genotoxicity assays

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of survival</th>
<th>Ou' frequency (× 10²)</th>
<th>Cumulative transformation (foci/total dishes)</th>
<th>Dishes with type II or III foci/total dishes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone, 0.5%</td>
<td>100</td>
<td>2</td>
<td>0/100</td>
<td>0/100</td>
</tr>
<tr>
<td>MNNG</td>
<td>84 ± 21</td>
<td>305</td>
<td>1/40</td>
<td>0/40</td>
</tr>
<tr>
<td>MCA</td>
<td>96 ± 11</td>
<td>35/118</td>
<td>52/118</td>
<td>87/118 (14.7)</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mg/ml, 24 h</td>
<td>73</td>
<td>1/40</td>
<td>0/40</td>
<td>1/40 (0.5)</td>
</tr>
<tr>
<td>1.0 mg/ml, 24 h</td>
<td>72</td>
<td>0/39</td>
<td>0/39</td>
<td>0/39 (0)</td>
</tr>
<tr>
<td>2.0 mg/ml, 24 h</td>
<td>32</td>
<td>1/35</td>
<td>0/35</td>
<td>1/35 (0.37)</td>
</tr>
<tr>
<td>3.0 mg/ml, 24 h</td>
<td>25</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20 (0)</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mg/ml, 24 h</td>
<td>49 ± 29</td>
<td>2</td>
<td>2/40</td>
<td>4/40 (1)</td>
</tr>
<tr>
<td>1.0 mg/ml, 24 h</td>
<td>16 ± 24</td>
<td>2</td>
<td>3/40</td>
<td>3/40 (1.5)</td>
</tr>
<tr>
<td>2.0 mg/ml, 24 h</td>
<td>9 ± 117</td>
<td>7</td>
<td>6/35</td>
<td>6/35 (3.4)</td>
</tr>
<tr>
<td>Phenacetin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mg/ml, 24 h</td>
<td>85 ± 24</td>
<td>3</td>
<td>3/54</td>
<td>0/54</td>
</tr>
<tr>
<td>1.0 mg/ml, 24 h</td>
<td>57 ± 25</td>
<td>2</td>
<td>17/65</td>
<td>0/65</td>
</tr>
<tr>
<td>2.0 mg/ml, 24 h</td>
<td>45 ± 19</td>
<td>3</td>
<td>12/40</td>
<td>0/40</td>
</tr>
</tbody>
</table>
| * Average of 2 experiments. 
| Cumulative transformation of 2 to 5 experiments using 20 dishes per point. 
| * Mean ± SEM of 3 to 4 experiments except where noted; plating efficiency of untreated cells ranged from 19 to 31%. 
| Numbers in parentheses, total number of foci normalized on the basis of 20 dishes. 
| Precipitates out of solution at this concentration.

Phenacetin. Treatment of 10T² cells with 0.5, 1.0, and 2.0 mg/ml of phenacetin caused dose-dependent 15, 43, and 55% decreases in plating efficiency, respectively (Table 1). Phenacetin was not detectably mutagenic at these concentrations but was much less toxic than acetaminophen at concentrations of 0.5, 1.0, and 2.0 mg/ml, respectively. In addition, the transforming activity of phenacetin occurred at much lower concentrations than for acetaminophen. Phenacetin was 1.1-, 3.0-, and 1.6-fold more potent than acetaminophen at concentrations of 0.5, 1.0, and 2.0 mg/ml, respectively. However, acetaminophen did consistently induce a low but concentration-dependent number of type II morphologically transformed foci. For ease of comparison, the number of foci normalized per 20 dishes is shown in parentheses in the column labeled total foci.

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### Table 2 Assay of acetaminophen and phenacetin for ability to initiate and promote cell transformation

<table>
<thead>
<tr>
<th>Initiator</th>
<th>Treatment</th>
<th>Total foci (type II + III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone, 0.5%</td>
<td>TPA, 125 μg/ml</td>
<td>0/20 (0)</td>
</tr>
<tr>
<td>MCA, 1.0 μg/ml, 24 h</td>
<td>TPA</td>
<td>13/20 (1.3)</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>0.5 mg/ml, 24 h</td>
<td>TPA</td>
</tr>
<tr>
<td>1.0 mg/ml, 24 h</td>
<td>TPA</td>
<td>3/40 (1.5)</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>0.5 mg/ml, 24 h</td>
<td>TPA</td>
</tr>
<tr>
<td>1.0 mg/ml, 24 h</td>
<td>TPA</td>
<td>17/65 (4.6)</td>
</tr>
<tr>
<td>MCA</td>
<td>0.1 μg/ml, 24 h</td>
<td>TPA</td>
</tr>
<tr>
<td>0.1 μg/ml, 24 h</td>
<td>TPA</td>
<td>6/20</td>
</tr>
<tr>
<td>0.001 mg/ml Acetaminophen</td>
<td>0.005 mg/ml</td>
<td>2/20</td>
</tr>
<tr>
<td>0.001 mg/ml Phenacetin</td>
<td>0.005 mg/ml</td>
<td>2/20</td>
</tr>
<tr>
<td>0.001 mg/ml Phenacetin</td>
<td>0.005 mg/ml</td>
<td>2/20</td>
</tr>
<tr>
<td>0.001 mg/ml Phenacetin</td>
<td>0.005 mg/ml</td>
<td>2/20</td>
</tr>
<tr>
<td>0.001 mg/ml Phenacetin</td>
<td>0.005 mg/ml</td>
<td>2/20</td>
</tr>
</tbody>
</table>

The breakdown of the types of foci induced by the various treatments was: acetone-TPA, 1 type II; MCA (0.1 μg/ml)-TPA, 4 type II and type III; Acetaminophen (1.0 μg/ml)-TPA, 3 type II; Phenacetin (1.0 μg/ml)-TPA, 4 type II and type III; MCA (0.1 μg/ml)-TPA, 4 type II and type III. Numbers in parentheses, total number of type II and type III foci normalized to a total of 20 dishes.

Significant to P < 0.05, MCA with TPA compared to MCA without TPA. Significant to P < 0.05, MCA with TPA compared to acetone with TPA.
continuous exposure of MCA-initiated cells to 0.001, 0.005, or 0.01 mg/ml of acetaminophen or phenacetin had no consistent effect on focus formation. Concentrations of acetaminophen and phenacetin greater than 0.01 mg/ml actually inhibited MCA-induced focus formation (not shown). Hence, acetaminophen and phenacetin did not function as initiators or promoters of cell transformation in these assays.

We also determined whether acetaminophen and phenacetin were cotransforming with MCA by adding them simultaneously with MCA to cells for 24 h. Neither acetaminophen nor phenacetin was synergistically cocarcinogenic with MCA (Table 3). In fact, the highest concentration of acetaminophen (1.0 mg/ml) actually inhibited MCA transformation by 64%. Addition of a presumed ultimate cytotoxic metabolite, NABQI, did not increase but actually decreased MCA-induced transformation by 82% (Table 3).

Metabolic Activation and Activity of Metabolites

To determine whether metabolic activation of acetaminophen and phenacetin was necessary for induction of foci by these compounds, we utilized a modification of the transformation assay which incorporates a 3-h treatment in the presence of an S9 activation mix (38). Cyclophosphamide was included in these experiments as a positive control for S9 activation, since our laboratory previously showed that metabolic activation is an absolute requirement for the transforming activity of this compound (38). We also compared the yield of S9-activated transformation between the standard assay and the Nesnow assay. Table 4 shows data representative of several experiments. The yield of MCA-induced transformed foci/plate increased 4.2-fold in the Nesnow assay versus the standard assay. How our own data with S9 activation indicated that metabolites of acetaminophen and phenacetin may be active toxic and carcinoogenic compounds, we next tested a battery of known, readily available metabolites in purified form for ability to induce cytotoxicity and transformation. Fig. 1 shows the compounds tested (circled), and Fig. 2 shows the range of cytotoxicity for each compound. All compounds induced dose-dependent cytotoxicity. The LC₅₀ values varied greatly between compounds; acetamide was the least cytotoxic metabolite, yielding a LC₅₀ of approximately 300 mM. LC₅₀ values for the other compounds were approximately 5 mM for acetaminophen, phenacetin, and p-phenetidine; 0.5 mM for N-hydroxy-phenacetin; and 5 μM for 4-aminophenol, benzoquinone, p-nitosophenol, and N-acetyl-p-benzoquinoneimine. The compounds were then tested at their LC₅₀ for ability to induce morphological transformation using the modified transformation assay of Nesnow et al. (39) to maximize sensitivity of detection. None of the metabolites tested induced focus formation in these experiments (data not shown).

Biological Characterization of Closed Transformed Foci

To characterize the biological properties of acetaminophen- and phenacetin-transformed cell lines, several foci induced by each compound were cloned, expanded into cell lines, and studied. We noted that acetaminophen- and phenacetin-induced foci were usually significantly smaller than those induced by MCA but sufficiently large and distinct to clone from transformation assays (see Fig. 3 for representative foci). Four acetaminophen- and phenacetin-induced once cloned cell lines (labeled ACT-1, ACT-2, PHN-1, and PHN-2) formed weak type II foci when passaged as nonsubcloned cultures and allowed to remain confluent for 5 to 6 wk (Fig. 4). These four clones, however, did not exhibit faster growth rates or increased saturation densities compared to clone 8 cells and did not grow in soft agar (data not shown). Furthermore, continuous exposure of these cell lines to 0.125 μg/ml of TPA had only a moderate effect on the morphology of two of the four cell lines, no effects on any of the growth parameters of any of the cell lines, and did not cause any of the clones to grow in soft agarose (data not shown). Attempts to enhance the differential properties between phenacetin- and acetaminophen-induced transformed...
Fig. 1. Metabolic scheme of phenacetin and acetaminophen compiled from the literature. The circled compounds were tested for transformation at their 50% lethal dose concentration.

Fig. 2. Cytotoxicity of various metabolites of acetaminophen and phenacetin to C3H10T½ cells. □, p-aminophenol; ○, benzoquinone; △, p-nitrosophenol; ▲, N-acetyl-p-benzoquinoneimine; ▼, N-hydroxyphenacetin; ◈, p-phenetidine; ◆, acetaminophen; †, phenacetin; Δ, acetamide.

Fig. 3. Macroscopic comparison of typical type II and type III foci induced by MCA to the weak type II foci induced by acetaminophen and phenacetin.

Fig. 4. Response of cell lines cloned from foci induced by acetaminophen (ACT-1 and ACT-2), phenacetin (PHN-1 and PHN-2), or MCA (MN1) to TPA.

cloned because of their dramatically altered morphology compared to that of 10T½ cells.

DISCUSSION

The prevalent use, and potential for abuse, of all therapeutic drugs necessitates rigorous testing and study of such drugs for carcinogenic potential. The utility of the assay for morphological transformation in C3H10T½ mouse embryo cells to detect and study mechanisms of action of chemical mutagens and carcinogens has been well documented (30, 31; reviewed in Refs. 32 and 37). Therefore, we used this assay to study phenacetin, acetaminophen, and aspirin for ability to cause cell transformation. Phenacetin has been implicated as a human carcinogen of the lower urinary tract (2), and acetaminophen might be suspected to have similar carcinogenic potential since it is a major metabolite of phenacetin in many animal species, has a related metabolic scheme (reviewed in Ref. 6), and has activity in some animal carcinogenicity studies (20–22). Aspirin is not known to be carcinogenic to humans or experimental animals and was inactive in our studies, except that it was...
cytotoxic at high concentrations. Therefore, this discussion will focus on acetaminophen and phenacetin.

Both phenacetin and acetaminophen are nephrotoxic and hepatotoxic in vivo at high doses, and these toxicities have been observed in patients with tumors thought to be caused by drug abuse (reviewed in Ref. 2). Thus, we first determined the cytotoxic concentration ranges of these drugs to use for mutation and transformation assays. The therapeutic p.o. doses of these drugs range from 500 to 1000 mg daily, and humans ingesting 1000 mg of acetaminophen have maximum blood levels of 30 to 40 μg/ml of serum (43). Abusers of these drugs probably have much higher blood levels, and since 80 to 90% of these drugs is conjugated and excreted in the urine, the local (nephron) concentration may be much higher than the blood level. We found that 0.5 to 2.0 mg/ml (500 to 2000 μg/ml, 5 to 10 mM) were required to induce cytotoxicity in 10T½ cells. We note that the concentrations of 3 to 7 mM acetaminophen that were required to cause cytotoxicity in primary hepatocyte cultures (23) are close to our values of 5 to 10 mM in 10T½ cells.

Another useful aspect of the mammalian 10T½ transformation assay is the ability to measure mutation to ouabain resistance in the same cells used to measure morphological transformation. Indeed, for some chemicals such as benzo(a)pyrene, N-acetoxy-acetylamino fluorene, and MCA, there is a good correlation between concentrations that cause mutation and those that cause transformation (31). In our studies neither aspirin, acetaminophen, nor phenacetin caused base substitution mutations to ouabain resistance even at high cytotoxic concentrations.

While bacterial mutagenesis systems are predominantly used for mutagenesis testing, approximately 55% of the tested carcinogens are nonmutagenic (1). In this light, it is important that the 10T½ cell transformation system is effective at detecting some nonmutagenic organic carcinogens, such as 5-aza-cytidine (35, 44), and nonmutagenic inorganic carcinogens, such as nickel and arsenic compounds. Using the 10T½ cell transformation assay, we found that both acetaminophen and phenacetin, but not aspirin, induced a low, but dose-dependent, number of type II morphologically transformed foci. Interestingly, increasing the number of cells at risk by using the modified protocol of Nesnow et al. (39) for treatment had no significant effect on the yield of transformed foci except for S9-activated phenacetin. The yield of transformed foci induced by another nonmutagenic carcinogen, nickel sulfide, was similarly unaffected by increasing the number of cells at risk. Furthermore, acetaminophen and phenacetin were inactive as initiators of TPA-promoted cell transformation, as promoters of MCA-initiated cells, and as cotransforming agents with MCA. Since many investigators tend to equate initiation with mutation, it may not be surprising that these nonmutagenic compounds were poor initiators and cocarcinogens, since they were unable to induce base-substitution mutations (Table 1).

As described in the "Introduction," the pharmacological and toxicological activity of these compounds is related to a complex metabolic scheme outlined in Fig. 1. Since C3H/10T½ cells have retained P448 activity (reviewed in Refs. 32 and 37) but lack N-hydroxylase activity (45), and since both parent compounds were weakly active without exogenous metabolic activation, our results in C3H/10T½ cells are consistent with the hypothesis that the ultimate toxic intermediate is a quinoneimine generated by hydrolysis of conjugation products or via P450-mediated epoxidation (reviewed in Ref. 6). However, purified quinoneimine NABQI was highly cytotoxic but did not induce transformation by itself and did not enhance the transformation induced by low concentrations (0.1 μg/ml) of MCA. It is possible that, while the postulated product of the P450-mediated metabolism of acetaminophen and phenacetin (i.e., NABQI) was cytotoxic but not carcinogenic, by-products of the reaction (i.e., active oxygen species) may be ultimate carcinogenic species (27, 28, 46). It is also possible that extracellular NABQI may not reach oncogenically relevant sites and may require generation in the vicinity of DNA in order to transform cells. Interestingly, Dybing et al. (47) showed that NABQI induced DNA single strand breaks in Reuber hepatoma cells but was unable to cause mutations in Salmonella typhimurium.

Historically in this laboratory approximately 50% of typical type II foci are tumorigenic in nude mice, whereas 80% of type III foci are tumorigenic (30; reviewed in Refs. 32 and 37). Since foci induced by acetaminophen and phenacetin were atypical (weak) type II foci, it was important for us to clone and characterize these foci. None of the foci cloned once from transformation assays could be recloned on the basis of transformed colony morphology. Even though the mixed clones reformed weak type II foci when maintained at confluence, they did not exhibit any other classical parameters of neoplastic transformation, such as increased saturation density or anchorage independence. While the significance of these types of foci is currently unknown, they may represent an early stage of cell transformation. However, the relative unresponsiveness of the cell lines to TPA indicates that the morphologically transformed phenotype of these cells would represent a very early, non-TPA-responsive stage of transformation.

In summary we have shown that: (a) aspirin was completely nongenotoxic to C3H/10T½ cells; and (b) acetaminophen and phenacetin were nonmutagenic, but S9-mediated metabolism of both of these compounds resulted in the induction of weak morphological transformation that was nonneoplastic in nature. None of seven putative metabolic end-products of these compounds induced transformation. These results are consistent with the epidemiological evidence presented in the accompanying paper by Ross et al. (48), showing that ingestion of various combinations of these analgesics and caffeine resulted in small (approximately 2-fold) increases in the risk of renal pelvic cancer. We suggest that, if acetaminophen and phenacetin are carcinogenic to humans, either other factors such as endogenous promoters or cocarcinogens not studied here act to further transform the weakly transformed cells induced by phenacetin or acetaminophen, or other metabolic schemes such as peroxidase or prostaglandin synthase-mediated catalysis (47, 49), or other mechanisms such as the recently demonstrated recruitment of activated macrophages to the site of toxicity (50, 51), might play an important role in tumor induction.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Sidney Nelson, Department of Medicinal Chemistry, University of Washington, for generous gifts of N-hydroxyphenacetin and N-acetyl-p-benzoquinonemine; Dr. Paul Hochstein of the Institute for Toxicity and Dr. Ronald Ross, Dr. Dave Garabrant, and Dr. Mimi C. Yu of the Department of Preventive Medicine at the University of Southern California for helpful criticism of the manuscript; and Sarah Olivo and Gloria Barreras for typing the manuscript.

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GENOTOXICITY OF PHENACETIN, ACETAMINOPHEN, AND ASPIRIN


Study of the Ability of Phenacetin, Acetaminophen, and Aspirin to Induce Cytotoxicity, Mutation, and Morphological Transformation in C3H/10T½ Clone 8 Mouse Embryo Cells


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