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Estragole DNA adduct accumulation in human liver HepaRG cells upon repeated in vitro exposure

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HIGHLIGHTS

- Estragole DNA adducts accumulate in cells in vitro upon repeated exposure.
- Upon dietary exposure 6–57 years would be required to reach 10−100 adducts/10^8 nts.
- Persistent nature causes substantial adduct levels upon prolonged dietary intake.

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ABSTRACT

Accumulation of N^2-(*trans*-isoestragol-3′-yl)-2′-deoxyguanosine (E-3′-N^2-dG) DNA adducts derived from the alkenylbenzene estragole upon repeated dose exposure was investigated since the repair of this adduct was previously shown to be inefficient. To this end human HepaRG cells were exposed to repeating cycles of 2 h exposure to 50 μM estragole followed by 22 h repair to mimic daily exposure. The E-3′-N^2-dG DNA adduct levels were quantified by LC–MS/MS after each cycle. The results show accumulation of E-3′-N^2-dG DNA adducts at a rate of 17.53 adducts/10^8 nts/cycle. This rate at the dose level calculated by physiologically based kinetic (PBK) modeling to result in 50 μM was converted to a rate expected at average human daily intake of estragole. The predicted time estimated to reach adduct levels reported at the BMD10 of the related alkenylbenzene methyleugenol of 10−100 adducts /10^8 nts upon average human daily intake of estragole amounted to 8–80 (in rat) or 6–57 years (in human). It is concluded that the persistent nature of the E-3′-N^2-dG DNA adducts may contribute to accumulation of substantial levels of DNA adducts upon prolonged dietary exposure.

1. Introduction

The group of food-borne alkenylbenzenes includes the compounds estragole, safrole and methyleugenol, generally present in herbs and spices like tarragon, nutmeg, basil and/or anise (European Food Safety Authority (EFSA), 2012; Van Den Berg et al., 2011). These compounds raise a health concern because of their DNA adduct formation and carcinogenicity (Miller et al., 1983), inducing hepatic tumors at high dose repeated exposure in rodents (Jamuna, 2010; Miller et al., 1983; Scientific Committee on Food (SCF), 2001). The mode of action underlying tumor formation includes metabolism of the parent alkenylbenzene by cytochromes P450 and sulfotransferases to 1′-sulfoxy metabolites that are able to form DNA adducts (Phillips et al., 1981; Randerath et al., 1984; Wiseman et al., 1985). Although formation of DNA adducts may result in mutations and increase the risk of developing cancer, the DNA adduct formation may also induce DNA repair and be a reversible process (Klaunig and Kamendulis, 2010). The ultimate hazard and risk posed by DNA adduct formation will thus depend on the efficiency of repair during the time before subsequent exposures. Our previous studies on cellular repair of the major estragole and safrole DNA adducts (E-3′-N^2-dG and S-3′-N^2-dG respectively) showed the repair of these DNA adducts to be inefficient with 80–90 % of the adducts still remaining in HepaRG cells or primary hepatocytes after 48 h and/or 4 h repair (Yang et al., 2020a,b). These observations are in line with in vivo studies where the DNA adducts of safrole were reported to persist in the liver of orally exposed CD-1 mice with only limited amounts being removed 30 days after a single dose (Gupta et al., 1993). This implies that DNA adduct levels are not only likely to increase with increasing dose levels, but also upon repeated exposure. Upon repeated exposure with insufficient repair between subsequent

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exposures, accumulation of DNA adduct levels with increasing days of exposure would be expected. The aim of the present study was to characterize this potential accumulation of DNA adduct levels upon repeated exposure to estragole. To mimic daily exposure HepaRG cells were exposed to estragole in a repeated 2 h exposure 22 h repair regimen, and the levels of the major E-3′-N^2-dG adduct were quantified using LC–MS/MS. The estragole concentration used for these experiments was selected based on outcomes of a physiologically based kinetic (PBK) model based evaluation of the dose dependent liver blood concentrations using the models described by Punt et al. (2008, 2009). From the results obtained, the rate of bioaccumulation of the DNA adducts upon daily exposure could be estimated. This result was used to estimate the time required, at normal human dietary intake levels of estragole, to accumulate levels of E-3′-N^2-dG DNA adducts in the range of the estimated level of liver DNA adducts of the related alkenylbenzene methyleugenol at its BMD10 (Paini et al., 2011).

2. Material and method

2.1. Chemical and reagents

Estragole, human insulin, bovine spleen phosphodiesterase II (SPDE II), venom phosphodiesterase I (VPDE I), nuclease P1, phosphatase alkaline (AP), Tris(hydroxymethyl)aminomethane (Tris), and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma (St. Louis, Missouri, USA). Phosphate buffered saline (PBS) (pH 7.4) was purchased from Gibco (Paisley, UK). Williams E Medium, and penicillin-streptomycin (P/S) were purchased from Gibco (Grand Island, New York, USA). Fetal Bovine Serum (FBS) was purchased from Bodinco BV (Alkmaar, Netherlands). Hydrocortisone 21-hemisuccinate was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). Dimethylsulfoxide (DMSO), hydrochloric acid (HCl), zinc sulfate ($ZnSO_4$), and sodium acetate were purchased from Merck (Darmstadt, Germany). Acetonitrile (ACN) was purchased from Biosolve (Dieuze, France). RNeasy Lysis buffer (RLT) was purchased from QIAGEN (Hilden, Germany). Formic acid was purchased from VMR (Fontenay-sous-Bois, France).

2.2. Cellular model

HepaRG cells provide a frequently used model for studying hepatotoxicity of especially compounds that require cytochrome P450 mediated bioactivation (Szabo et al., 2013). The HepaRG cell line is derived from human hepatocellular carcinoma cells (Gripon et al., 2002) and can differentiate into hepatocyte-like morphology when treated with DMSO (Marion et al., 2010). In our previous study (Yang et al., 2020a), it was already shown that upon exposure of the HepaRG cells to estragole detectable levels of estragole derived DNA adducts can be formed. The undifferentiated HepaRG cell line was purchased from Biopredic international (Saint Grégoire, France) and cultured in growth medium consisting of William E Medium supplemented with 10 % FBS (Sigma, St. Louis, MI, USA), 100 IU/mL P/S, 5×10^{-5} M hydrocortisone 21-hemisuccinate and 5 μg/mL human insulin at the density of 2×10^5 cells/flask for 2 weeks at 37 °C and 5% (v/v) CO_2 in a humidified atmosphere. After 2 weeks growth, differentiation medium was used to facilitate cell differentiation into cells with hepatocyte-like morphology with extra 1.7 % DMSO added to the growth medium for the following 2 weeks. All media were refreshed every other day. HepaRG cells were cultured in T-25 flasks (Greiner Bio-One, Frickenhausen, Germany) and maintained until the first week after full differentiation before being used for detection of DNA adduct formation upon repeated exposure.

2.3. PBK modeling

In order to find a suitable concentration of estragole that will result in formation of detectable levels of E-3′-N^2-dG DNA adducts and has biological meaning, PBK model based evaluation of the dose dependent liver blood concentrations were undertaken using the models described by Punt et al. (2008, 2009) predicting the bioactivation and detoxification of estragole in rat and human respectively. Both models were used to calculate the dose-dependent maximum liver blood concentration of estragole, and from these data a suitable concentration used for the in vitro repeated exposure experiments was derived.

2.4. DNA adduct formation upon repeated exposure

Based on the PBK model results the concentration of estragole selected for the experiments was 50 μM. Detailed information underlying this selection is shown in the result section. For repeated exposure, eight T-25 flasks of differentiated HepaRG cells were exposed to 50 μM estragole for 2 h. All the test compounds were added to exposure medium from a 1000 times concentrated stock solution in DMSO (final DMSO concentration amounting to 0.1 %). After 2 h incubation, cells were washed by PBS once and the exposure medium was changed back to the differentiation medium without test compound for 22 h to facilitate DNA repair. This 2 h exposure and 22 h repair is considered as one cycle. The same process was repeated for another three cycles and after each time

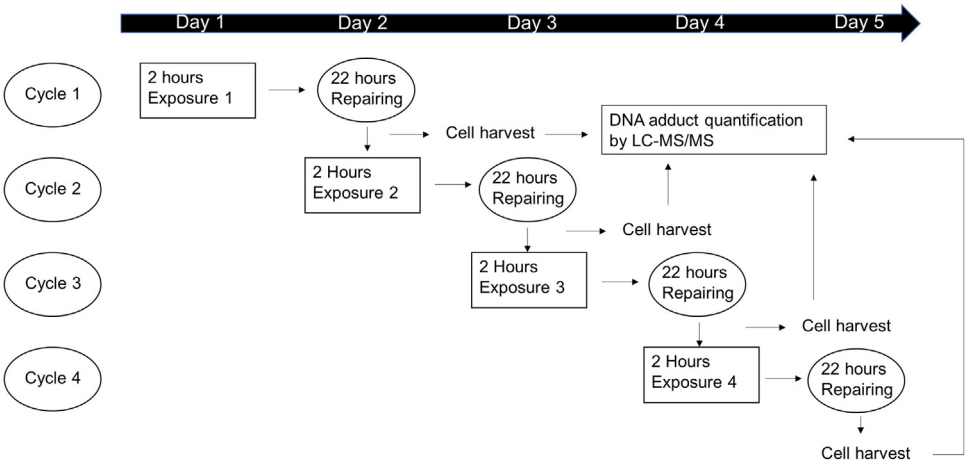

Fig. 1. Schematic presentation of the repeated exposure experiment.

of repair, two flasks of HepaRG cells were randomly chosen and collected for quantification of the E-3′-N^2-dG adduct levels. Fig. 1 presents a schematic overview of the repeated exposure experimental design. Since the differentiated HepaRG cells did not proliferate during the repair period (data not shown), no correction for DNA adduct dilution due to cell proliferation was required. Upon harvesting, cells were washed twice by resuspending in PBS and centrifugation at 211g for 5 min twice. After the second centrifugation, the supernatant was removed and the cells were lysed via the addition of 200 µl RNeasy Lysis Buffer (RLT buffer) before DNA isolation.

2.5. DNA isolation and digestion

DNA isolation was performed using the QIAamp DNA Mini Kit protocol for cultured cells (Hilden, Germany). The range of the cell number for DNA isolation was between 2×10^6 and 5×10^6 cells. The isolated DNA concentration was determined through Nanodrop™One (Thermo scientific, Waltham, MA, USA) and purity of the isolated DNA was measured based on the absorbance ratio A260/A280 with a value of 1.8–2.0 considered as sufficiently pure. After isolation, DNA samples were freeze-dried overnight and stored at −80 °C until digestion. Dried samples were dissolved in nanopure water, adjusted to 50 µg per 30 µl before digestion. DNA digestion was performed as previously described (Yang et al., 2020a). Briefly, samples were incubated with 40 µl PI-buffer (300 mM sodium acetate, 1 mM $ZnSO_4$, pH 5.3), 20 µl SPDE II solution (0.0004 U/µl in water), and 10 µl nuclease P1 (0.5 µg/µl in water) at 37 °C for 4 h. After 4 h incubation, 40 µl PA-buffer (500 mM Tris−HCl, 1 mM EDTA, pH 8.0), 20 µl VPDE I solution (0.00026 U/µl in water), and 1.6 µl AP (200 units) were added and samples were incubated at 37 °C for another 2 h.

2.6. Synthesis of E-3′-N^2-dG adduct and LC–MS/MS method for detection and quantification of E-3′-N^2-dG adducts

E-3′-N^2-dG adduct synthesis was performed as previously reported (Yang et al., 2020a). LC–MS/MS detection and quantification of the E-3′-N^2-dG adducts was adapted from Paini et al. (2010) using a Shimadzu Nexera XR LC-20CE SR UPLC system coupled with a Shimadzu LCMS-8040 mass spectrometer (Kyoto, Japan). The samples (5 µL) were loaded onto a reverse phase C18 column (1.7 µm 2.1 × 50 mm) with a flow rate of 0.3 mL/min and the column temperature set at 40 °C. The mobile phase consisted of ultrapure water with 0.1 % (v/v) formic acid and acetonitrile containing 0.1 % (v/v) formic acid. The initial condition was 5% acetonitrile for 1 min, followed by a linear gradient to 100 % acetonitrile in 8 min which was maintained for 0.5 min, after which the gradient went back to the initial conditions in 0.1 min and remained at this condition for the rest of the running time. The total running time of each sample was 10 min. E-3-N^2-dG eluted at 5.99 min. The MS-MS analysis was carried out using a Shimadzu LCMS-8040 triple quadrupole with electrospray ionization (ESI) interface. The instrument was operated in positive mode in the multiple reaction monitoring (MRM) mode with a spray voltage of 4.5 KV. E-3′-N^2-dG was monitored at the [M+H]+ of precursor to product 414.2 → 298.2, 414.2 → 164.1, and 414.2 → 147 *m/z*. The level of DNA adducts was quantified using a calibration curve where the peak area of a known concentration of the synthesized DNA adduct was plotted against the corresponding DNA adduct concentrations. Calibration curves in buffer and cellular matrix were similar. The limits of detection and quantification of the E-3′-N2-dG DNA adducts were 0.0025 pmol and 0.008 pmol respectively. The amount of the DNA adducts detected in the samples was related to the total amount of digested DNA in each sample, and calculated from the Nanodrop output in ng/mL using a molar extinction coefficient for double stranded DNA of 50 L × mol^{-1} cm^{-1}. E-3′-N^2-dG DNA adduct levels were expressed as the number of E-3′-N^2-dG adducts per 10^8 nucleotides (nts) based on the assumption of 1.98×10^{15} nucleotides / µg DNA.

2.7. Estimation of the number of daily cycles required to reach the estimated level of DNA adducts at the BMD10 for the related alkenylbenzene methyleugenol upon repeated daily exposure at realistic human dietary exposure levels

From the repeated exposure experiment a linear equation describing the cycle dependent increase in E-3′-N^2-dG adduct levels in the HepaRG cells could be derived. The slope of the linear equation represents the increase in the E-3′-N^2-dG adduct level / 10^8nts / cycle upon exposure of the cells to 50 µM estragole. The slope thus obtained was used to estimate the rate of E-3′-N^2-dG DNA adduct accumulation per cycle upon exposure to the dose at normal dietary levels using linear extrapolation from the dose level that is predicted by the PBK model to result in an estragole liver blood concentration of 50 µM. This linear extrapolation is supported by previous studies that revealed i) that the level of 1′-sulfooxyestragole responsible for the DNA adduct formation increased linear with the dose from dose levels as low as dietary human intake levels up to the BMDL10 for tumor formation in rats (Rietjens et al., 2010), ii) a linear E-3′-N^2-dG adduct formation in primary rat hepatocytes with increasing concentration of 1′-hydroxyestragole and also linear dose-dependent E-3′-N^2-dG adduct formation in rats in vivo predicted by physiologically based translation of these in vitro data to the in vivo situation (Paini et al., 2010) and iii) a linear dose dependent increase in E-3′-N^2-dG adduct formation in estragole exposed rats (Paini et al., 2012). Except estragole, another alkenylbenzene methyleugenol, also showed an increased linear relation between the level of 1‘-sulfooxymethyleugenol that contributes to the DNA adduct formation and the dose of methyleugenol from the low level of human dietary intake up to the BMDL10 for tumor formation in both in vivo and in vitro (Al-Subeihi et al., 2012). The value thus obtained for the increase in the E-3′-N^2-dG adduct level / 10^8nts / cycle was used to calculate the number of days required to reach 10-100 adducts/10^8 nts, the estimated level of methyleugenol liver DNA adduct formation at its BMD10 for liver tumor formation (Paini et al., 2011).

3. Results

3.1. PBK modeling based selection of the in vitro exposure concentration

Fig. 2 presents the human and rat PBK model based prediction of the dose dependent concentration of estragole in venous blood of the liver for dose levels up to 100 mg/kg bw. The graph reveals an increase in the estragole liver venous blood concentration with the dose of estragole. From this curve it was derived that at the BMD_{10} value for rat, the dose level causing 10 % tumor incidence, reported to amount to 47 mg/kg bw/day (Paini et al., 2010), the liver venous blood concentration of estragole is predicted to amount to 71 µM in human and 44 µM in rat respectively. Based on these results the estragole concentration selected for the repeated exposure experiment was chosen at 50 µM corresponding to a dose level of 52 mg/kg bw in rat and 36 mg/kg bw in human.

3.2. DNA adduct formation upon repeated exposure

HepaRG cells were exposed to 50 µM estragole repeatedly for 4 cycles with each cycle consisting of 2 h of exposure and 22 h of repair (Fig. 3) after which the level of E-3′-N^2-dG DNA adducts was

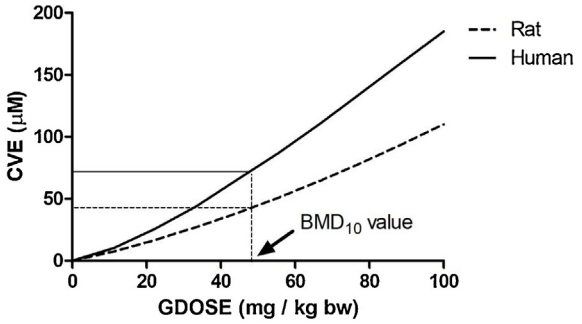

Fig. 2. The PBK model based prediction of the dose dependent venous liver blood concentration of estragole in human (straight line) and rat (dotted line). The CVE values at the BMD10 of 47 mg/kg bw/day (Paini et al., 2010) are also indicated.

quantified. Fig. 3 shows the results obtained revealing a gradual increase in the level of E-3′-N^2-dG DNA adducts with increasing number of cycles reflecting E-3′-N^2-dG DNA adduct accumulation upon repeated exposure. The data can adequately (R^2 = 0.94) be described by a linear increase with the number of cycles described by Eq. (1):

Number of E-3′-N^2-dG DNA adducts/10^8 nts = 17.53x Number of cycles. (1)

The slope of this relationship reflects the accumulation rate expressed in the number of E-3′-N^2-dG DNA adducts/10^8 nts/cycle at 50 μM estragole and amounts to 17.53 E-3′-N^2-dG DNA adducts/ 10^8nts/ cycle.

3.3. Estimation of the number of daily cycles of estragole exposure required to reach adduct levels at the BMD10 for liver tumor incidence of the related alkenylbenzene methyleugenol at the estimated human daily intake of estragole

The estimated level of DNA adducts reported to occur at the BMD10, the dose level causing 10 % tumor incidence above background values (EFSA et al., 2017) of the related alkenylbenzene methyleugenol was used for comparison. This value was reported to amount to of 10−100 /10^8nts (Paini et al., 2011). According to the data presented in Fig. 3 obtained at a concentration of 50 μM estagole indicating a rate of E-3′-N^2-dG DNA adduct accumulation of 17.53 adducts/ 10^8nts/ cycle it would take 5–6 cycles to reach a E-3′-N^2-dG DNA adduct levels amounting to 100/10^8 nts, while at this level of exposure a level of 10/10^8 nts would already be achieved within the first cycle. The PBK modeling indicated this concentration of 50 μM to be reached in venous liver blood at a dose level of 52 mg/kg bw in rat and 36 mg/kg bw in human, which implies exposure in the range of the BMD10 and substantially higher than normal human dietary intake. The human daily dietary intake of estragole was estimated by the EU Scientific Committee on Food (SCF) and the US Flavor and Extract Manufacturers Association (FEMA) to amount to values of 0.07 and 0.01 mg/kg bw/day, respectively. This relatively high level of intake estimated by the SCF was based on the theoretical maximum use levels of estragole in 28 food categories and consumption data for these food categories based on 7-day dietary records of adult individuals, which also included pure compound addition. However, since estragole has been demonstrated to be genotoxic and carcinogenic, in the EU the addition of estragole as a pure compound to food has been regulated (Scientific Committee on Food (SCF), 2001). Therefore, the value of 0.07 reported by the SCF likely overestimates current exposure levels (Punt et al., 2016). Therefore, a human daily dietary estragole exposure of 0.01 mg/kg bw/day was considered to provide a realistic estimate.

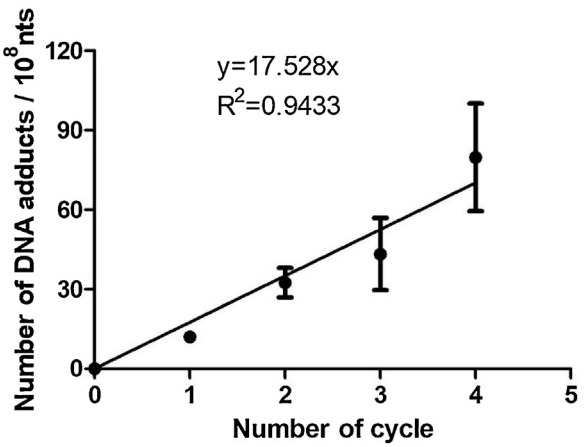

Fig. 3. Relation between the number of E-3′-N^2-dG DNA adducts/10^8 nucleotides detected in the HepaRG cells upon increasing number of daily cycles of 2 h exposure to 50 μM estragole and 22 h repair.

Assuming a linear relationship between the rate of DNA adduct accumulation per cycle and the dose level, the rate of accumulation at 0.01 mg/kg bw/day would amount to 0.0034 and 0.0048 E-3′-N^2-dG DNA adducts/ 10^8nts/ cycle in rat and human respectively. These rates for formation of E-3′-N^2-dG DNA adducts estimated to occur at realistic human dietary intake levels imply that a level of 10−100 adducts /10^8 nts would be reached in 8–80 and 6–57 years in rat and human respectively.

4. Discussion

In present study, the potential accumulation of E-3′-N^2-dG DNA adduct levels upon repeated exposure of human liver cells to estragole in vitro was characterized in order to obtain insight in the consequences of the limited efficiency of the repair of this major estragole DNA adduct (Yang et al., 2020a). To this end first a rat or human PBK model-based estimation of the venous liver blood concentration at dose levels in the range of the BMD_{10} for tumor formation in rat was obtained to define the experimental estragole concentration to which the HepaRG cells were exposed. In line with what was reported before (Punt et al., 2009) the species difference in the kinetics of estragole was limited. For the repeated exposure experiment HepaRG cells were used instead of primary hepatocytes, although primary human hepatocytes are considered as “gold standard model” for xenobiotic metabolism and hepatoxicity (Choi et al., 2015; Gerets et al., 2012; Hart et al., 2010). However, the short lifespan of human hepatocytes (Hart et al., 2010) hampers their application in repeated exposure scenario’s., Upon full differentiation, HepaRG cell cultures have been reported to be comparable to primary human hepatocytes in terms of drug metabolism capacity (Gerets et al., 2012), and E-3′-N^2-dG DNA adduct formation upon exposure to estragole was recently shown to be only 2-fold less efficient in differentiated HepaRG cells than in primary rat hepatocytes (Yang et al., 2020a). Moreover, in both cell lines the E-3′-N^2-dG DNA adducts were shown to be not efficiently repair (Yang et al., 2020a). This supports the use of HepaRG cells as an adequate model to study the E-3′-N^2-dG DNA adduct accumulation upon repeated exposure, also because they show less batch-to-batch variability than primary hepatocytes (Gerets et al., 2012). Furthermore, they do not proliferate within the time frame of the repeated dose experiment so that DNA adduct levels are not diluted by DNA replication.

Upon repeated exposure to estragole, in the HepaRG cells accumulation of E-3′-N^2-dG DNA adducts was readily observed. The accumulation of E-3′-N^2-dG DNA adducts showed a linear increase with the number of cycles allowing calculation of a rate of

E-3′-N^2-dG DNA adduct accumulation per cycle. This rate amounted to 17.53 adducts/ 10^8nts/ cycle at 50 μM estragole. At this rate of accumulation, levels of 10–100 adducts/10^8 nts, would be reached in 5–6 cycles (days). This level of 10–100 adducts/10^8 nts was chosen as a reference value given that exposure to the structurally related alkenylbenzene methyleugenol at its BMD10 for liver tumor formation was estimated to result in this level of DNA adducts (Paini et al., 2011). Given that the comparable value estimated for the alkenylbenzene safrole was one order of magnitude higher (Paini et al., 2011) using the value of 10–100 adducts/10^8 nts provides a conservative reference value. Converting the rate observed in the present study for estragole induced DNA adduct formation at 50 μM estragole, predicted by PBK modeling to be achieved in liver venous blood at dose levels of 52 mg/kg bw in rat and 36 mg /kg bw in human, to a rate at a human daily dietary intake of 0.01 mg/kg bw in a linear way, results in a rate for adduct accumulation of 0.0034 and 0.0048 E-3′-N^2-dG DNA adducts/ 10^8nts/ cycle in rat and human respectively. Assuming such a linear relationship between the estragole concentration or dose and DNA adduct formation is supported by studies in the literature reporting a linear concentration or dose response relationship for DNA adduct formation in primary rat hepatocytes exposed to 1′-hydroxyestragole (Paini et al., 2010) and also in rats exposed to estragole (Paini et al., 2012). It is also supported by the linear dose-dependent formation shown for the DNA reactive 1′-sulfooxymetabolite from dose levels in the low range of the virtual safe dose up to dose levels as high as the $BMDL_{10}$ for tumor formation (Rietjens et al., 2010). At an accumulation rate of 0.0034 to 0.0048 E-3′-N^2-dG DNA adducts/ 10^8nts/ cycle it would take 8–80 or 6–57 years to reach E-3′-N^2-dG DNA adduct levels in the range of 10–100 adducts/10^8 nts in rat and human respectively. Furthermore, it is of importance to note that in a recent study, the concentration dependent E-3′-N^2-dG DNA adduct formation in HepG2-CYP1A2 cells was measured at low concentrations of estragole (0–1 μM) (Schulte-Hubbert et al., 2020). The PBK model predicts these concentrations to be present in liver blood at oral dose levels of 0–1.5 mg/kg bw. The authors reported that at exposure concentrations increasing up to 1 μM E-3′-N^2-dG DNA adduct formation increases in a sublinear way with an apparent threshold at 0.5 μM. This implies that the number of days likely required to reach the level of 10–100 adducts/10^8 may take longer than what has been estimated based on linear extrapolation. This because the PBK model predicts that at estimated dietary daily intakes of 0.01–0.07 mg/kg bw/day the liver blood concentrations of estragole amount to value of 0.01 to 0.04 μM and are thus likely below this apparent threshold of 0.5 μM for adduct formation.

In the study reported by Herrmann et al. (2013) methyleugenol DNA adducts have been detected in human liver samples. Methyleugenol DNA adducts (N^2-MIE-dG and N^6-MIE-dA) were present at detectable levels in non-tumorous liver tissue samples of 28 out of 30 subjects (age 19–69 years). The median methyleugenol DNA adduct levels (13 / 10^8 nts for N^2-MIE-dG and N^6-MIE-dA combined) detected in human non-tumorous liver tissue samples was also in the range of the 10–100 / 10^8nts. These human experimental data on adduct levels of this related alkenylbenzene in human liver likely resulting from dietary exposure, are thus in line with the results obtained in the present study for estragole via repeated exposure of HepaRG cells in vitro.

5. Conclusion

In this study, the rate of accumulation of E-3′-N^2-dG DNA adducts in HepaRG cells repeatedly exposed to estragole in vitro was quantified. The rate of E-3′-N^2-dG adduct accumulation amounted to 17.53 E-3′-N^2-dG DNA adducts/ 10^8nts/ cycle at 50 μM estragole from which the rate at realistic human dietary intakes levels was estimated to amount to 0.0034 and 0.0048 E-3′-N^2-dG DNA adducts/ 10^8nts/ cycle in rat and human respectively. At this rate of accumulation adduct levels estimated to occur at the BMD10 value of the related alkenylbenzene methyleugenol of 10–100 adducts/10^8 nts were predicted to be reached upon prolonged dietary exposure. It is concluded that the persistent nature of the E-3′-N^2-dG DNA adducts may contribute to accumulation of substantial levels of DNA adducts upon prolonged dietary exposure.

Declaration of Competing Interest

The authors report no declarations of interest.

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References

Al-Subeihi, A.A., Spenkelink, B., Punt, A., Boersma, M.G., van Bladeren, P.J., Rietjens, I. M., 2012. Physiologically based kinetic modeling of bioactivation and detoxification of the alkenylbenzene methyleugenol in human as compared with rat. Toxicol. Appl. Pharmacol. 260 (3), 271–284.
Choi, J.M., Oh, S.J., Lee, S.Y., et al., 2015. HepG2 cells as an in vitro model for evaluation of cytochrome P450 induction by xenobiotics. Arch. Pharm. Res. 38 (5), 691–704.
EFSA, Scientific Committee, Hardy, A., Benford, D., et al., 2017. Update: use of the benchmark dose approach in risk assessment. EFSA J. 15 (1) e04658.
European Food Safety Authority (EFSA), 2012. Compendium of botanicals reported to contain naturally occuring substances of possible concern for human health when used in food and food supplements. EFSA J. 10 (5), 2663.
Gerets, H., Tilmant, K., Gerin, B., et al., 2012. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. Cell Biol. 28 (2), 69–87.
Gripon, P., Rumin, S., Urban, S., et al., 2002. Infection of a human hepatoma cell line by hepatitis B virus. Proc. Natl. Acad. Sci. U. S. A. 99 (24), 15655–15660.
Gupta, K.P., van Golen, K.L., Putman, K.L., Randerath, K., 1993. Formation and persistence of safrole-DNA adducts over a 10,000-fold dose range in mouse liver. Carcinogenesis 14 (8), 1517–1521.
Hart, S.N., Li, Y., Nakamoto, K., et al., 2010. A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. Drug Metab. Dispos. 38 (6), 988–994.
Herrmann, K., Schumacher, F., Engst, W., et al., 2013. Abundance of DNA adducts of methyleugenol, a rodent hepatocarcinogen, in human liver samples. Carcinogenesis 34 (5), 1025–1030.
Jamuna, P., 2010. Evaluation of certain food additives. Sixty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). WHO technical report series No. 952. 2009. World Health Organization. Geneva, pages 208. J. Food Sci. Technol. 47 (4), 465.
Klaunig, J.E., Kamendulis, L.M., 2010. Comprehensive Toxicology, second ed. Elsevier, pp. 117–138.
Marion, M.-J., Hantz, O., Durantel, D., 2010. The HepaRG Cell Line: Biological Properties and Relevance as a Tool for Cell Biology, Drug Metabolism, and Virology Studies Hepatocytes. Springer, pp. 261–272.
Miller, E.C., Swanson, A.B., Phillips, D.H., Fletcher, L., Liem, A., Miller, J.A., 1983. Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. Cancer Res. 43 (3), 1124–1134.
Paini, A., Punt, A., Viton, F., et al., 2010. A physiologically based biodynamic (PBBD) model for estragole DNA binding in rat liver based on in vitro kinetic data and estragole DNA adduct formation in primary hepatocytes. Toxicol. Appl. Pharmacol. 245 (1), 57–66.
Paini, A., Scholz, G., Marin-Kuan, M., et al., 2011. Quantitative comparison between in vivo DNA adduct formation from exposure to selected DNA-reactive carcinogens, natural background levels of DNA adduct formation and tumour incidence in rodent bioassays. Mutagenesis 26 (5), 605–618.
Paini, A., Punt, A., Scholz, G., et al., 2012. In vivo validation of DNA adduct formation by estragole in rats predicted by physiologically based biodynamic modelling. Mutagenesis 27 (6), 653–663.
Phillips, D.H., Miller, J.A., Miller, E.C., Adams, B., 1981. Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1′-hydroxyestragole. Cancer Res. 41 (1), 176–186.
Punt, A., Freidig, A.P., Delatour, T., et al., 2008. A physiologically based biokinetic (PBBK) model for estragole bioactivation and detoxification in rat. Toxicol. Appl. Pharmacol. 231 (2), 248–259.

Punt, A., Paini, A., Boersma, M.G., et al., 2009. Use of physiologically based biokinetic (PBBK) modeling to study estragole bioactivation and detoxification in humans as compared with male rats. Toxicol. Sci. 110 (2), 255–269.

Punt, A., Paini, A., Spenkelink, A., et al., 2016. Evaluation of interindividual human variation in bioactivation and DNA adduct formation of estragole in liver predicted by physiologically based kinetic/dynamic and monte carlo modeling. Chem. Res. Toxicol. 29 (4), 659–668.

Randerath, K., Haglund, R.E., Phillips, D.H., Reddy, M.V., 1984. 32 P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. Carcinogenesis 5 (12), 1613–1622.

Rietjens, I.M., Punt, A., Schilter, B., Scholz, G., Delatour, T., van Bladeren, P.J., 2010. In silico methods for physiologically based biokinetic models describing bioactivation and detoxification of coumarin and estragole: implications for risk assessment. Mol. Nutr. Food Res. 54 (2), 195–207.

Schulte-Hubbert, R., Küpper, J.-H., Thomas, A.D., Schrenk, D., 2020. Estragole: DNA adduct formation in primary rat hepatocytes and genotoxic potential in HepG2-CYP1A2 cells. Toxicology 444, 152566.

Scientific Committee on Food (SCF), 2001. Opinion of the Scientific Committee on Food on Estragole (1-Allyl-4-methoxybenzene). . http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf.

Szabo, M., Veres, Z., Baranyai, Z., Jakab, F., Jemnitz, K., 2013. Comparison of human hepatoma HepaRG cells with human and rat hepatocytes in uptake transport assays in order to predict a risk of drug induced hepatotoxicity. PLoS One 8 (3) e59432.

Van Den Berg, S.J., Restani, P., Boersma, M.G., Delmulle, L., Rietjens, I.M., 2011. Levels of Genotoxic and Carcinogenic Ingredients in Plant Food Supplements and Associated Risk Assessment. .

Wiseman, R.W., Fennell, T.R., Miller, J.A., Miller, E.C., 1985. Further characterization of the DNA adducts formed by electrophilic esters of the hepatocarcinogens 1′-hydroxysafrole and 1′-hydroxyestragole in vitro and in mouse liver in vivo, including new adducts at C-8 and N-7 of guanine residues. Cancer Res. 45 (7), 3096–3105.

Yang, S., Diem, M., Liu, J.D., et al., 2020a. Cellular levels and molecular dynamics simulations of estragole DNA adducts point at inefficient repair resulting from limited distortion of the double-stranded DNA helix. Arch. Toxicol. 1–17.

Yang, S., Liu, J.D., Diem, M., et al., 2020b. Molecular dynamics and in vitro quantification of safrole DNA adducts reveal DNA adduct persistence due to limited DNA distortion resulting in inefficient repair. Chem. Res. Toxicol. 33 (9), 2298–2309.