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Research Article

# Biphasic hCAR Inhibition-Activation by Two Aminoazo Liver Carcinogens

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**Abstract.** Detailed dose-response data recently archived by the National Center for Biotechnology Information (NCBI) identified 853 human CAR (hCAR) agonists by quantitative high-throughput screening (qHTS) assays applied to >9,000 chemicals tested at ≥14 concentrations using $n$ = 3–48 replicates. By re-examining NCBI data on 746 agonists with replicate data sets each satisfying additional quality criteria, ~95% had average values of agonist-specific Hill-model slopes estimated by NCBI that exceed 1 (i.e., exhibited an overall sublinear low-dose dose-response), and two unambiguously biphasic hCAR inhibitor-agonists were identified, 4-aminoazobenzene ($n$ = 37) and *ortho*-aminoazotoluene ($n$ = 3), both of which also cause rodent liver tumors. Although evidently rare among hCAR agonists, such biphasic responses add to evidence that nuclear receptors can exhibit complex patterns of low-dose response, consistent with previous observations and theoretical predictions for endpoints governed by ultrasensitive molecular switches. The pronounced biphasic hCAR response pattern observed for 4-aminoazobenzene is particularly noteworthy insofar as it was identified with statistical power that exceeds that of most if not all other receptor-mediated biphasic cellular responses to any single-chemical exposure reported to date.

**Keywords:** 4-aminoazobenzene, *o*-aminoazotoluene, constitutive androstane receptor (CAR), nuclear receptor, nonmonotonic dose-response.

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## 1. Introduction

The constitutive androstane receptor (CAR, NR1I3) is a moderately promiscuous nuclear receptor and xenosensor expressed primarily in hepatocytes. Normally phosphorylated and complexed with heat shock protein 90 (HSP90) and cytosolic CAR retention protein (CCRP) in cytosol, CAR can become activated, e.g., in mice by binding to a ligand, such as 1,4-bis[2-(3,5- dichloropyridyloxy)]benzene (TCPOBOP), or in mice or humans by being dephosphorylated via phenobarbital-mediated recruitment of protein phosphatase 2 (PP2A), after which CAR translocates to the nucleus where it heterodimerizes with nuclear receptor RXR and then interacts with promoter complexes of target genes that regulate many physiological processes including lipid metabolism, glucose metabolism, hormonal regulation, cell growth, wound healing, and apoptosis [1–7]. CAR is thought to promote liver tumors in some rodents by stimulating downstream (e.g., CYP2b, Wisp, FoxM1, cMyc) receptors, multidrug transporters and resistance genes, and related epigenetic modifications (e.g., regions of altered DNA methylation) and microRNA dysregulation (e.g., miR-182 and miR-802 upregulation and miR-122 downregulation) that facilitate hepatocellular proliferation and associated shifts in energy and growth-directed metabolism [2, 5, 8–22], particularly when coupled with $\beta$-catenin activation [23].

Mouse CAR (mCAR) is activated by nongenotoxic mouse liver tumor promoters such as phenobarbital and phenytoin via indirect activation, and via direct CAR or CAR-coactivator binding by TCPOBOP, di(2-ethylhexyl)phthalate (DEHP), and statins [3, 5]. Activation of human CAR (hCAR), e.g., by phenobarbital, chlordane, or 6-(4-chlorophenyl)imidazo-[2,1-*b*,1,3]-thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl) oxime (CITCO), triggers patterns of downstream effects that overlap in many ways but differ in some respects (e.g., by excluding cell proliferation-specific and enhancing apoptotic gene activity), from those elicited by mCAR [2, 3, 24–28]. CAR is also activated by phthalate reproductive toxicants [29–31]. In contrast, CAR antagonists or inverse agonists reported to attenuate and/or inhibit basal levels of CAR activity include: the mCAR inhibitors endogenous androstanol and the pheromone androstenol; the peripheral benzodiazepine receptor ligand PK11195, which activates human CAR (hCAR) in human cell lines; the potent agonist of liver X receptor (LXR) and the human pregnane X receptor (hPXR) TO901317, which inhibits human, mouse, and rat CAR; the soybean and legume phytoestrogen coumestrol, which inhibits hCAR; the antifungal agent ketoconazole, which inhibits hCAR and mCAR; and the hCAR-inhibiting type II diabetes drug metformin [32].

Mechanistic and statistical models describe possible J- or U-shaped, biphasic patterns of dose-response, such as those governed by ultrasensitive molecular switches involving nuclear receptors [33–38]. Such biphasic, apparently receptor-mediated dose-response patterns have been reported and reviewed [39–43]. Highly significantly biphasic dose-response patterns were shown recently to describe detailed sets of activation data for two highly conserved ultrasensitive molecular switches [44, 45], nuclear factor erythroid 2-like factor 2 transcription factor (Nrf2), and heat shock protein 70 (HSP70), each of which also interact with CAR [46–48]. The present study examined detailed activation data for 853 hCAR agonists identified from a total of >9,000 chemicals screened using a quantitative high-throughput screening (qHTS) luciferase reporter assay—recently archived by the National Center for Biotechnology Information (NCBI) [49]—to determine if responses exhibited by those identified CAR agonists include any clearly biphasic (inhibition-activation) patterns.

## 2. Materials and Methods

### 2.1. Data on hCAR activation in human HepG2 cells

*hCAR Activation in Human HepG2 Cells.* Detailed, qHTS concentration-response data for *in vitro* hCAR activation in a line of human liver HepG2 cells containing a double-stable CYP2B6-driven luciferase reporter were recently archived by NCBI [49]. This HepG2-CYP2B6-hCAR cell line was constructed as previously described in detail [50, 51] to express both the full-length hCAR protein, and a luciferase signal driven by the promoter of the prototypical hCAR-target CYP2B6, by co-transfecting the pEF6/V5-hCAR expression plasmid and a pGL4.17[*luc2*/*Neo*]-CYP2B6-2.2kb construct (containing phenobarbital- responsive and xenobiotic-responsive enhancer modules, PBREM and XREM) into HepG2 cells, followed by continual culture in selective media and then by selection of a single colony verified to contain both plasmids. The fact that constitutive activation in immortalized cells is a hallmark of CAR makes the identification of CAR activators extremely challenging—a drawback addressed in applications

of HepG2-CYP2B6-hCAR cells to quantify CAR activation by using PK11195 (a commonly used ligand of the peripheral benzodiazepine receptor) as a potent and selective deactivator of hCAR; PK11195 competes directly with CITCO (by disrupting recruitment of co-activators such as steroid receptor coactivator-1 and glucocorticoid receptor interacting protein-1 to hCAR) and thus can effectively lower the high basal hCAR activity observed in HepG2-CYP2B6-hCAR cells [50]. Thus, as previously described [27, 50–53], results for each tested chemical list reporter-signal intensities measured after 24 hours of exposure to each of 13 or 14 log-spaced chemical concentrations delivered in a dimethyl sulfoxide (DMSO) vehicle to which was added hCAR antagonist PK11195 (at 2.5 μM/well) to repress otherwise relatively high hCAR basal activity in HepG2 cells, using 3–48 replicates per concentration depending on the chemical tested. Raw well-specific plate reads were each normalized to a percent-activity scale in which 100% represents the maximal activity measured using the positive-control hCAR agonist CITCO, and 0% represents control activity measured in DMSO/PK11195-only wells placed in the first four columns of each 1,536-well plate. Each replicate set of agonist-specific NCBI hCAR activation data includes, among other reported information: (i) an estimated corresponding slope ($n_{Hill}$) from a Hill model fit to that data set, (ii) a corresponding value of $R^2$ (the fraction of total response variance explained by the NCBI-fitted Hill model), and (iii) a model-fit Descriptor. The latter Descriptor was "Single" if a tested chemical was characterized as an "Activator" (i.e., agonist) based on substantially elevated activity observed only at a single test concentration.

## 2.2. hCAR activation data analysis and modeling

Each set of replicate NCBI activation data for each of its 853 identified hCAR agonists was first screened first by applying all of three inclusion criteria (agonist classification = Activator, Hill model fit $R^2 > 0.9$, and Descriptor ≠ Single), and then by applying a fourth criterion that at least three agonist-specific replicates remained after applying the first three criteria (i.e., $n \geq 3$) for each agonist. Replicate data sets for a total of 746 agonists satisfied these selection criteria, or ~87.5% of the 853 hCAR agonists identified by NCBI. For all 746 of these agonists, NCBI-estimated $n_{Hill}$ values were characterized as a cumulative likelihood distribution to assess the overall magnitude of apparent nonlinearity in activation response. Log(concentration)-activation patterns for each of the 746 agonists were then plotted and visually screened for apparent patterns of biphasic inhibition-activation response. Apparent biphasic candidates were fit to a five-parameter mixed-lognormal response model by inverse-variance-weighted nonlinear least-squares regression, implemented using *Mathematica* 11.1® software [54]. Goodness of fit was then characterized by $R^2$ and a p-value ($p_{fit}$) from a corresponding chi-square goodness-of-fit test with degrees of freedom (df) equal to the number of data points fit minus the number of fitted parameters. Negativity of initial slope-parameter estimates was in each case assessed by corresponding t-test [54].

# 3. Results

Of the 853 hCAR agonists identified by NCBI [49], 746 were determined to meet the additional criteria for unambiguous dose-response characterization described in Materials and Methods.

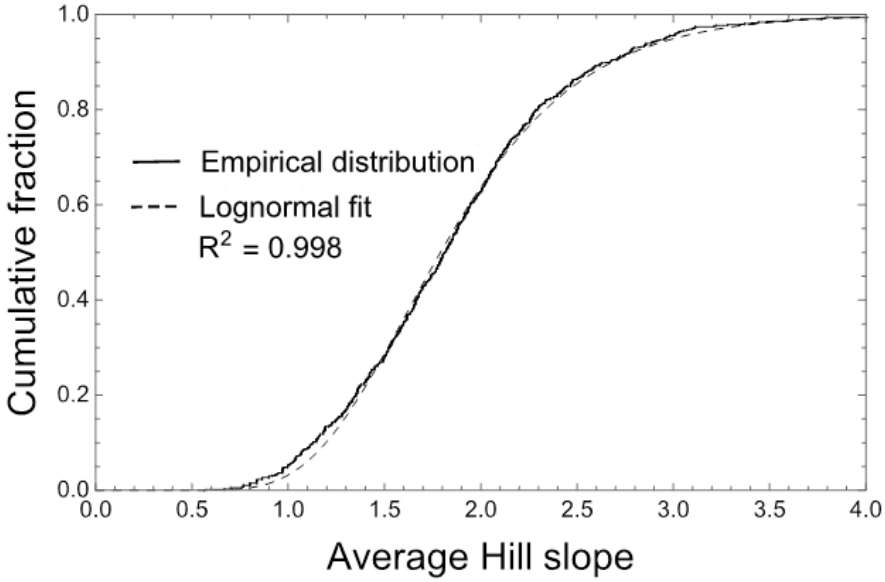


**Figure** 1: Cumulative distribution (solid curve) of NCBI-estimated Hill slope coefficient ($n_{Hill}$) values for those 746 hCAR activators, among 853 designated by NCBI [49] as hCAR activators in HepG2 cells based on that analysis of *in vitro* luciferase-reporter assay results for >9,000 tested chemicals, which met additional activation criteria described in Materials and Methods. The parameters (±1 standard error) of the corresponding estimated lognormal distribution (dashed curve) are GM = 1.79 ± 0.00095 and GSD = 1.37 ± 0.0013, where GM and GSD denote geometric mean and geometric standard deviation, respectively.

The cumulative distribution of $n_{Hill}$ slope values estimated by NCBI [49] for this set of 746 agonists was determined to be approximately lognormal, with a geometric mean (GM) and geometric standard deviation (GSD) of approximately 1.8 and 1.4, respectively (Figure 1). These $n_{Hill}$ estimates have an arithmetic mean (±1 standard deviation) value of 1.86 ± 0.61, and a large majority (~95%) of them exceed 1, indicating a general sublinear pattern of activation dose-response. A detailed examination of agonist-specific dose-response patterns for these 746 agonists indicated two clearly biphasic patterns, those for 4-aminoazobenzene (*para*-aminoazobenzene, Aniline Yellow, C.I. Solvent Yellow, CAS RN 60-09-3, PubChem Chemical Identifier [CID] 6051) (Figure 1) and for *ortho*-aminoazotoluene (*o*-aminoazotoluene, Solvent Yellow 3, CAS RN 97-56-3, CID 7340) (Figure 2). Fits obtained to hCAR-activation data sets for these two agonists are discussed below.

The following 5-parameter biphasic inhibition-activation model was fit to hCAR activation data in relation to 4-aminoazobenzene concentration $C$, as shown in Figure 1:

$$\%\text{Activity} = a - b\,\Phi[\ln(C/\text{GM})/\ln(\text{GSD})] + c\,\Phi[\ln(C/100)/\ln(\text{GSD})] \quad (1)$$

in which Φ denotes the cumulative standard normal distribution function. This data set includes $n = 37$ replicates reported at each of 13 concentrations. Parameter estimates ±1 standard error (SE) obtained for this fit and associated fit statistics are: $a = -(0.723 \pm 0.142)\%$, $b = (2.93 \pm 0.376)\%$, GM = (1.67 ± 0.0594)μM, GSD = 4.09 ± 0.266, and $c = (77.6 \pm 3.25)\%$ ($p_{fit} = 0.45$, $R^2 = 0.996$). The estimated Y-intercept ($a$), inhibition slope ($b$), and activation slope ($c$) each differ significantly from zero ($p = 0.00094$, 0.000052, and $<10^{-7}$, respectively), as do estimates for parameters GM ($p = 0.023$) and GSD ($p < 10^{-6}$), by 2-tail t-tests. A corresponding 4-parameter activation-only model, in which the rightmost term of Equation 1 is omitted and its "– $b$" replaced by "+ $b$", explains nearly as much % Activity variability exhibited in this data

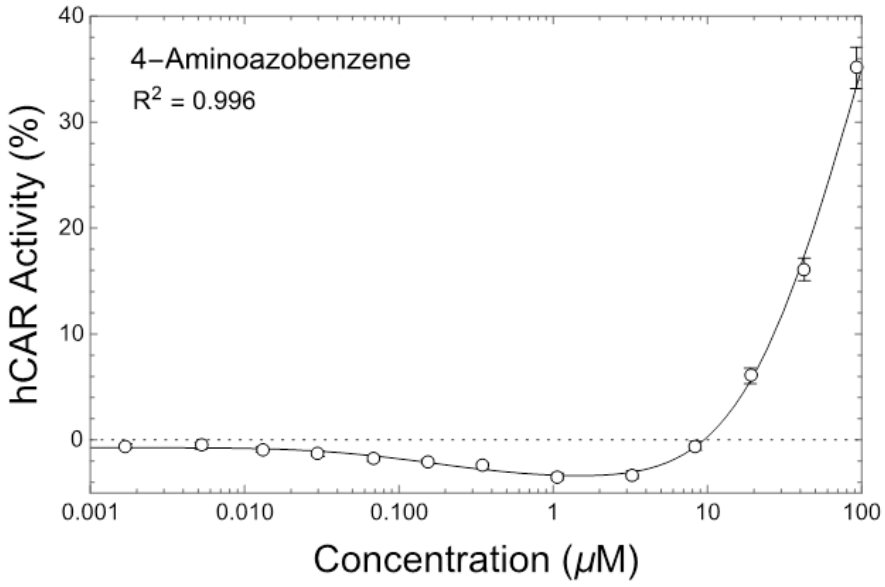


**Figure** 2: Model fit (curve) to data (open points) on hCAR activation in luciferase-reporter human HepG2 cells cultured for 24 hours at the indicated 4-aminoazobenzene concentration, which were reported normalized to a scale of 0% for DMSO control wells (plotted at 0 μM) and 100% (the maximum CITCO-induced activity). Error bars = ±1 standard deviation of the mean (SDM), involving 37 replicates/concentration. Dotted line = background in DMSO-exposed cells.

set ($R^2 = 0.990$) but nevertheless is clearly inconsistent with the mean response pattern due to its relatively small error at each concentration ($p_{fit} = \sim 0$).

The following 4-parameter biphasic inhibition-activation model was fit to hCAR activation data in relation to *o*-aminoazotoluene concentration *C*, as shown in Figure 2:

$$\begin{aligned} \%\text{Activity} &= -b\,\Phi[\ln(C/\text{GM}_0)/\ln(3/2)] + c\,\Phi[\ln(C/\text{GM})/\ln(2)] \\ &\quad +5c\,\Phi[\ln(C/[25\text{GM}])/\ln(2)] \end{aligned} \tag{2}$$

This data set includes $n = 3$ replicates reported at each concentration. Parameter estimates ±1 SE obtained for this fit and associated fit statistics are: $b = (4.54 \pm 0.621)\%$, $\text{GM}_0 = (0.108 \pm 0.0191)\mu\text{M}$, $c = (23.26 \pm 1.26)\%$, and $\text{GM} = 3.02 \pm 0.265$ ($p_{fit} = 0.49$, $R^2 = 0.997$). The estimated inhibition slope ($b$) differs significantly from zero ($p = 0.000045$), as do estimates for parameters $\text{GM}_0$ ($p = 0.00031$), $c$ ($p < 10^{-7}$), and GM ($p = \sim 10^{-6}$), by 2-tail t-tests. A corresponding 3-parameter activation-only model (like that described above but with no Y-intercept term), explains nearly as much % Activity variability exhibited in this data set ($R^2 = 0.981$), but nevertheless is clearly inconsistent with the mean response pattern due to its relatively small error at each concentration ($p_{fit} = \sim 0$).

## 4. Discussion

The two hCAR activators, 4-aminoazobenzene (AAB) and *o*-aminoazotoluene (OAT), were identified in this study to exhibit significantly biphasic patterns of hCAR inhibition-activation. The biphasic pattern observed for 4-aminoazobenzene is particularly noteworthy insofar as it was identified with statistical power that exceeds that of most if not all other biphasic receptor-mediated responses to any single-chemical exposure documented to date [39–45].

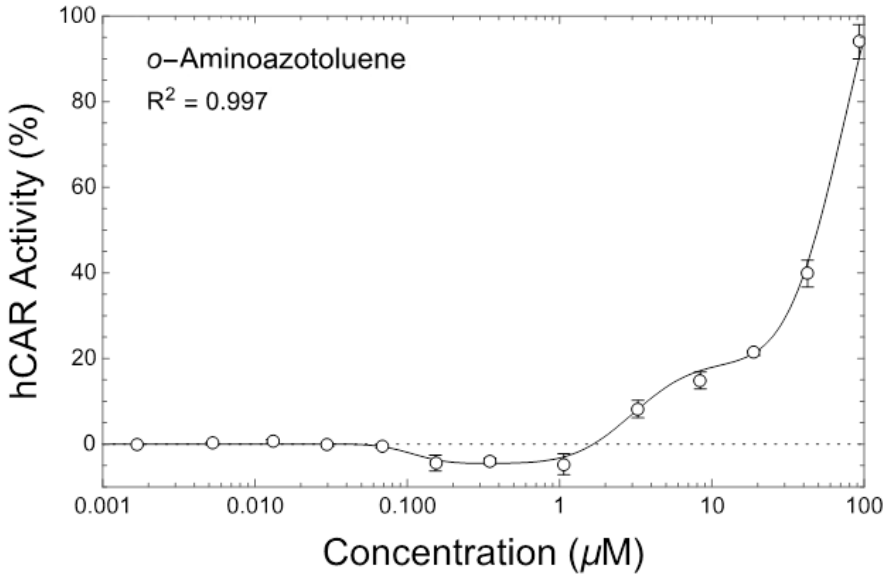


**Figure** 3: Model fit (curve) to data (open points) on hCAR activation in luciferase-reporter human HepG2 cells cultured for 24 hours at the indicated *o*-aminoazotoluene concentration (see Figure 2 legend). Error bars = ±1 SDM, involving 3 replicates/concentration. Dotted line = background in DMSO-exposed cells.

Although specific mechanisms underlying biphasic hCAR activation patterns identified here for two hCAR activators remain to be elucidated, their relatively low frequency (2, or ~0.25%) among the 746 activation patterns examined in this study suggests that these biphasic patterns reflect an apparent CAR function to balance competing signals, that typically are integrated as different signaling chemicals bind competitively and/or otherwise interact at key regulatory domains within this receptor. Under this interpretation, such integration mediates a cellular "choice" between two alternative (basal or negatively activated, vs. activated) modes of downstream signaling that (perhaps more so in some tissues than others) determine or influence cellular metabolic and/or proliferative status. Such chemical-specific balancing by hCAR is clearly indicated by potent chemical-specific anti-activation of this receptor [50], and by the application of the potent and selective hCAR deactivator PK11195 to reduce the high basal hCAR activity observed in HepG2-CYP2B6-hCAR cells used in the activation assay described in Methods. Evidently, chemicals such as AAB and OAT have a relatively rare capacity to both antagonize and activate hCAR substantially, and to do the former at sufficiently lower concentrations than the latter to yield a demonstrably biphasic pattern of overall activation. The signal-balancing basis of biphasic hCAR activation hypothesized here for hCAR appears to differ markedly from that of more subtle biphasic activation patterns observed for Nrf2 and HSP70 receptors functioning as ultrasensitive molecular switches that trigger suites of cytoprotective gene expression in response to specific (e.g., oxidative or heat) stress conditions, but which at sub-threshold trigger levels appear to dampen switch-activation likelihood, perhaps to suppress energy-draining "false alarms" under conditions of transient or marginal stress [44–48].

Both aminoazo compounds identified here to have a biphasic hCAR response have long been known to induce rodent liver tumors [55–59] (see Appendix). As noted in the Introduction,

constitutive androstane receptor (CAR) activation by rodent liver tumor promoters such as phenobarbital act at least in part by stimulating downstream receptors and related epigenetic modifications that trigger hepatocellular proliferation and supporting shifts in energy and growth-directed metabolism. In contrast, CAR antagonist/inhibitors inhibit basal CAR activity levels. Among the 746 hCAR activator data sets discussed, possible nonlinear, nonmonotonic patterns of hCAR activation were previously suggested for the mouse liver carcinogens toxaphene (CAS RN 8001-35-2, CID 5284469 or 102000395), aldrin (CAS RN 465-73-6, 124-96-9, 309-00-2; CID 2087, 24860538), and isodrin (CAS RN 465-73-6, 124-96-9, 309-00-2; CID 10066, 60196420) [[60], see Figures 1, S5 and S6 of that study]. In contrast, approximately linear and lognormal patterns of hCAR activation were observed for the structurally related mouse liver carcinogens chlordane (CAS RN 57-74-9, CID 5993) and dieldrin (CAS RN 60-57-1, 72-20-8, 128-10-9; CID 3038, 969491), respectively [[60], see Figures S7 and S8 of that study].

It remains to be determined whether and to what extent mouse or rat CAR activation exhibits a biphasic dose-response pattern *in vitro* and *in vivo*, and what role CAR plays in driving or modulating AAB and/or OAT tumorigenicity in those species, analogous to the role it has been demonstrated through studies comparing CAR-knockout and wild-type mice to play in tumors associated with the nongenotoxic rodent tumor promotors phenobarbital and toxaphene [60–62]. However, the aryl hydrocarbon receptor (AHR) may also play an important and possibly CAR-related role in liver tumors associated with AAB and OAT, because these chemicals are also AHR agonists [63–65], CAR is (including in hepatocytes) upregulated by AHR [66], CAR and AHR activation correlate with susceptibility to OAT-induced liver tumors [65], and (like CAR) AHR plays central, diverse, and signal-integrating roles in cellular (including hepatocellular) development, energy metabolism, inflammation, enzyme induction, endocrine, and other systems that can modulate tumor likelihood [67–69]. Reports of biphasic AHR activation currently appear to be limited to subtle indications observed for galangin and reversitrol [70, 71], although more systematic investigation using qHTS methods might reveal additional and more pronounced examples.

The biphasic nature *per se* of hCAR activation by AAB and OAT, and the fact that these two chemicals can also elevate rodent liver tumor incidence, appear to be coincidental with no present evidence of any causal connection. However, if future studies indicate that AAB and OAT (and perhaps other similarly rare biphasic CAR activators) elevate liver tumors by CAR-dependent mechanisms, their biphasic activation patterns would clearly be important and complicating considerations that bear directly on potential cancer risks posed by related environmental exposures.

## Appendix

Concerning AAB tumorigenicity, dietary AAB administration did not result in liver tumors as 0.056% in the diet administered to albino rats for 8 months followed by two months of basal diet [72], or as 0.106% in the diet (5.34 mg/kg) administered to Sprague-Dawley rats for 9 months [73]. However, hepatic tumors were induced in 40% of Wistar rats fed 0.2–1% AAB the diet for up to 28 months [58]. By one year after neonatal ICR/JCL mice were exposed to AAB, they developed neoplastic lesions of liver, lung and lymphoreticular tissues [74]. In male 12-day-old C57BL/6 X C3H/ HeF1 (B6C3F1) mice administered single i.p. doses of 0.017–0.15 μmol AAB

per g body weight (equivalent to a maximum transient concentration range of approximately 17–150 μM, assuming a 5-g mouse pup body weight with a 0.2-L/kg volume of distribution), hepatoma multiplicity was approximately linearly related to dose, with an average of 11 hepatomas/mouse observed at 10 months in the high-dose group; in contrast, female B6C3F1 mice were resistant to tumor induction under these conditions, and similar administration of AAB to male F344 rats with or without co-administration of 0.1% of phenobarbital in drinking water for 1–24 months did not induce a significant number of hepatic tumors [75]. In this study, 24 hours after 12-day-old male B6C3F1 mice and F344 rats were injected i.p. with 0.3 μmol AAB per g body weight, ~40-fold more hepatic *N*-(deoxyguanosin-8-yl)-AAB-DNA adducts were detected in these mice than in similarly exposed rats [75]. The International Agency for Research on Cancer (IARC) currently classifies AAB as a possible (class 2B) human carcinogen [76].

Concerning OAT tumorigenicity, by 15 months after newborn (~17-g) male and female A/Jax mice were given a single subcutaneous injection of 0, 0.4, or 0.7 mg OAT, ~42–50% of males had one or more liver tumors, whereas females had about a third as many and unexposed mice had none; the OAT-exposed mice also showed a ~4-fold elevation in pulmonary tumors [59]. Although OAT induces liver tumors potently in several mouse strains (CBA, SWR, DBA/2, A/He, and DD), much less or no observed hepatotumorigenic potency was observed in other strains (AKR and CC57Br) and in rats [77, 78]. OAT was first shown to induce CAR and CAR-dependent liver cell proliferation in mice [65, 79]. Onset of liver cell proliferation in mice hepatectomized and then treated with OAT was more delayed (60–80% inhibited) in strains more susceptible to OAT-induced liver tumors compared to <15% inhibition of cell proliferation exhibited in less-susceptible strains using the same protocol and OAT treatment [79]. IARC currently classifies OAT as a possible (class 2B) human carcinogen [76].

AAB and OAT each exhibit *in vitro* mutagenic activity in the Ames test using *Salmonella typhimurium* strain TA 98 activated by liver enzymes, where such activity was suppressed by adding pentachlorophenol (PCP) and induced by adding 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to the activating enzymes [80]. However, tumor multiplicity exhibited in tumor-susceptible mouse strains neonatally exposed to OAT was increased with PCP pretreatment but was reduced with TCCD metabolic-activation pretreatment; in contrast, PCP pretreatment was observed to inhibit AAB-induced carcinogenic activity in three strains of mice [81].

## Competing Interests

The author prepared the paper during the normal course of his employment by Exponent (Health Sciences), which is a consulting firm that, among other services, provides advice on toxicological and risk analysis issues to private and public clients. Formulation of scientific questions addressed, review of the literature, synthesis and integration of scientific information, and conclusions drawn in the paper are the exclusive professional product of the author and are not necessarily those of Exponent or any of its clients. Only Exponent reviewed the submitted paper and funded its preparation.

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