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Oral exposure to drugs with immune-adjuvant potential induces hypersensitivity responses to the reporter antigen TNP-OVA

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ABSTRACT

Immune-mediated drug hypersensitivity reactions are important causes of black box warnings and drug withdrawals. Despite the high demand for pre-clinical screening tools no validated *in vitro* or *in vivo* models are available.

In the current study we used a previously described oral administration model using Trinitrophenyl-Ovalbumin (TNP-OVA) as an antigen to report immuno-adjuvating effects of the analgesic drug acetaminophen (APAP) and its non hepatotoxic regioisomer (AMAP), the antibiotic ofloxacin (OFLX), the anti-epileptic drug carbamazepine (CMZ), and the anti-diabetic drug metformin (MET). Furthermore, APAP and AMAP were tested in a popliteal lymph node assay (PLNA) combined with TNP-OVA as reporter antigen.

C3H/HeOuJ mice were dosed by oral gavage with diclofenac (DF), APAP, AMAP, OFLX, MET or CMZ. On the first exposure day, the mice received an intraperitoneal injection with TNP-OVA. Fifteen days later, they were ear-challenged with TNP-OVA and delayed type hypersensitivity (DTH) responses were assessed 24 hours later. One week after challenge, the ear-draining lymph node was removed and TNP-specific antibody-secreting cells were determined. DF, APAP, CMZ and OFLX showed a significant increase in DTH responses to ear-injection with TNP-OVA, whereas AMAP and MET did not. C57BL/6 mice were slightly less responsive to APAP and DF after oral gavage and importantly both AMAP and APAP were negative in the RA-PLNA.

The present work shows that the oral exposure model using reporter antigens and the RA-PLNA may serve to screen the immune-adjuvant potential of new chemical entities during pre-clinical drug development.

Key words: Immune Mediated Drug Hypersensitivity, preclinical testing, allergenic drugs, oral administration, Reporter Antigen

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INTRODUCTION

Immune-mediated drug hypersensitivity reactions (IDHR) are among the most frequent reasons for failure of new drugs. IDHR may cause patients to be admitted to hospital with life threatening effects like drug-induced liver injury or toxic epidermal necrolysis. Unfortunately, due to their low frequency, IDHR are often unnoticed until a drug has been marketed and used in the general population for some time (Naisbitt *et al.* 2000; Pourpak *et al.* 2008). Clearly, there is a need for predictive models to test the sensitizing capacity of new drugs during early stages of development.

The aetiology of IDHR is very complex. Low molecular weight drugs as such are generally not immunogenic, *i.e.* are unable to cause drug-specific sensitization. However, these compounds or metabolites thereof may be able to bind to proteins to form drug-protein adducts or otherwise induce neo-antigens, which can subsequently be presented to T cells by antigen presenting cells or recognized by B cells. In addition, costimulatory help that may arise from immune-adjuvant effects is indispensable for optimum immunization.

The popliteal lymph node assay (PLNA) in its original version measuring increases in draining lymph node weight or cellularity upon subcutaneous footpad injection of a drug, appears to enable prediction of a drug's immune-stimulating capacity (Pieters 2001; Weaver *et al.* 2005). Addition of reporter antigens (RA) in the PLNA further improves this assay by allowing discrimination between the adjuvant (using TNP-OVA) or sensitizing (using TNP-Ficoll) capacity of drugs (Gutting *et al.* 1999; Pieters 2001). However, although the RA-PLNA can be used as a straightforward screening test for new compounds, chemicals are injected subcutaneously and induce a local response. Hence, this way excludes major influences of gastrointestinal and hepatic metabolism on the local response.

Previous studies demonstrate the ability of an oral mouse model using RA, to identify the immune-adjuvant or sensitizing capacity of drugs (Gutting *et al.* 2002a; Nierkens *et al.* 2005).

63 In the current study we extended this data by testing an additional selection of drugs in
64 combination with TNP-OVA as RA. As positive compounds the analgesic drug
65 acetaminophen (APAP), the antibiotic ofloxacin (OFLX), the non-steroidal anti-inflammatory
66 drug diclofenac (DF) and the anti-epileptic drug carbamazepine (CMZ) were used. As control
67 compound for APAP its non-hepatotoxic regioisomer 3'-hydroxyacetanilide (AMAP) and as
68 an ADR-negative compound the anti-diabetic drug metformin (MET) were selected.

69 Although some IDHR have been reported for APAP, this compound was mainly chosen for
70 its well-described adverse reactions in the liver. At therapeutic doses, APAP is metabolized
71 by cytochrome P450 to NAPQI, and subsequently detoxified by glutathione. When overdosed,
72 NAPQI depletes the liver of glutathione, allowing binding of metabolites to hepatic proteins
73 causing toxicity (James *et al.* 2003). The isomer of APAP, AMAP, has also been shown to
74 form reactive metabolites. However, it does not induce liver toxicity and glutathione depletion
75 to the extent APAP does, and therefore was used as control compound for APAP (Rashed *et*
76 *al.* 1990; Salminen *et al.* 1998).

77 DF is a widely used drug, which incidentally induces severe hepatotoxicity. This compound is
78 capable to form drug-protein adducts (Boelsterli 2003; Naisbitt *et al.* 2007). Furthermore,
79 being a non-selective cyclooxygenase (COX) inhibitor, DF can cause gastrointestinal
80 irritation and ulceration, possibly resulting in an inflammatory environment. Moreover, DF
81 has previously been tested in the (RA)-PLNA and oral models using either TNP-OVA or
82 TNP-Ficoll and is regarded as positive control compound in these models (Gutting *et al.*
83 2002c; Gutting *et al.* 2002b; Nierkens *et al.* 2005).

84 Adverse reactions caused by CMZ involve the liver, but are often also skin-related. This
85 compound has been shown to form reactive intermediates and drug-protein adducts
86 (Lillibridge *et al.* 1996; Ju and Uetrecht 1999; Njoku 2010). In addition, CMZ and CMZ

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metabolite-specific T cells have been identified in CMZ-sensitized patients (Mauri-Hellweg *et al.* 1995; Wu *et al.* 2006).

Fluoroquinolones, e.g. ofloxacin (OFLX) exhibit gastro-intestinal and skin-related side effects (Rubinstein 2001). As for CMZ, fluoroquinolones-specific T cells have also been observed in allergic patients (Schmid *et al.* 2006).

We have so far mainly used C3H/HeOuJ mice for the oral TNP-OVA approach, and furthermore found that BALB/c mice appeared to be less suitable. However, since an important read-out parameter appeared to be Th1-dependent DTH responses, we compared the C57BL/6 strain, claimed to be biased towards cellular immune-responses, with the C3H/HeOuJ strain, known to be biased towards humoral responses.

The results described here show that the combination of a RA-PLNA and an oral administration protocol using reporter antigens may be able to determine immune-adjuvant effects of new chemical entities and can be a useful tool in preclinical screening.

MATERIALS & METHODS

Mice

Four to six weeks old female C3H/HeOuJ (C3H) or C57BL/6Jlco (B6) mice from Charles River (France and Germany, respectively) were used. Mice were specific pathogen-free and maintained under barrier conditions in filter-topped macrolon cages with wood chip bedding at a mean temperature of 23 ± 2 °C, 50-55 % relative humidity and a 12 hour light/dark cycle. Drinking water and standard laboratory food pellets were provided *ad libitum*. The experiments were conducted according to the guidelines, and with permission from the animal experiments committee of Utrecht University.

Chemicals

Chemicals were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands) unless stated otherwise. TNP-OVA and TNP-BSA was obtained from Biosearch Technologies (Novato, CA).

All compounds for subcutaneous injection were dissolved in saline and compounds for oral administration were dissolved in distilled water (Aqua B. Braun, Melsungen, Germany). CMZ was dissolved in 1 % carboxymethylcellulose (CMC) in H₂O.

RA-PLNA

Naïve B6 mice were injected subcutaneously into the hind footpad (toe-to-heel) with a freshly prepared mixture of APAP (0.3 or 1 mg) or AMAP (0.3 or 1 mg) together with a sub-sensitizing dose (10 µg) of TNP-OVA in 50 µl saline. Seven days after drug injection, mice were sacrificed by cervical dislocation and the popliteal lymph node (PLN) was removed and separated from adherent fatty tissue. PLN were placed in ice-cold complete RPMI 1640 supplemented with 2.5 % fetal calf serum and 1 % penicillin/streptomycin (RPMI/2.5 %) and

single cell suspensions were prepared, washed and resuspended in 0.5 ml RPMI/2.5 %. Cell numbers were determined using a Coulter Counter (Beckman Coulter) and adjusted to 1×10^6 cells/ml.

Oral exposure protocols

C3H mice received OFLX, APAP, AMAP, CMZ or MET by oral gavage (200 μ l) in different regimens chosen using the maximum tolerable dose as described in the datasheet of the compounds and used in literature. OFLX was dosed at 100, 300 or 1000 mg/kg, MET was dosed at 50, 100 or 500 mg/kg and APAP and AMAP was administered at 30, 100 or 300 mg/kg for 7 consecutive days. CMZ was dosed 50 or 100 mg/kg for 7 days, or at 250 mg/kg on day 1 (Figure 1).

To study strain differences C3H mice and B6 mice received APAP (150 mg/kg) for 7 days or a single dose (75 mg/kg) of DF on day 1 (Figure 1).

On the first day of drug exposure mice were injected intraperitoneally with a sub-sensitizing dose (10 μ g in 200 μ l saline) of TNP-OVA to assure systemic responses. Mice were challenged with TNP-OVA (10 μ g in 20 μ l saline) in the ear on day 15 to assess delayed type hypersensitivity (DTH) responses. Ear thickness was measured under isofluran anaesthetics using a digital microcalliper (Mitutoyo, Japan).

One week after challenge, the mice were sacrificed by cervical dislocation. The auricular lymph node (ALN) was removed and separated from adherent fatty tissue. Single cell suspensions of the ALN were made as described for the PLN.

T-cell depletion

C3H mice were treated with DF or APAP according to the oral administration protocol described in Figure 1. In addition, mice received i.p. injections with 250 μ g T-cell depleting

antibody (anti-CD3 clone 17A2) in 200 μ l saline on day 13 and 14. The antibody was purified from culture supernatant using ammonium sulphate gradient on thiophilic agarose resin.

ELISpot assay

ELISpot assay, to detect TNP-specific antibody secreting cells, was performed as described previously (Schielen *et al.* 1995). In brief, 5×10^5 cells were added to TNP-BSA coated Immobilon-P membranes (Immobilon PVDF transfer, Millipore, Etten-Leur, the Netherlands). After cell incubation (4 hours, 37 °C), AP-conjugated IgG1 or IgG2a antibodies (Southern Biotechnology Associates (SBA), Inc., Birmingham, AL) were added and incubated overnight. After several washing steps, membranes were stained with para-nitroblue tetrazolium (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 5-bromo-4-chloro-3-indolylphosphate toluidine salt in dimethylformamide to visualize TNP-specific antibody spots. Spots were counted by sight by at least two independent observers.

Statistical analysis

Multiple comparisons of group means were analysed using one-way-analyses of variance (ANOVA) with Bonferonni as *post-hoc* test. A value of $p < 0.05$ was considered significantly different compared to controls (Graphpad prism 4 for Windows, Graphpad software Inc.)

RESULTS

APAP nor AMAP stimulate antibody responses to TNP-OVA in the RA-PLNA

The immune-adjuvant capacity of APAP and AMAP was examined in the RA-PLNA using TNP-OVA as reporter antigen. Neither APAP nor AMAP was able to induce a significant increase in PLN cellularity (Figure 2A). Furthermore, the amount of TNP-specific IgG1-producing ASC was not increased by either chemical (Figure 2B). The lack of a response to APAP and AMAP observed in the RA-PLNA indicates that the metabolizing capacity in the footpad is insufficient to detect the adverse immune effects of this compound in this assay.

APAP, CMZ, DF and OFLX, but not AMAP and MET stimulate CD3-dependent TNP-OVA specific DTH responses

In contrast to the RA-PLNA, the described oral exposure model does take metabolism into account. Four compounds known to cause ADR and two ADR-negative compounds were administered orally to C3H mice. To determine RA-specific DTH responses, mice treated to drugs and TNP-OVA, received a subcutaneous TNP-OVA injection into the ear. Twenty four hours later the increase in ear thickness was determined. Animals exposed to APAP showed a significant dose dependent increase in DTH responses (Figure 3A), whereas its non-hepatotoxic regioisomer AMAP did not cause an increase in DTH responses at any dose (Figure 3B). Oral OFLX treatment showed a significant increase in the RA-specific DTH response after exposure to 300 mg/kg but not after exposure to 1000 mg/kg OFLX (Figure 3C). The negative compound MET did not induce a DTH response at any dose (Figure 3D), although the control group did have a high background response.

192 Exposure to DF and the highest concentration of CMZ caused a significant increase in RA-
193 specific ear swelling when compared to control animals (Figure 3E & 3F).

194 T-cell depletion (using anti-CD3) resulted in a diminished DHT response in DF or APAP
195 treated mice, which is indicative of the T-cell dependence of the DTH response measured
196 (Figure 4). These results show that of the selection of compounds tested; only those linked to
197 any kind of toxicity induce a T-cell dependent DTH response.

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199 ***Oral exposure to drugs may result in TNP-OVA specific Antibody Secreting Cells***

200 Seven days after ear challenge with the RA, TNP-OVA-specific IgG1 antibody responses in
201 the ALN were determined using the ELISpot assay.

202 Exposure to APAP resulted in increases in RA specific IgG1 ASC in some animals (Figure
203 5A), whereas AMAP exposure did not show changes in IgG1 levels (Figure 5B). Of all other
204 compounds, only DF caused a significant increase in the amount of IgG1 ASC (Figure 5C-F).

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206 ***Oral administration of a drug induces higher DTH responses in C3H mice compared to B6*** 207 ***mice***

208 To compare RA-specific responses of different mouse strains, B6 mice and C3H mice were
209 subjected to the oral model described in Figure 1.

210 In C3H mice, the DTH response towards TNP-OVA was significantly increased compared to
211 controls when mice were treated with either DF or APAP (Figure 6A). B6 mice also displayed
212 a DTH response towards the RA after oral administration of DF or APAP, but this response
213 was not significant. Treatment with DF and APAP resulted in increased numbers of IgG1
214 secreting cells, which were only significant in B6 mice (Figure 6B). In contrast to the results
215 described in figure 5F and previous experiments (data not shown), exposure of C3H to DF in
216 this experiment did not result in significantly increased numbers of IgG1 secreting cells,

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217 which is indicative of the variability of this parameter. These results suggest that the strenght
218 drug induced responses are strain-dependent.

DISCUSSION

In this study the PLNA and a previously described oral sensitization model, both in combination with TNP-OVA as reporter antigen, have been used to test the immune-adjuvant capacity of an additional selection of pharmaceuticals (Pieters 2001; Nierkens *et al.* 2005; Weaver *et al.* 2005). Previously, it has been discussed that immunostimulation of TNP-specific responses in these models allows assessment of adjuvant or danger-inducing activity of (new) compounds.

Of the pharmaceuticals used in this study APAP, OFLX, DF and CMZ, but not MET and AMAP, were clearly able to induce increased responses towards TNP evidenced in particular by moderate dose-dependent increases of DTH responses to TNP-OVA. Importantly these responses were T-cell dependent and hence can be regarded as relevant DTH responses (Posadas and Pichler 2007; Rozieres *et al.* 2009).

In contrast to the DTH responses, TNP-specific antibody responses measured by ELISpot were less pronounced, and although the ELISpot as such is regarded a sensitive assay, the high variability of this assay hinders drawing clear conclusions. The use of TNP-Ficoll as reporter antigen to register neo-antigen specific T cells, or the inclusion of other read-out parameters might be an improvement to the oral exposure model. Importantly, the use of a RA allows testing numerous drugs using the same read-out system, but the presence of drug-specific (T-cell) responses remains undetermined. Of the compounds we tested here, DF, OFLX and CMZ have previously been shown to be able to induce drug-specific T cells in drug-sensitized humans (Naisbitt *et al.* 2003; Beeler *et al.* 2006; Schmid *et al.* 2006; Wu *et al.* 2006). Isolated T cells from sensitized patients were stimulated with the culprit drug or its metabolite, resulting in the production of a specific set of cytokines secreted by these cells (Lochmatter *et al.* 2009). Furthermore, the expression of certain T cell activation markers like CD69 were found to be upregulated on T cells of sensitized patients, when stimulated with the

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3 244 specified drug (Beeler *et al.* 2008). In addition to the DTH and number of antibody secreting
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8 246 of cells with drugs or their metabolites could be included as complementary parameters in the
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10 247 oral administration model. To identify effects caused by the drug itself or by metabolites
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12 248 thereof, mice could be challenged with proteins conjugated with the different compounds or
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15 249 their metabolites.
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17 250 The use of DF in this study was based on previous studies, and our results confirmed that this
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19 251 drug is able to induce robust DTH responses towards the RA upon oral administration
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21 252 (Nierkens *et al.* 2005). However, the mechanism by which DF stimulates responses towards
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23 253 the RA is yet unknown. DF is able to increase cell numbers in the draining lymph node after
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25 254 sc injection into the footpad, despite the fact that metabolism is absent in the paw (Gutting *et*
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27 255 *al.* 1999; Gutting *et al.* 2002b; Gutting *et al.* 2002a). Furthermore, incubation of isolated
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29 256 splenocytes or several hepatocyte cell lines with DF resulted in the induction cell death (data
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31 257 not shown and (Wang *et al.* 2002)). This indicates that the parent compound is able to induce
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33 258 cell stress or inflammation, possibly attracting metabolising macrophages. Whether
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35 259 glucurodination of DF by liver metabolism and the subsequent enterohepatic circulation of
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37 260 protein-coupled metabolites are also involved after oral exposures remains to be established.
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39 261 Furthermore, the direct pharmacological effects of oral exposure to DF might be of influence
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41 262 on the response. Recently, we have shown that DF stimulates the allergic response to peanut
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43 263 in a mouse model. In that study we observed that DF, being a COX inhibitor, causes damage
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45 264 to the epithelial lining of the gut and the stomach thereby possibly causing a local response
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47 265 (Bol-Schoenmakers *et al.* 2010).
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49 266 The immune-adjuvant effects of APAP appeared to be dependent on the route of exposure.
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51 267 APAP was not able to enhance TNP-OVA responses in the RA-PLNA, but the compound
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53 268 showed a dose dependent DTH response to TNP-OVA when used in the oral exposure model.
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This indicates that metabolism is required for the toxic effect of APAP, which is lacking in the paw. In line with this, APAP (up to 2 mM) was not able to induce direct cell death in H1G1 mouse hepatocytes (data not shown). AMAP did not induce a response in the RA-PLNA or the oral model, despite the fact that AMAP is also metabolized. Metabolites of AMAP and APAP have been shown to bind to different proteins in hepatocytes, with APAP derivatives binding to mitochondrial proteins, and AMAP derivatives binding merely to cytosolic proteins (Rashed *et al.* 1990; Roberts *et al.* 1990; Tirmenstein and Nelson 1991). In addition, APAP is able to deplete mitochondrial glutathione and cause disruption of calcium homeostasis to a higher extent than AMAP (Tirmenstein and Nelson 1989). Apparently, the metabolites of APAP and AMAP are toxicologically different, resulting in their different adjuvant effects. Notably, AMAP has not been tested as a drug in the general population, and we can therefore not completely rule out the possibility of this compound to induce adverse reactions. However, in present experiments, it does not act as an adjuvant, and is therefore considered a negative compound.

Similar to DF also OFLX was able to induce a response in the PLNA, and also in a modified lymph node assay using the ear draining lymph node as read-out (Nierkens *et al.* 2004). Therefore, this compound most likely does not require metabolism to become immune stimulating in the PLNA. In contrast to all other positive compounds studied, OFLX showed a higher DTH response at a dose of 300 mg/kg, than after a dose of 1000 mg/kg. Similar results have been shown previously for DF and D-Pen in combination with TNP-Ficoll (Gutting *et al.* 2003; Nierkens *et al.* 2005). This illustrates that determination of the correct dosing and duration of administration of the compound is of crucial importance in further exploration of this model.

CMZ injection into the paw resulted in increased cellularity in the PLNA, indicative of an adjuvant capacity of this drug (Kammüller *et al.* 1989). CMZ is also metabolized after oral

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294 administration, and similar to DF and OFLX both metabolites and the parent compound could
295 act as immune-adjuvant.

296 As described previously, the dosing and subsequent challenge with TNP-OVA is very delicate,
297 and this may be the reason of the high background DTH response observed after metformin
298 exposure (Nierkens *et al.* 2005). Consequently, these high background levels hamper the
299 conclusions that can be drawn following oral administration of this compound. However,
300 since no significant differences were observed between treatment groups metformin is
301 regarded a negative compound.

302 As shown in the present and in previous studies using the (RA)-PLNA or oral administration
303 models, the mouse strain influences on the outcome of the experiments (Kammüller *et al.*
304 1989; Nierkens *et al.* 2005). When exposed to DF or APAP, B6 mice showed a DTH response,
305 but C3H mice showed higher DTH responses towards the same compounds. In contrast, the
306 number of TNP-specific antibody secreting cells was higher in B6 compared to the number of
307 antibody secreting cells in C3H mice. Although strain-specific responses have been found
308 frequently in studies on drug induced immune responses, it is remarkable that a strain claimed
309 to be a typical Th1 responder shows higher antibody responses than typical Th2 responding
310 C3H mice (Pieters and Nierkens 2007).

311 In addition to effects due to genetic background, we have previously demonstrated that the
312 chemical itself might be as important in determining the immunological outcome (Albers *et al.*
313 1998). For example, DF is capable of inducing intestinal damage and COX inhibition, and e.g.
314 APAP in high doses results in liver damage. Although the doses for all compounds have been
315 chosen carefully, possible pharmacological effects of the compounds should be taken into
316 account when analysing the data.

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3 317 In conclusion, the present work shows that the oral exposure model using RA and the RA-
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FIGURE LEGENDS

Figure 1. Oral exposure protocol to assess the immune-adjuvant capacity of drugs.

The immunosensitizing capacity of DF, APAP, AMAP, OFLX, CMZ and MET to TNP-OVA was assessed by treating C3H mice orally with the specified drugs. On the first day of exposure mice were injected ip with TNP-OVA (10 µg). After fifteen days, mice were ear-challenged with TNP-OVA. The same protocol was used to compare strains.

Figure 2. Immune-adjuvant capacity of APAP and AMAP.

B6 mice were injected sc with 0.3 or 1 mg APAP or AMAP in the hind footpad together with a sub-sensitizing dose of TNP-OVA (10 µg). After seven days the popliteal lymph node was removed, and subsequently lymph node cellularity (A) and the amount of IgG1 ASC (B) was determined.

Figure 3. DTH response measured by ear swelling.

C3H mice were orally treated with 30/100/300 mg/kg APAP/AMAP (A, B), 100/300/1000 mg/kg OFLX (C), 50/100/500 mg/kg MET (D) or 50/100 mg/kg CMZ (E) for seven consecutive days. DF 75 mg/kg and 250 mg/kg CMZ were given only on day 1. Administration of the drug was performed by oral gavage and the RA by i.p. injection. At day 15 ear thickness was measured after which mice received a s.c. injection in the ear with TNP-OVA. After 24 h, ear thickness was measured again to determine the DTH response. * p<0.05, ** p<0.01, *** p<0.001 significantly different compared to vehicle controls.

Figure 4. T-cell dependence of DTH responses measured by ear swelling.

C3H mice received one dose of 75 mg/kg DF or 150 mg/kg APAP for seven consecutive days. Administration of the drug was performed by oral gavage and the RA by ip injection. On day

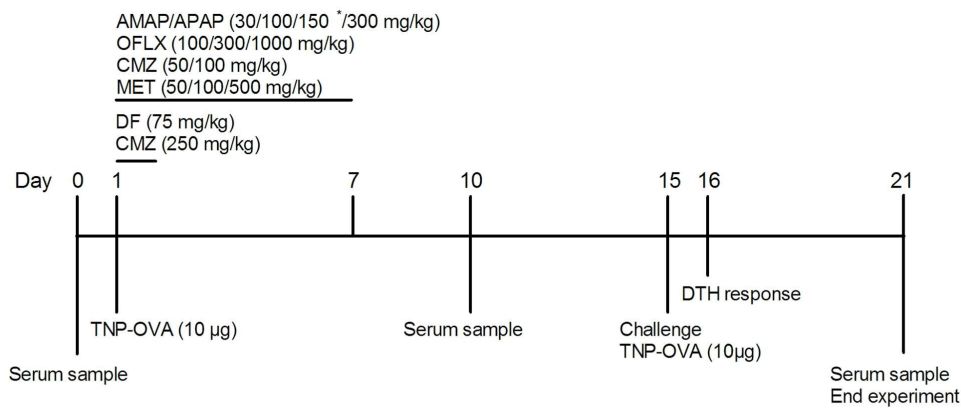
13 and 14, T cells were depleted by ip injection with an anti-CD3 antibody. At day 15 ear thickness was measured after which mice received a s.c. injection in the ear with 10 µg TNP-OVA (20 µl total volume). After 24 h, ear thickness was measured again to determine the DTH response. * p<0.05.

Figure 5. TNP-specific IgG1 ASC in after oral pre-treated with DF, OFLX, MET, CMZ, APAP or AMAP in combination with TNP-OVA.

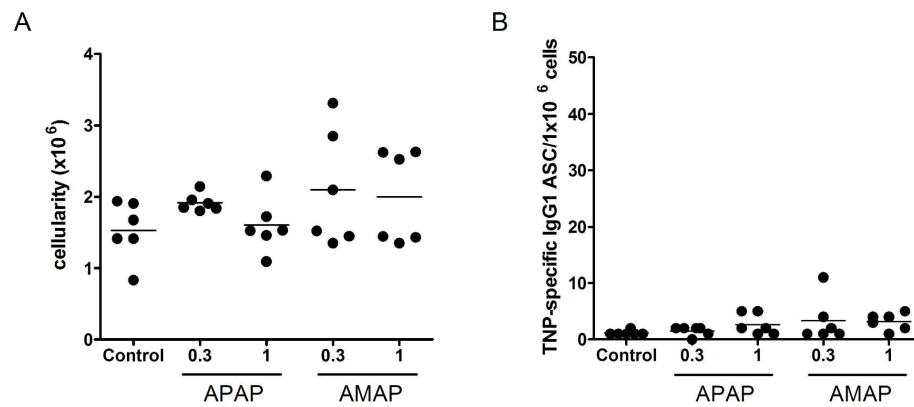
C3H mice were treated with (A) APAP or AMAP (30/100/300 mg/kg), (C) OFLX (100/300/1000 mg/kg), (D) MET (50/100/500 mg/kg) or (E) CMZ (50/100 mg/kg) on 7 consecutive days or DF (75 mg/kg) or CMZ (250 mg/kg) on day 1 by oral gavage. Mice were challenged with the RA on day 15. Seven days after challenge mice were sacrificed and RA specific IgG1 ASC were determined using ELISpot. *** p<0.001.

Figure 6. RA specific immune responses in different mouse strains.

B6 and C3H mice were orally exposed to either DF (1 day, 75 mg/kg), or APAP (7 days, 150 mg/kg). After 15 days the mice were sc challenged in the ear with the RA, and 24 hours later the DTH response was measured (A). One week after challenge the ear draining lymph node was removed and the amount of RA-specific ASC was determined by ELISpot (B). ** p<0.01.



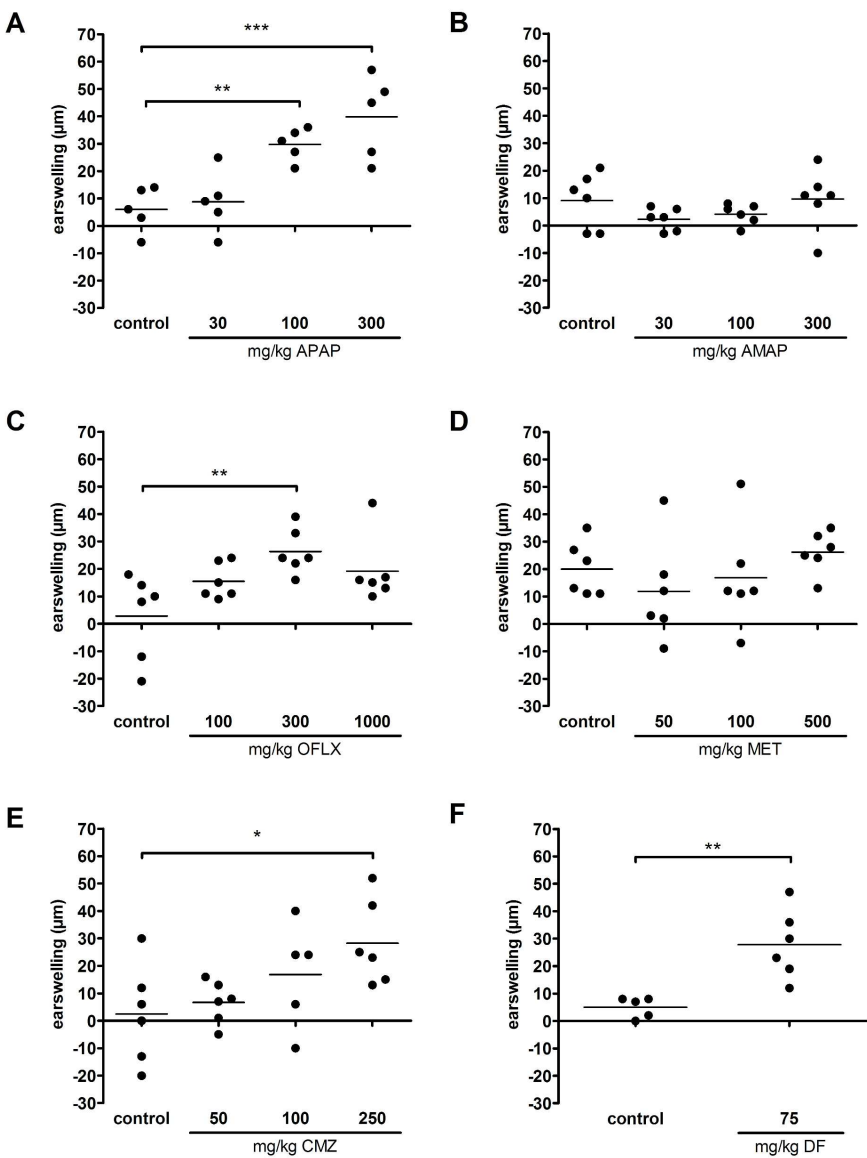
Oral exposure protocol to assess the immunosensitizing capacity of drugs.
The immunosensitizing capacity of DF, APAP, AMAP, OFLX, CMZ and MET to TNP-OVA was assessed by treating C3H mice orally with the specified drugs. On the first day of exposure mice were injected ip with TNP-OVA (10 µg). After fifteen days, mice were ear-challenged with TNP-OVA. The same protocol was used to compare strains.
85x37mm (600 x 600 DPI)



Immunosensitizing capacity of APAP and AMAP

B6 mice were injected sc with 0.3 or 1 mg APAP or AMAP in the hind footpad together with a sub-sensitizing dose of TNP-OVA (10 μ g). After seven days the popliteal lymph node was removed, and subsequently lymph node cellularity (A) and the amount of IgG1 ASC (B) was determined.

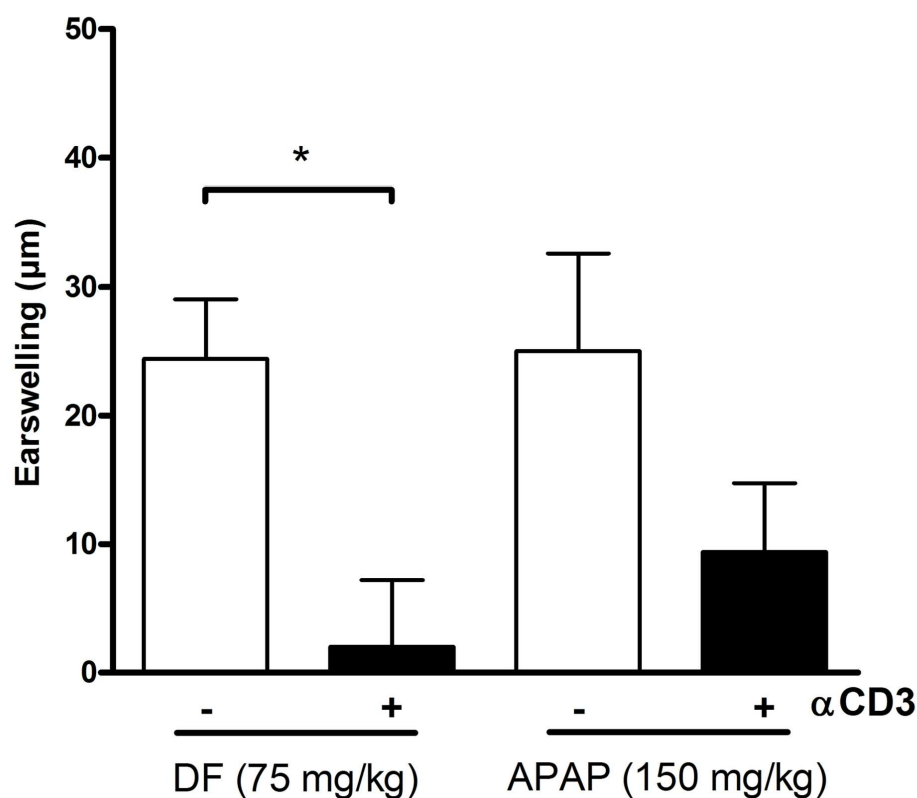
177x80mm (600 x 600 DPI)



DTH response measured by ear swelling

C3H mice were orally treated with 30/100/300 mg/kg APAP/AMAP (A, B), 100/300/1000 mg/kg OFLX (C), 50/100/500 mg/kg MET (D) or 50/100 mg/kg CMZ (E) for seven consecutive days. DF 75 mg/kg and 250 mg/kg CMZ were given only on day 1. Administration of the drug was performed by oral gavage and the RA by i.p. injection. At day 15 ear thickness was measured after which mice received a s.c. injection in the ear with TNP-OVA. After 24 h, ear thickness was measured again to determine the DTH response. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different compared to vehicle controls.

171x215mm (600 x 600 DPI)

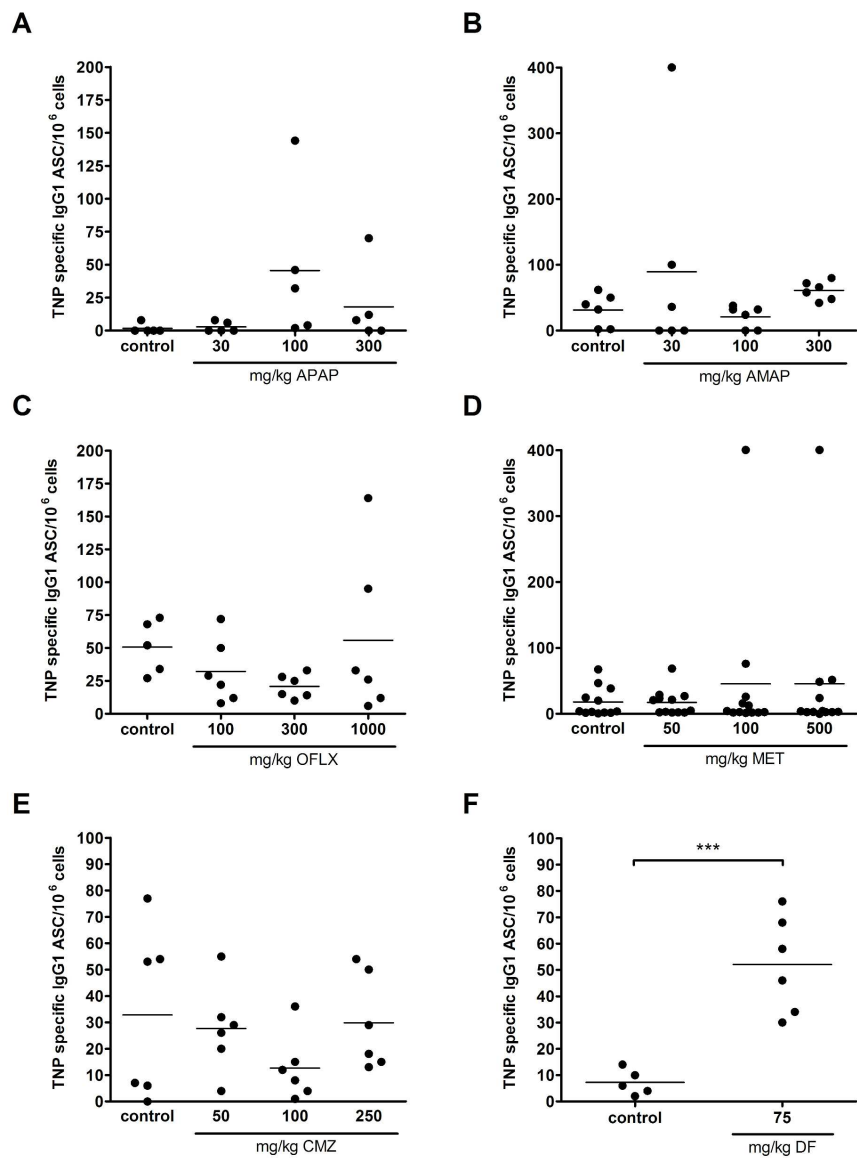


T-cell dependence of DTH responses measured by ear swelling

C3H mice received one dose of 75mg/kg DF or 150mg/kg APAP for seven consecutive days. Administration of the drug was performed by oral gavage and the RA by i.p. injection. On day 13 and 14, T cells were depleted by ip injection with an anti-CD3 antibody or PBS. At day 15 ear thickness was measured after which mice received a s.c. injection in the ear with 10 μg TNP-OVA (20 μl total volume). After 24 h, ear thickness was measured again to determine the DTH response.

* $p < 0.05$.

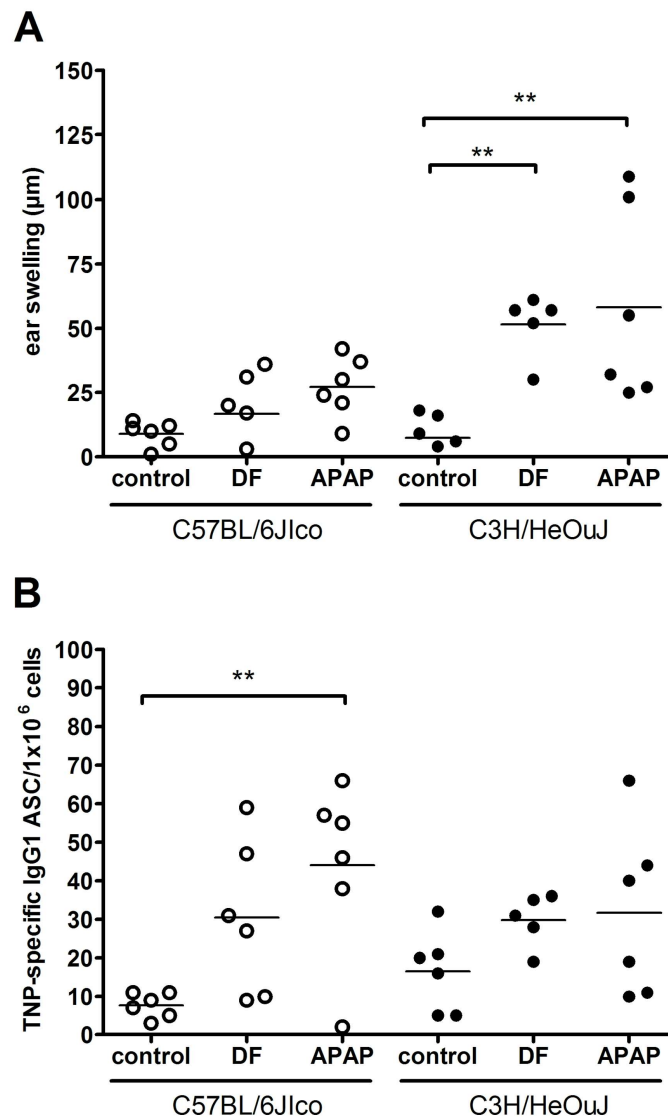
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TNP-specific IgG1 ASC in after oral pre-treated with DF, OFLX, MET, CMZ, APAP or AMAP in combination with TNP-OVA

C3H mice were treated with (A) APAP or AMAP (30/100/300 mg/kg), (C) OFLX (100/300/1000 mg/kg), (D) MET (50/100/500 mg/kg) or (E) CMZ (50/100 mg/kg) on 7 consecutive days or DF (75 mg/kg) or CMZ (250 mg/kg) on day 1 by oral gavage. Mice were challenged with the RA on day 15. Seven days after challenge mice were sacrificed and RA specific IgG1 ASC were determined using ELISpot. *** p<0.001

167x215mm (600 x 600 DPI)



RA specific immune responses in different mouse strains

B6 and C3H mice were orally exposed to either DF (1 day, 75 mg/kg), or APAP (7 days, 150 mg/kg). After 15 days the mice were sc challenged in the ear with the RA, and 24 hours later the DTH response was measured (A). One week after challenge the ear draining lymph node was removed and the amount of RA-specific ASC was determined by ELISpot (B). ** $p < 0.01$.

88x130mm (600 x 600 DPI)