Letter to the Editor

Thierry Delatour

Answer to Prof. Pfohl-Leszkowicz's Letter to the Editor

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We appreciate that Prof. Pfohl-Leszkowicz *et al.* [1] paid much attention to our work [2]. We considered their comments carefully and would like to take the opportunity of the present letter to bring some clarifications towards concerns raised.

(i) Prof. Pfohl-Leszkowicz *et al.* raise concerns about the LOD of our method. Unfortunately, however, their comments are not supported by any definition of this parameter. In the field of analytical sciences, the LOD is the lowest concentration of an analyte in a sample that can be detected, *i. e.* it specifies whether an analyte is above or below a certain value. The LOD is commonly based on the lowest concentration of an analyte in a sample that generates a response with a S/N of 3:1. This definition, which was applied in our work, is commonly used within the scientific community [3–6] and consequently was not questioned during the peer-review process.

The chromatographic profile of a blank DNA hydrolysate supplemented with $^{15}N_5$-dGuoOTA (Figure 5) exhibits a very low signal in the trace *m/z* 633 → 429 (dGuoOTA) with a S/N <2:1 (signal not apparent anymore if half the intensity is considered). According to the definition mentioned above, this shows that the contribution of the non-labelled adduct as an impurity of the $^{15}N_5$-dGuoOTA is below the LOD. However, a S/N at 3:1 is obtained when DNA hydrolysates (500 μg DNA equivalent) are supplemented with 10 fmol of dGuoOTA (second chromatographic profile in Figure 5), demonstrating that the LOD of our LC-MS/MS method is 10 fmol dGuoOTA on-column (corresponding to 3.5 adducts per 10^9 nucleotides using 500 μg DNA). In contrast, dGuoOTA in kidney DNA obtained from rats treated with a known carcinogenic dose was consistently below the LOD (*i. e.* S/N <3:1), both after 28- and 90-day treatment (as Prof. Pfohl-Leszkowicz *et al.* correctly pointed out "this impurity is not detected in the rat kidney samples (Figure 6)").

Prof. Pfohl-Leszkowicz *et al.* calculated a LOD based on the sum of the amount of dGuoOTA added to the DNA hydrolysate (10 fmol) and the non-labelled contribution of $^{15}N_5$-dGuoOTA, which they estimated to be *ca.* 12.5 fmol. However, this approach is not correct. Using isotope dilution mass spectrometry-based methods, the contribution of a potential impurity in the mass transition of interest is not considered, unless the impurity is present in detectable amounts, *i. e.* the intensity of the signal originating from the impurity is above a S/N of 3:1. In the absence of a detectable signal in a blank sample, the LOD is determined by spiking the sample with the standard. Obviously, only the amount of analyte added to the sample can be used to determine the lowest concentration detected. Since equal amounts of internal standard (in this case, $^{15}N_5$-dGuoOTA) are added to both standard dilutions and samples, the presence of trace amounts of dGuoOTA in the internal standard does not present a problem for quantification.

Prof. Pfohl-Leszkowicz *et al.* claim that the transition *m/z* 633 → 517 is"less specific" to the adduct than transition *m/z* 633 → 429. It is unfortunate that Prof. Pfohl-Leszkowicz *et al.* confuse specificity, selectivity and characteristic product ion. Any signal is characteristic as long as the pertaining transition is observed in the product-ion spectrum (CIDS), while the selectivity (and not specificity) is improved only with additional information related to the presence of the analyte (for instance, an additional transition monitored). In the case of dGuoOTA, both transitions *m/z* 633 → 429 and *m/z* 633 → 517 are characteristic (both product ions at 517 and 429 Th are observed in the CIDS) while the selectivity is not altered if, irrespective to the choice, only one of these two transitions is monitored. In contrast, the selectivity is improved when two transitions are recorded instead of a single one. Therefore, the absence of a signal at transition *m/z* 633 → 517 observed in Figure 6 (for OTA-treated rat at a dose of 210 μg/kg b.w. for 90 days) is a strong argument against the presence of dGuoOTA in the sample. It should be pointed out that, under our acquisi-

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tion conditions and according to chromatographic profiles exhibited in Figure 4, the intensity of the signal obtained with transition *m/z* 633 → 517 is approximately 80% of the *m/z* 633 → 429. Consequently, the presence of dGuoOTA in the kidney DNA of the 90-day-OTA-treated rat would have generated an obvious signal at the transition *m/z* 633 → 517.

(ii) In the second part of their letter, Prof. Pfohl-Leiszkowicz *et al.* discuss the genotoxicity of OTA and conclude that OTA-mediated DNA-damage may be responsible for tumour formation in the rat. We do concur with this statement provided that genotoxicity is broadly defined as the potential to produce DNA-damage. However, the issue addressed in our paper was not to determine if OTA causes DNA-damage. As mentioned by Pfohl-Leiszkowicz *et al.*, it is well documented and acknowledged that OTA increases oxidative stress and consequently DNA-damage. The actual question was whether OTA forms covalent DNA-adducts in rat kidney *in vivo*. No DNA-adduct was found suggesting that OTA-mediated DNA damage does not involve direct DNA-binding. From a risk assessment perspective this result is important. It indicates that, although OTA may be genotoxic, OTA is likely to act through a thresholded mechanism such as oxidative stress. This conclusion was recently endorsed by international expert groups including the contaminant panel of the European Food Safety Authority [7] and the WHO Joint Expert Committee on Food additives [8].

In summary, our results indicate that the adduct characterized by Dai *et al.* [9] is not observed in kidney DNA of rats OTA-treated with a limit of detection at 3.5 adducts *per* 10^9 nucleotides. This confirms that OTA does not act through DNA-adduct formation. The actual mode of action involved is still unclear but is likely to involve a number of integrated mechanisms including oxidative stress, cell proliferation and activation of specific cell signalling pathways.

The authors have declared no conflict of interest.

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