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A quantum chemical study of the reactivity of acetaminophen (paracetamol) toxic metabolite N-acetyl-*p*-benzoquinone imine with deoxyguanosine and glutathione

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ABSTRACT

Acetaminophen (APAP) forms some reactive metabolites that can react with DNA. APAP is a potentially genotoxic drug and is classified as a Group 3 drug according to International Agency for Research on Cancer (IARC). One of the possible mechanisms of APAP genotoxicity after long term of use is that its reactive quinone imine (QI) metabolite of acetaminophen (NAPQI), can chemically react with DNA after glutathione (GSH) depletion. A quantum chemical study of the reactions between the NAPQI and deoxyguanosine (dG) or GSH was performed. Activation energies ($\Delta G^{\ddagger}$) for the reactions associated with the 1, 4-Michael addition were calculated on the M062X/6-311++G (d,p) level of theory. We modeled the reaction with dG as a multi-step process. The first step is rate-limiting ($\Delta G^{\ddagger} = 26.7$ kcal/mol) and consists of formation of a C–N bond between the C3 atom of the QI moiety and the N7 atom of dG. The second step involves proton transfer from the C3 moiety to the nitrogen atom of the QI with $\Delta G^{\ddagger}$ of 13.8 kcal/mol. The depurination reaction that follows has a $\Delta G^{\ddagger}$ of 25.7 kcal/mol. The calculated $\Delta G^{\ddagger}$ for the nucleophilic attack of the deprotonated S atom of GSH on the C3 atom of the NAPQI is 12.9 kcal/mol. Therefore, the QI will react with GSH much faster than with DNA. Our study gives mechanistic insight into the genotoxicity of the APAP metabolite and will be useful for estimating the genotoxic potential of existing drugs with a QI moiety. Our results show that clinical application of APAP is safe, while in the case of severely depleted GSH levels APAP should be administered with caution.

1. Introduction

Acetaminophen (APAP or paracetamol) is one of the most popular and widely used analgesic and antipyretic drugs, with superior overall gastrointestinal profile safety compared to other non-steroidal anti-inflammatory drugs (NSAID). It can be found in the form of over-the-counter remedies or prescription medicines. Despite its extensive use, its precise mode of action is still a matter of debate. Although it is commonly stated that APAP acts centrally by inhibition of cyclooxygenase isoenzyme 3 (COX-3) [1], recent data suggests it inhibits both COX-1 and COX-2 isoforms [2]. Two recent studies have also demonstrated a preferential COX-2-inhibition by APAP under different clinically relevant conditions [3,4]. APAP is used in large quantities and is available both alone and in combinations with other drugs (e.g. NSAID, codeine, caffeine, opiates) [5–10]. Despite rigorous pharmacological and toxicological tests associated with the registration of a new drug it should be clear that every drug is associated with side effects. It remains a major challenge to properly understand side effects in order to minimize them in terms of dosing and interactions with other drugs [11]. APAP has been the subject of several *in vitro* and *in vivo* studies concerning endocrine disruption [12–14]. Some of these studies concluded that APAP is toxic and interacts with reproduction and development. They reported impaired sperm parameters and testicular germ cell cancer in humans [15,16]. The International Agency for Research on Cancer (IARC) last reviewed epidemiological data for APAP in 1999, especially for its carcinogenicity, hepatotoxicity, nephrotoxicity, testicular damage, reproductive and development toxicity [17]. However, studies have provided insufficient evidence to prove or disprove carcinogenicity for APAP in

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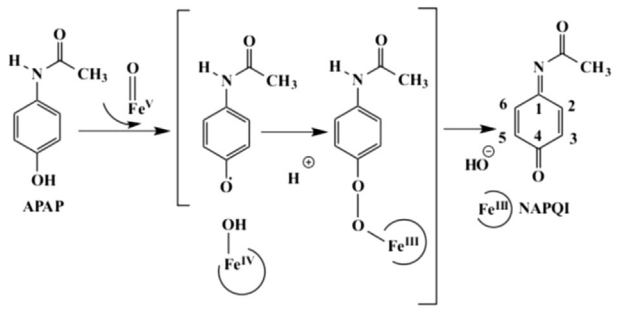
humans and experimental animals.

Until now, a great number of *in vitro* and *in vivo* assays have been performed to evaluate the carcinogenicity and genotoxicity of APAP that can be attributed to the reactive metabolites that can react with DNA, nucleophilic groups of proteins, and glutathione (GSH). APAP oxidation is mediated by cytochrome P450 enzymes (CYP1A1, 1A2 and 3A4), with CYP2E1 being the most active in the formation of the reactive quinone imine (QI), N-acetyl-*p*-benzoquinone imine (NAPQI) [18,19]. NAPQI is a highly reactive metabolite as an electrophile, which can bind covalently to endogenous nucleophiles like GSH or protein thiols close to their site of formation [20]. Literature data show that QIs react with DNA [21,22]. However, less than 5% of the dose represents CYP-catalyzed oxidation (Scheme 1) [23,24]. For a complete understanding of the genotoxicity of APAP, it is also crucial to know the mechanisms of the chemical reaction between a reactive APAP metabolite and DNA while *in vitro* and *in vivo* tests of genotoxicity have not been conclusive. All *in vitro* tests for genotoxicity in bacteria (*S. typhimurium, E. coli*) or mammalian cells (HPRT locus in V-79 hamster cells, C3H/10T$^1/_2$ mouse embryo fibroblasts, Chinese hamster ovary (CHO–K1) cells, mouse lymphoma TK fluctuation assay) have shown that APAP does not induce point mutations [25,26] even after metabolic activation. It was also not mutagenic to insects (*Drosophila Melanogaster*) [17,27]. The cytogenetic effect of APAP was determined with *in vitro* studies of chromosome aberrations in CHO cells, V79 cells and primary peripheral human lymphocytes (all positive) and chromatid exchange *in vivo*. Published studies based on micronucleus assays (mouse bone marrow *in vivo*) are not enough to draw conclusions on the ability of APAP to induce micronuclei *in vitro* or *in vivo* [25,28]. On the other side APAP induces sister chromatid exchanges (SCE) in human cells *in vitro* and *in vivo*. Moreover, *in vitro* studies of DNA single strand breaks (SSB) have been performed in CHO cells, V79 cells, rat hepatoma cells and with human fibroblasts and peripheral lymphocytes, while the *in vivo* studies have been done in mice and rats. All studies have shown that a reactive APAP metabolite is responsible for the formation of SSB in liver DNA [25]. Replicative and reparative DNA synthesis is probably inhibited through inactivation of ribonucleotide reductase *in vitro* and *in vivo* by an active species of APAP, which until now has not been characterized. However, studies of carcinogenicity have not been conclusive: in some animal species APAP promotes preneoplastic changes (mouse liver) and neoplastic kidney changes were found in a male F344 rat, but APAP also had an inhibitory effect on the development of preneoplastic foci in rat liver. In addition, APAP was carcinogenic for IF mice, but not for B6C3F$_1$ and NIH mice. The carcinogenic effect was obvious in the Leeds strain study, but no carcinogenic effect was reported for Sprague–Dawley and F344 rats [26]. The follow-up of these studies is that APAP has been classified as Group 3, which means that APAP is not carcinogenic, while further research is needed in order to properly understand its genotoxicity.



Scheme 1. One of the metabolic pathways of acetaminophen (APAP). The following abbreviations are used (see text also): APAP, acetaminophen; NAPQI, N-acetyl-*p*-benzoquinone imine. Please note that 5% of APAP undergoes this metabolic pathway [30,31].

In addition to the studies mentioned above, it is important to clearly explain and understand the chemical mechanism of genotoxicity. One of the possible mechanisms of APAP genotoxicity after long term of use is that its reactive metabolite, NAPQI, can chemically react with DNA after GSH depletion [25]. *In vivo* GSH depletion also leads to increased levels of reactive oxygen species that damage DNA [29]. Experimentally it is very difficult to distinguish between these two effects since one can only measure the total GSH concentration.

QIs are electrophilic reactive intermediates that may undergo redox cycling and generation of reactive oxygen species [32]. They react with nucleophiles such as reduced GSH and nucleic acids by a 1, 4-Michael addition similarly to the quinone (Q) metabolites of bisphenol A (BPA) [33–35]. Covalent binding to cellular macromolecules and other thiol groups can occur when hepatic GSH is depleted [36]. Various experimental studies have identified adduct formation of NAPQI with GSH [37–39]. By assuming that NAPQI reacts with the protein SH groups by the same mechanism, the GSH-NAPQI adduct formation can serve as a model for these reactions. Moreover, DNA adducts formed by the APAP metabolite have not been structurally characterized [40]. Therefore, a detailed quantum-chemical study of the multi-step reaction mechanism for both reactions is desirable.

In this study we considered the chemical reactivity of the APAP QI metabolite NAPQI using quantum chemical methods on the M062X/6-311++G (d,p) level. We calculated the Born-Oppenheimer surface stationary points that provided the activation energy and geometric parameters of the reactive species. With inclusion of the polar environment using the solvent reaction field method we calculated the free energies of activation that are directly linked to the reaction rate constants. We studied the reaction mechanism between NAPQI and dG as a model of its reactions with DNA followed by depurination. In addition, we estimated the free energy cost for GSH SH group deprotonation, followed by a 1, 4-Michael addition with NAPQI. We demonstrated that formation of the C–N bond in the 1, 4-Michael addition represents the rate-limiting step. The calculated barriers are critically compared with the experimental barriers for related species (Qs and epoxides). Experimental data for the reaction between NAPQI and GSH are in very good agreement with the calculated barrier.

2. Computational methods

The Born-Oppenheimer hypersurface stationary points for the reactions between NAPQI and dG, were first calculated on the M062X functional developed by Truhlar and coworkers [41,42] in conjunction with the 6-31G* basis set, followed by the more flexible 6-311++G (d,p) basis set in order to geometrically re-optimize all stationary points. Vibrational analysis was performed in the harmonic approximation. Minima on the potential energy surface (PES) correspond to reactants and products, and the saddle points correspond to transition states. The thermal energy corrections were included in the harmonic approximation. The free energies of hydration were calculated by solvent reaction field (SCRF) method of Tomasi and coworkers [43] as implemented in Gaussian 09 [44]. Protonation states were assigned according to the physiological pH value of 7.4 with the analogy of protonation states of DNA moieties. In Scheme 2 all species were considered neutral except the final products where cation and anion were a consequence of heterolytic bond cleavage. In Scheme 3 the total charge of the system is −2, which corresponds to both carboxylic groups being deprotonated, a protonated amino group and deprotonated SH group entering the reaction.