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Inhibition of cytokine production by the herbicide atrazine Search for nuclear receptor targets

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Abstract

The hematological toxicity of the commonly used triazine herbicides is a cause for concern. In a search for molecular targets of these compounds, as their effects paralleled those seen with dexamethasone (DEX), we first looked for interaction with the glucocorticoid receptor. In contrast to the effects on proliferation and cytokine production of DEX, those induced by atrazine were not prevented by the glucocorticoid antagonist RU486. Also, whereas DEX was able to inhibit the promoter activity of genes regulated by NF-κB, atrazine failed to do so. We next looked for interaction with members of the peroxisome proliferator-activated receptor (PPAR) family. No peroxisome proliferation was observed in the liver or kidneys of mice treated with atrazine. Moreover, no PPAR-mediated induction of promoter activity was seen on targets of PPARα, PPARγ, or PPARδ. Similarly, neither atrazine nor simazine were able to stimulate RORα-mediated promoter activity. Finally, no binding of atrazine to the AR was observed. In conclusion, the effects of atrazine-type herbicides most probably do not result from interaction with the above-mentioned nuclear receptors.

Keywords: Atrazine; Glucocorticoid; Retinoid-related orphan receptor; Androgen receptor; PPAR; RU486; Cytokine; Lymphocyte

1. Introduction

Atrazine is the most commonly used herbicide in the US and probably in the world. It is a known ''endocrine disrupter'', interfering with sexual development, at very low doses, in animals and possibly responsible for an increased incidence of prostate cancer in man [1,2]. The LD_{50} values of triazine herbicides for mammals are relatively high (up to several gram/kilogram) and, with the exception of contact hypersensitivity, there are few confirmed reports of toxicity following normal professional exposure. The toxicity of atrazine on the hematopoietic and the immune systems is poorly documented. The increased incidence of non-Hodgkin's lymphoma in farmers, the correlation between pesticide use and the incidence of cancer and more generally ''agriculturally related cancers'' point to a role for pesticides in the development or the progression of cancer [2,3]. Most herbicides have only very weak mutagenic or oncogenic properties but they might favor the development or progression of lymphoma by reducing immune defenses. At high exposure atrazine is toxic for blood-forming organs and the immune system [4,5]. Recently, we have reported effects of micromolar concentrations (less than 1 ppm) of the triazine herbicides atrazine and simazine on cytokine production by human peripheral blood mononuclear cells (PBMC) after exposure *in vitro* [6]. We have now extended these observations and tested the hypothesis that atrazine affects leukocyte function *via* nuclear receptors such as the glucocorticoid receptor (GR),

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Abbreviations: AR, androgen receptor; DEX, dexamethasone; DHT, dihydrotestosterone; ELISA, enzyme-linked immunosorbent assay; GR, glucocorticoid receptor; IFN-γ, interferon-γ; IL, interleukin; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PPAR, peroxisome proliferator-activated receptor; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; rh, recombinant human; ROR, retinoid-related orphan receptor; RU486, mifepristone; TNF, tumor necrosis factor.

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the androgen receptor (AR), members of the PPAR family and the retinoid-related orphan receptor (ROR) α. Other investigators have conclusively shown that the effects of atrazine are not mediated through the estrogen receptor [7,8]. In contrast, weak binding of atrazine to the AR has been reported and was, therefore, tested again [9]. The GR was investigated first in view of the similarity of effects induced by atrazine and the synthetic glucocorticoid DEX [6]. Glucocorticoids have anti-inflammatory and immunosuppressive properties. Indeed, they interfere with nuclear factor-κB (NF-κB) and AP-1, and in this way repress the expression of inflammation-related genes such as interleukin (IL)-6, IL-8, ICAM, and E-selectin, that contain NF-κB and AP-1 response elements [10–17]. Several reports now indicate that activation of PPARs also has effects on leukocytes [18,19]. As some herbicides activate the PPARα or PPARγ, we tested whether atrazine too might activate a member of the PPAR family [20–22]. Finally, possible modulation by triazine herbicides of the RORα, which affects leukocyte function too, was tested [23].

2. Materials and methods

2.1. Reagents

Reagent grade atrazine [2-chloro-4-ethylamino-6-(isopropylamino)-*S*-triazine] (99.0% pure) and simazine [2-chloro-4,6-bis(ethylamino)-*S*-triazine] (99.2% pure), both from Riedel-de-Haën, were first dissolved in DMSO, then diluted in PBS. Dilutions were made in such a way that the final concentration of DMSO in all conditions would be 1%. Solutions were sterilized by filtration. DEX, from Calbiochem, was dissolved in PBS and mifepristone (RU486), a gift from R. Sitruk-Ware, was dissolved in ethanol. The production and the characterization of recombinant murine tumor necrosis factor (TNF) have been described previously [24].

2.2. Plasmids

The IL-6 promoter-containing plasmid p1168hu.IL6P-luc+ was described previously [24,25]. The plasmids p98.IL8P-luc+ and p133.IL8P-luc+ containing respectively 98 or 133 bp of NF-κB-responsive promoter fragments of the human IL-8 promoter were provided by N. Mukaida (Kanazawa Cancer Research Insititute) [26]. pELAMP-luc+, containing the E-selectin promoter, was a gift from D. Goeddel (Tularik Inc.) [27].

2.3. Cells and culture

Murine fibrosarcoma L929sA and rabbit kidney RK13 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum and 5% fetal bovine serum (L929sA) or 10% fetal bovine serum (RK13), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Sixteen hours before induction, cells were seeded in multi-well dishes at a density that results in about 90% confluency at the time of the experiment.

Blood was obtained from six healthy adults. PBMC were isolated using Lymphoprep (Nycomed Pharma) and kept in serum-free medium as described [6,28]. PHA (HA15, Murex), 2 μg/mL, and test substances (or vehicle) were added at the start of the culture. After 3 days, supernatants were collected and kept at −20° for enzyme-linked immunosorbent assays (ELISA). ^{3}H-Thymidine incorporation was measured in parallel cultures.

2.4. Transfection and reporter gene analysis

For the GR study: L929sA cells were stably transfected using the calcium phosphate coprecipitation technique [24]. Apart from the relevant luciferase construct, cells were also transfected with the plasmid pPGKβGeobpA, constitutively expressing a neomycin-resistant β-galactosidase fusion protein under control of the mouse 3-phosphoglycerate kinase promoter (given by P. Soriano, Fred Hutchinson Cancer Research Center). After induction, cells were lysed with lysis buffer (Tropix). Luciferase assays were performed according to the manufacturer's instructions (Promega). Detection of β-galactosidase activity was achieved with a chemiluminescent reporter assay Galacto-Light kit (Tropix). Light emission was quantified in a TiterTek Luminoscan UV 91-6 (Labsystems). The "induction factor" is the ratio of expression levels recorded under induced and non-induced conditions.

For the PPAR and RORα study: A similar strategy as for the GR receptor was used following procedures and with reagents described in detail [23,29,30].

2.5. ELISA

ELISAs were run in duplicate according to manufacturers' instructions (Biosource for interferon-γ (IFN-γ) and TNF-α, Pharmingen for IL-5, and R&D for IL-8). In view of the variability in cytokine production by PBMC from normal donors, results from herbicide (or DEX or RU486)-treated cultures were expressed as percent of control (vehicle only-treated) cultures from the same donor. Measurements were done in duplicate. The two-sided Mann–Whitney rank sum test was used for statistical analysis.

2.6. AR assay

The affinity of atrazine to the recombinant human androgen receptor (rhAR) was measured as described [31]. Briefly, to produce rhAR, a baculovirus insect cell expression system was used (Pharmingen). After transferring the human AR cDNA into baculoviruses, insect cells were infected with these viruses for 40 hr. After collection, cells were disrupted. After centrifugation, the cytosolic

receptor was recovered in the solution. Aliquots of 0.5 mL from the 1:50 diluted receptor preparation were incubated with a constant amount of 5×10^{-10} M ^{3}H-dihydrotestosterone (DHT) and with increasing concentrations of atrazine between 2 nM and 1.25 mM. Separation of bound and free ligands was carried out by incubation with 100 μL of dextran-coated charcoal (4% charcoal and 0.4% dextran) for 5 min and centrifugation. Four hundred milliliters of the supernatant were mixed with scintillation fluid and counted. For comparison, the competition curve of DHT was evaluated: the binding of labeled DHT was reduced to 50% by 1.09 nM DHT.

2.7. Animal treatment

Adult (2- or 7-month-old) CF1 mice obtained from the VITO breeding center were injected i.p. on days 1 and 4 with atrazine (100 mg/kg, 10 mg/mL) as described [32]. Control mice received the vehicle only (carboxymethylcellulose containing 0.5% Tween 80). On day 7, mice were killed by decapitation. Liver and kidneys were quickly removed from two treated (one from each age group) and two control mice and slices fixed in 4% formaldehyde, 1% calcium chloride in cacodylate buffer [33].

2.8. Peroxisome analysis

Peroxisomes were visualized by localization of catalase activity [33]. Seven micrometer cryostat sections were observed by light microscopy. Sixty micrometer sections were embedded in plastic. Random micrographs were taken from 2 μm sections by light microscopy and from ultra-thin sections by electron microscopy. Blinded light micrographs of control and treated animals were ranked by visual estimation of peroxisome number. In electron micrographs at magnification of 9600 times, the number of peroxisomes was counted per area; at higher magnification, shape and clustering of closely adjacent organelles was monitored.

3. Results and discussion

3.1. Effects of atrazine on leukocyte proliferation and cytokine production

We have previously shown that atrazine inhibits cell proliferation and cytokine production, as does DEX [6]. Therefore, we proposed that these effects of atrazine might be mediated by the GR. Experiments were thus repeated in the presence or absence of the glucocorticoid inhibitor RU486. As shown in Tables 1–4, this compound (10^{-7} M) significantly prevented DEX-induced effects, but not atrazine-related effects. RU486 on its own is able to inhibit proliferation and cytokine production ([34] and Tables 1–4). Even so, it was clearly able to prevent inhibitory effects of DEX. Also, in contrast to DEX, atrazine did not reduce IL-8 production. These experiments suggest that effects of atrazine on PBMC are not mediated through the GR.

Table 1
Effect of DEX and RU486, alone or in combination, on proliferation of PBMC[a]

	No RU486	10^{-8} M RU486	10^{-7} M RU486
No DEX	100 ± 0	66 ± 17	67 ± 22
10^{-10} M DEX	85 ± 11	61 ± 14	51 ± 17[b]
10^{-9} M DEX	58 ± 16	70 ± 19	49 ± 14
10^{-8} M DEX	12 ± 10	24 ± 18	53 ± 13[b]
10^{-7} M DEX	4 ± 2	5 ± 4	22 ± 17

[a] Percent of control (no DEX, no RU486); mean ± SD from six donors.
[b] Significant, compared to the cultures treated with the same concentration of DEX in the absence of RU486 ($P < 0.01$).

Table 2
Effect of atrazine and RU486, alone or in combination, on proliferation of PBMC[a]

	No RU486	10^{-8} M RU486	10^{-7} M RU486
No atrazine	100 ± 0	72 ± 26	70 ± 28
3×10^{-8} M atrazine	101 ± 9	81 ± 23	54 ± 9[b]
3×10^{-7} M atrazine	78 ± 9	82 ± 12	59 ± 7[b]
3×10^{-6} M atrazine	57 ± 21	51 ± 20	55 ± 20
1.5×10^{-5} M atrazine	57 ± 24	48 ± 21	40 ± 19

[a] Percent of control (no atrazine, DMSO 1% final, no RU486); mean ± SD from six donors.
[b] Significant, compared to the cultures treated with the same concentration of atrazine in the absence of RU486 ($P < 0.01$).

3.2. Lack of effects of atrazine on NF-κB or AP-1

Possible interaction of atrazine with the GR was investigated also at the molecular level. To test whether the

Table 3
Effect of DEX and RU486, alone or in combination, on cytokine production by PBMC[a]

	IFN-γ	TNF-α	IL-5	IL-8
10^{-7} M RU486	92 ± 19	75 ± 19	63 ± 9	99 ± 26
10^{-8} M DEX	31 ± 15	35 ± 17	13 ± 6	31 ± 6
10^{-8} M DEX + 10^{-7} M RU486	84 ± 14[b]	75 ± 10[b]	52 ± 11[b]	76 ± 21[b]

[a] Percent of control (no DEX, no RU486); mean ± SD from six donors.
[b] Significant, compared to the cultures treated with the same concentration of DEX in the absence of RU486 ($P < 0.01$).

Table 4
Effect of atrazine and RU486, alone or in combination, on cytokine production by PBMC[a]

	IFN-γ	TNF-α	IL-5	IL-8
10^{-7} M RU486	101 ± 32	79 ± 16	68 ± 13	78 ± 13
3×10^{-6} M atrazine	55 ± 15	51 ± 18	88 ± 25	123 ± 8
3×10^{-6} M atrazine + 10^{-7} M RU486	59 ± 22	37 ± 10	54 ± 16	90 ± 38

[a] Percent of control (1% DMSO but no atrazine, no RU486); mean ± SD from six donors.

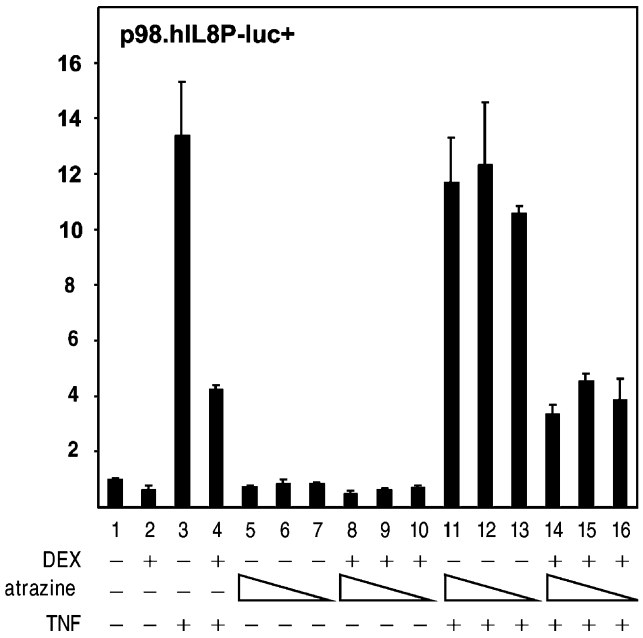

Fig. 1. Atrazine does not affect TNF-induced IL-8 promoter activity, in contrast to DEX. Stable cell pools transfected with an IL-8 promoter-driven reporter gene construct, p98.IL8P-luc+ were left untreated or were induced 2500 IU/mL TNF and treated with 1 μM DEX and three concentrations of atrazine (3, 0.3, and 0.03 μM), alone or in combination, as indicated by the graph's legend. Atrazine and DEX were added 2 hr before TNF, which was left on the cells for 6 hr. Lysates were assayed for reporter gene expression and the data were normalized for β-galactosidase expression levels.

mode of action of atrazine is comparable to that of glucocorticoids, we investigated its effects on NF-κB-driven reporter genes, in comparison with DEX. To this purpose, L929sA cells were stably transfected with constructs carrying a luciferase reporter gene, driven by the promoters of IL-8 (two variants) (Fig. 1 and data not shown), E-selectin, and IL-6 (data not shown). Fig. 1 shows that TNF, (an inflammatory stimulus activating the reporter genes) is capable of stimulating the 98 bp variant of the IL-8 promoter 13-fold (lane 3 vs. lane 1). DEX at a concentration of 10^{-6} M is able to efficiently downregulate this TNF-induced reporter gene activity (lane 4) by targeting its activating NF-κB elements [35,36]. As expected, atrazine or DEX on their own, as well as a combination of both reagents, have no effect on the IL-8 promoter activity (lane 2 and lanes 5–10). Importantly, atrazine, at concentrations ranging from 0.03 to 3×10^{-6} M, has no effect on TNF-induced IL-8 promoter-driven gene expression (lanes 11–13). Nor was atrazine able to revert the inhibitory effect of DEX on TNF-mediated IL-8 reporter gene expression (lanes 14–16) [37]. Testing a longer variant of the IL-8 promoter, p133.IL8P-luc+ yielded the same overall results (not shown). To test whether the results obtained with the IL-8 promoter were promoter-specific or a more general phenomenon, we also tested the effect of atrazine on other proinflammatory gene construct, containing the E-selectin promoter (with NF-κB elements) or the IL-6 promoter (with NF-κB and AP-1-elements), that are both inducible by TNF. As with IL-8 promoter constructs, DEX efficiently inhibited TNF-activated E-selectin and IL-6 promoter activities [38] and again no substantial effects could be recorded with atrazine. Also the activities arising from the combined activities of DEX and TNF remained unchanged when atrazine was included in the induction mixes. Thus, by assessing its negative cross-talk potential with the transcription factors NF-κB and AP-1, we confirm that atrazine does not exhibit glucocorticoid-like properties and does not interact with the GR. Other authors have found that NF-κB is a target of the herbicide propanil, which is not structurally related to atrazine [39].

3.3. Peroxisome proliferation in mice

As some herbicides were recently shown to act through PPAR, we investigated possible effects of atrazine on peroxisome proliferation [20–22]. Atrazine was injected i.p. into male mice and liver and kidneys were studied 7 days after the first injection. Effects on peroxisomes, if any, were not comparable to those of known proliferators [40,41]. Indeed, by light or electron microscopy, no consistent differences in peroxisome numbers were found between control and treated mice (3- or 7-month-old). Variation between 3- or 7-month-old was not significant either (Mann–Whitney non-parametric test). In the kidney, the large-type peroxisomes were limited to the proximal tubule, convoluted and straight parts, as expected; in the distal tubule they were very small and few, and were not studied here. Tails and peroxisome clusters suggesting proliferation were sometimes observed, in control and treated animals alike.

3.4. Promoter induction by PPARα, PPARγ, or PPARδ, and RORα

When cotransfected with the appropriate reporter vector in RK13 cells, the Gal4-hPPAR chimeras was activated by the reference compounds (Wy14643, a fibrate, for PPARα, BRL49653, rosiglitazone, for Gal4-hPPARγ, L-165041, a phenoxyacetic acid derivative, for Gal4-hPPARδ) but not by any concentration of atrazine tested (0.03×10^{-6}–3×10^{-6} M). In view of these negative results, a possible effect through the RORα nuclear receptor was also evaluated. Again, atrazine (0.03×10^{-6}–3×10^{-6} M) as well as the related triazine herbicide simazine (0.1×10^{-6}–1×10^{-6} M) failed to affect reporter gene expression (results not shown).

3.5. AR binding

In the concentration range between 2×10^{-9} and 6.25×10^{-4} M atrazine showed no binding to the rhAR. Only at the highest evaluated concentration (1.25×10^{-3} M) was atrazine able to decrease the ^{3}H-DHT binding to 86%.

Using a very sensitive test, we thus could not confirm the results of Danzo [9] who found a decrease in ^{3}H-DHT binding to the rat AR to 70% of control values by incubation with 10^{-4} M atrazine. Our results indicate that the human AR cannot mediate the effects of atrazine in the low micromolar range. Among pesticides, so far only organotin compounds were found to activate the AR [42].

3.6. Perspectives

The lipophilic properties of atrazine pointed to nuclear receptors as possible targets. In leukocytes, the GR and the PPARs are key transcription factors in physiological and pharmacological situations. The AR, the estrogen receptor and the RORα can also modulate immune responses [23,43–45]. Atrazine is not an estrogen receptor activating compound, as indicated by yeast transactivation and receptor competition data [7,8,46]. The present study shows that AR, GR, PPARs, or RORα are not likely targets of atrazine either. The experiments with RU486 in PBMC indicate that neither atrazine, nor its metabolites generated in our culture system, act through the GR. In the case of the other receptors investigated here, only pure compounds were tested and it, therefore, remains possible that metabolites generated by PBMC (but not in the test system) bind one of these receptors even though no effect was detected with the parent compound. So far, interaction of atrazine has only been tested with a small number of the different nuclear receptors. The triazine herbicides could act through one of these nuclear receptors.

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