Some Plant Defense Stimulators can induce IL-1β production in human immune cells in vitro

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1. Introduction

Plant Defense Stimulators (PDS) are developed by agrochemistry as an alternative to classical pesticides that are highly criticized for their potential toxicity and impact on the environment. Various PDS are, thus, available for applications to numerous crops.

The mode of action of PDS has been suggested to be specific to the plant physiology. For instance, acibenzolar-S-methyl (ASM), the active molecule of BION 50 WG®, belongs to the benzothiadiazole family and is an analogue of the plant hormone salicylic acid (SA). ASM stimulates or primes the plant immune response in several plant species such as wheat (Triticum aestivum) ([1–3] and reference therein). A similar effect has been reported for phosphite, the active substance of LBG-01F34®. When used in pre-treatment, its primes the defense response induced by SA, inducing a stronger resistance of plants to pathogens. In potato, phosphite treatment restricts the development of the oomycete Phytophthora infestans likely through a SA-dependent process [4]. In Vacci-plant®, the active principle is laminarin, an oligosaccharide extracted from Laminaria digitata algae. Laminarin reduces the soft rot disease symptoms induced by the bacterial pathogen Erwinia carotovora subsp. carotovora in tobacco [5]. In grape, it was shown to induce defense responses including the production of reactive oxygen species, defense gene expression, phytoalexin production and to reduce infection by Botrytis cinerea and Plasmopura viticola, the causal agents of grey mold and mildew, respectively [6]. Finally, Prohexadione, the active compound of Regalis®, is a molecule that inhibits gibberellic acid biosynthesis, a typical plant hormone, and thus delays growth of apple tree. However, prohexadione has also a PDS activity. Treatment of apple trees with prohexadione induces activation of defense gene and a reduction of apple scab [7]. Furthermore, Regalis® reduces the occurrence
of fire blight caused by Erwinia amylovora and induces plant defense [8]. Given these modes of actions, it is generally thought that PDS might be safer than the classical pesticides used until now.

We recently showed that the PDS Stifenia® (or FENS60) can potentially induce an inflammatory response in humans [9]. Indeed, this product stimulates the production of inflammatory cytokines in human leukocytes in vitro and upregulates the expression of TNF-α and IL-1β in zebra fish larvae. In the present paper, we investigated the effects of four other commercially available PPs on the production of the pro-inflammatory cytokine IL-1β in human peripheral blood mononuclear cells (PBMC). In addition to the direct effect of PPs on IL-1β production, we also investigated a possible additive or synergistic effect between PPP and the bacterial wall component lipopolysaccharide (LPS) which is known to induce a strong pro-inflammatory response [9,10]. Finally, we tested the different compounds on 4 days old larvae of the fish Danio rerio to get information about the toxic doses on small aquatic animals.

2. Material & methods

2.1. Chemicals

BION-50 WG®, LBG-01F34®, Regalis® and Vacciplant® were purchased from Dijon Céréal (France), a provider for farmers. These compound are only partially soluble in water. Compounds were prepared at 17 mg of active substance/mL in RPMI medium (human cell culture) or water (zebra fish larvae). In the present paper, we investigated the effects of four other commercially available PPP on the production of the pro-inflammatory cytokine IL-1β in human peripheral blood mononuclear cells (PBMC). In addition to the direct effect of PPs on IL-1β production, we also investigated a possible additive or synergistic effect between PPP and the bacterial wall component lipopolysaccharide (LPS) which is known to induce a strong pro-inflammatory response [9,10]. Finally, we tested the different compounds on 4 days old larvae of the fish Danio rerio to get information about the toxic doses on small aquatic animals.

2.2. Human peripheral blood mononuclear cells

Buffy coats from healthy donors were obtained from Établissement Français du Sang (EFS, Besançon, France, Agreement N’DECO-14-0124). Peripheral blood mononuclear cells (PBMC) were prepared using Pancoll (density 1.077 g/mL, PAN-biotech GmbH, Germany) and Blood Sep Filter tubes (Dominique Dutscher, France). Briefly, 15 mL of Pancoll were collected into the lower part of a Blood Sep Filter tube by a short centrifugation. Then, 25 mL of buffy coat and 15 mL of DPBS (Dulbecco’s Phosphate-Buffered Saline, PAN-biotech GmbH, Germany) were added, gently mixed and centrifuged (400 g, 30 min, room temperature) without brake for the deceleration phase. The PBMC ring was collected, washed 3 times in DPBS without Ca²⁺ and Mg²⁺ and centrifuged (300 g, 10 min, 4 °C). Cells were suspended in 2–5 mL of DPBS depending on the size of the cell pellet and kept on ice. Viable PBMC were counted using trypan blue, suspended in RPMI medium supplemented with 10 % BSA Bovine Serum Albumin, w/v and 1% PSA Penicillin 100 units.mL⁻¹, Streptomycin 10 mg.mL⁻¹, Amphotericin B 25 µg.mL⁻¹ prepared in water) and then seeded at the density of 10⁵ cells per well in 150 µL of medium in 96-well plates.

2.3. Treatments

Because PBMC are floating cells, cells were seeded in 96 wells round bottom plates (Nunc®, Corning Inc.) (10⁵ cells per well) then treated by various concentrations of the different compounds dissolved in 10 µL vehicle (RPMI) within one hour after plating. The final well volume was adjusted to 170 µL and cells incubated for 20 h.

For each dose tested, 8 replicates from at least 3 different healthy human blood donors were used. These 8 replicates were tested for cell metabolic activity (XTT) and IL-1β production, as described below.

2.4. Cell metabolic activity

Cell metabolic activity was determined using the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay (Sigma-Aldrich, France) as already described in Teyssier et al. [9]. After centrifugation of the plates and removing of supernatant for IL-1β measurement, cells pellets were suspended in the reaction mixture (100 µL RPMI without phenol red and 20 µL containing 0.9 mg/mL XTT and 0.01 mM PMS) and incubated at 37 °C for 4 h. Absorbance was measured at 490 nm with a background subtraction at 660 nm using a microplate reader (Infinities M200 PRO, Tecan, France). The results are expressed as percentage of metabolic activity compared to control non-exposed cells.

2.5. Quantification of IL-1β

Production of the inflammatory cytokine IL-1β was measured on aliquots of the culture medium from each well 20 h after the treatment using ELISA assay (Human IL-1β ELISA Ready-SET-Go! eBiosciences, France) according to the supplier’s instructions. Reference curves were run in each sample plate. Each reference curve was established with

| Formulated product | Active substance and concentration in formulated product | EFSA journal | Recommended use in field treatment | Concentration of active substance | AOELb | Fish toxicityc |
|--------------------|-----------------------------------------------------------|--------------|-----------------------------------|---------------------------------|-------|---------------|
| BION 50GW          | Acibenzolar-S-methyl, 500 µg/kg                           | 2014;12(8):3691 | 3.2 – 5 g/L (0.32 % - 0.5 %, v/v) | 1.6 - 2.5 g/L | 0.03 mg/kg bw/day | 1.1 mg/L |
| LBG-01F34          | Potassium phosphonates, 730 µg/L                         | 2012;10(12):2963 | 67.2 – 336 mL/L (6.72 % - 33.6 %, v/v) | 49.05–245.3 g/L | 5 mg/kg bw/day | > 118 mg/L |
| Regalis            | Prohexadione, 100 g/kg                                   | 2010; 8(3):1555 | 0.3 – 12.5 g/L (0.03 % - 1.25 %, m/v) | 0.03 - 1.25 g/L | 0.35 mg/kg bw/day | > 100 mg/L |
| Vacciplant         | Laminarine, 37 g/L                                      | 2017;15(6):4836 | 1 - 3 mL/L (0.1 % - 0.3 %)         | 0.037 - 0.111 g/L | NRd | > 88 µg/L |

a Concentration of active compound regarding recommended concentration use.
b AOEL: acceptable operator exposure level (mg/kg of body weight/day) for active substance as mentioned in EFSA article.
c Fish toxicity for active substance on Oncorhynchus mykiss (rainbow trout): acute toxicity (LC50) at 96 h as mentioned in EFSA article.
d ANSES n° 2011-0627.
e ANSES n° 2002-0556.
f ANSES n° 2012-1952.
g ANSES n° 2012-2220.
h not required.
standard human IL-1β solution (500 pg/mL) extemporaneously prepared and sequentially diluted twice seven times in the assay buffer to reach 3.9 pg/mL. Duplicates of each concentration were run and Optical Density (OD) of the samples were plotted on a double logarithmic scale. Extrapolation of the concentrations of IL-1β had a highly right-skewed distribution and was log-transformed to normalize the distribution of model errors. In each model, the dependent variable was the log-transformed IL-1β, while experimental group and replicate nested in the experimental group were included as fixed terms. We also included cell metabolic activity as a covariate in each model to correct for a possible covariation between metabolic activity and IL-1β production. Finally, the identity of blood donor was included as a random factor to take into account the repeated nature of the data per donor. Degrees of freedom were approximated using the Satterthwaite method. Pairwise post-hoc comparisons were conducted on between group differences of least squares means and p values adjusted for multiple comparisons using the Schefé method.

Cell metabolic activity was expressed as percent change with respect to the control group, and analyzed with linear mixed effect models that included experimental group and replicate nested in the experimental group as fixed factors, and blood donor identity as a random factor. Degrees of freedom were approximated using the Satterthwaite method. Pairwise post-hoc comparisons were conducted on between group differences of least squares means and p values adjusted for multiple comparisons using the Schefé method.

The effect of PPP on the mortality of zebrafish larvae was tested using a non-parametric test (Kruskal-Wallis test). For each product concentration, we computed the proportion of dead larvae per replicate, and these values were used as the dependent variable in the model.

3. Results

The formulated products we tested are either sold as powders (BION-50WG®, Regalis®) or as liquid preparations (LBG-01F34®, and Vacciplant®) ready to be dissolved in water. However, most of them are not totally soluble in water. To study their effects on human PBMC or zebra fish larvae, we used aqueous soluble extracts obtained as described in Section "Materials and Methods". According to French Agency for Food, Environmental and Occupational Health and Safety (ANSES) documents (Table 1), we tested concentrations in the range of the recommended use-concentrations of these formulated products (Table 1, column 5).

3.1. Effect of LBG-01F34®

3.1.1. Cell metabolic activity

The linear mixed effect model indicated that PBMC in the different experimental groups had significantly different metabolic activity (Table 2). However, pairwise post-hoc comparisons showed that PBMC exposed at the three LBG doses (0.1, 0.3, and 1 mg/mL) had similar metabolic activity compared to the control group (Fig. 1A; Table S1). While PBMC treated with LPS had similar metabolic activity compared
3.2. Effect of Regalis®

3.2.1. Cell metabolic activity

The linear mixed effect model indicated that PBMC in the different experimental groups had different metabolic activity (Table 2, Fig. 2A). However, pairwise post-hoc comparisons showed that PBMC exposed to the two Regalis® doses (0.1 and 0.3 mg/mL) had similar metabolic activity compared to the control group (Fig. 2A; table S3); the only statistically significant pairwise comparison was between metabolic activity of PBMC treated with Regalis® at the dose of 0.3 mg/mL and PBMC treated with LPS (Fig. 2A; table S3).

There was no variation among replicates within experimental groups nor among blood donors in metabolic activity (Table 2).

3.2.2. IL-1β

The linear mixed effect model indicated that PBMC in the different experimental groups produced significantly different amount of IL-1β, and that, across groups, metabolic activity was positively correlated with IL-1β production (Table 3). Pairwise post-hoc comparisons showed that PBMC exposed to the two Regalis® doses had similar production of IL-1β compared to the control group (Fig. 2B; table S4). PBMC treated with LPS produced more IL-1β compared to the control group (Fig. 2B; table S4); however, adding Regalis® (at the two doses) to LPS-treated PBMC did not alter the IL-1β production compared to PBMC that were only exposed to LPS (Fig. 2B; table S4). There was no variation among replicates within experimental groups nor among blood donors in IL-1β production (Table 3).

3.3. Effect of BION-50WG®

3.3.1. Cell metabolic activity

The linear mixed effect model indicated that PBMC in the different experimental groups had different metabolic activity (Table 2, Fig. 3A). Pairwise post-hoc comparisons showed that PBMC treated with BION-50 WG at the 0.1 and 0.3 mg/mL had similar metabolic activity compared to the control group (Fig. 3A; table S5); while PBMC treated at the highest BION-50 WG dose (1 mg/mL) had slightly lower metabolic activity compared to the control group (Fig. 3A; table S5). PBMC treated with LPS had higher metabolic activity compared to the control group (Fig. 3A; table S5). PBMC treated with both LPS and BION-50 WG had lower metabolic activity compared to LPS-only treated PBMC (Fig. 3A; table S5 except at the dose of 0.3 mg/mL where PBMC treated with LPS and BION-50 WG had a reduced metabolic activity (Fig. 3A; table S5).

There was no variation among replicates within experimental groups nor among blood donors in metabolic activity (Table 2).

3.3.2. IL-1β

The linear mixed effect model indicated that PBMC in the different experimental groups produced significantly different amount of IL-1β, and that, across groups, metabolic activity was negatively correlated with IL-1β production (Table 3). Pairwise post-hoc comparisons showed that, whatever the dose, BION-50 WG induced a up-regulation of IL-1β production compared to the control group (Fig. 3B; table S6). LPS induced a strong up-regulation of IL-1β production (Fig. 3B; table S6). PBMC treated with both LPS and BION had much higher IL-1β production compared to the control group (Fig. 3B; table S6); whereas the pairwise comparisons between IL-1β production of PBMC co-treated with BION-50 WG and LPS vs. LPS alone showed that only at the dose of 0.3 μg/mL the difference was statistically significant (co-treated PBMC producing lower amount of IL-1β compared to LPS-only) (Fig. 3B; table S6).

There was no variation among replicates within experimental groups nor among blood donors in IL-1β production (Table 3).
Table 3

Linear mixed effect models exploring the effect of four plant protection products (LBG-01F34®, Regalis®, BION-50WG®, Vacciplant®) on PBMC IL-1β production (log-transformed). Each model included the experimental group, the metabolic activity, and the replicate nested within the experimental group as fixed effects and the blood donor identity as a random effect. The model investigating the effect of LBG-01F34® included 6 experimental groups (control, LBG-01F34® 0.1 mg/mL, LBG-01F34® 0.06 mg/mL + LPS, BION-50WG® 0.3 mg/mL, LBG-01F34® 0.3 mg/mL + LPS, LPS) and 8 replicates per experimental group. The model investigating the effect of Regalis® included 6 experimental groups (control, Regalis® 0.1 mg/mL, Regalis® 0.1 mg/mL + LPS, Regalis® 0.3 mg/mL + LPS, Regalis® 0.3 mg/mL + LPS, LPS) and 8 replicates per experimental group. The model investigating the effect of BION-50 WG® included 8 experimental groups (control, BION-50WG® 0.1 mg/mL, BION-50WG® 0.1 mg/mL + LPS, BION-50WG® 0.3 mg/mL, BION-50WG® 0.3 mg/mL + LPS, BION-50 WG® 1 mg/mL, BION-50 WG® 1 mg/mL + LPS, LPS) and 8 replicates per experimental group. The model investigating the effect of Vacciplant® included 4 experimental groups (control, Vacciplant® 0.02 mg/mL, Vacciplant® 0.02 mg/mL + LPS, LPS) and 8 replicates per experimental group. Degrees of freedom were approximated using the Satterthwaite method.

4. Discussion

Plant Protection Products (PPP) that we studied in this report act as plant defense stimulator and thus stimulate the plant innate immunity. The goal of this new agrochemical approach is to limit classical pesticide extensive use, as to reduce the noxiousness for human beings and the environment. However, innate immunity shares common pathways between plants and animals [11,12] and the compounds that are developed to stimulate plant defenses might also have side effects on animals and humans. In agreement with this view, we recently reported one of such side effects of the PDS Stifenia® on human PBMC and zebra fish larvae [9]. Stifenia® induced an over-production of the pro-inflammatory cytokine IL-1β, decreased the viability of cultured human PBMC, and activated the expression of inflammatory genes in zebra fish larvae. Here, we tested four additional DDS for which the biologically active compounds are known.
Our data investigated metabolism of XTT by human PBMC during 4 h after PPP treatments. This measurement of cellular metabolic activity could be considered as an indicator of cell viability. We also measured the released IL-1β in the culture medium, an indicator of the activation of the NLRP3 inflammasome involved in the innate immunity of humans [11]. NLRP3 is expressed in leucocytes [13,14] and is activated upon stimulation by various stimuli involved in innate immunity [15,16]. Upon activation, NLRP3 protein complex binds caspases-1 that cleave IL-1β inactive precursor to its short active form released outside of the cells. IL-1β is thus only produced by activated leucocytes. Since the antibody used in ELISA could cross-react with the non cleaved precursor form of IL-1β stored in the cytosol, it cannot be excluded that leaking of this form from cells presenting plasma membrane deterioration by the PPP could slightly overestimate the measured cytokine concentrations, especially when metabolic activity is found to be low. However, measure of IL-1β in the medium clearly indicates a cellular dysfunction.

Among the four PDS tested here, two of them, LBG-01F34® and Regalis®, did not have any effect on PBMC metabolic activity and IL-1β production in vitro. They neither synergize the inflammatory effect of LPS on the cells. Viability of PBMC treated by 1 mg/mL LBG-01F34® estimated with Trypan blue was not different from control (data not shown) and confirm the absence of toxicity at this concentration. These compounds might however have other effects on eukaryotic cells. On the one hand, phosphite (phosphonate) contained in LBG-01F34® is an ionic structure that naturally combines with sodium and sulfates. Metabolomic analysis of phosphite-treated Arabidopsis thaliana plants showed changes in several metabolite pools, such as aspartate, asparagine, glutamate, and serine [17]. These alterations in amino acid pools influence growth and differentiation of mouse nervous system (neurospheres) in vitro [18]. This mechanism could account for the toxicity that we observed on zebrafish larvae at 0.3 mg/mL Regalis®. For this product, we began to observe effects on the larvae at 0.1 mg/mL, a concentration similar to the EC50 measured on the rainbow trout Oncorhynchus mykiss using a different test (see Table 1). It is noteworthy that 1 mg/mL LBG-01F34® did not induce significant death in our Danio rerio assay, while this concentration is ten times the EC50 found in the rainbow trout test (Table 1).

Contrary to Regalis® and LBG-01F34®, BION-50 WG® had a pro-inflammatory effect on PBMC, inducing an overproduction of IL-1β whatever the dose tested. According to the manufacturer, BION-50 WG® contains 10–20 % silica to increase ASM penetration in plant leaves. Given that silica have known pro-inflammatory effects [19], it is possible that silica remaining in the supernatants that was used for the in vitro test might account for the up-regulated production of IL-1β in PBMC. However, these silica remains should be lower than the actual
amount to which farmers are exposed to. When tested on zebra fish larvae, this product began to disturb development at 2.5 mg/mL, a concentration twice those of EC50 in the test using rainbow trout.

Vacciplant® had a very strong effect on PBMC metabolic activity measured by the XTT assay. At the dose of 0.02 mg/mL, metabolic activity was reduced by 35% and at the dose of 0.06 mg/mL this reduction attained 76%. Since XTT is classically used as a viability/proliferation test, our results suggest a cytotoxic effect of Vacciplant®. This is confirmed by estimation of PBMC viability when they were treated with 0.03 mg/mL Vacciplant® by Trypan blue. It corresponded to only 8% of cells while 92% alive cells were found in controls (data not shown) and confirms the toxicity at this concentration. Vacciplant® also induced an overproduction of IL-1β. Vacciplant® is manufactured from laminarin, that is known to modulate animal innate immunity and to induce TNF-α and IL-6 mRNA in murine cultured cells [20]. It also inhibits phagocytosis in human macrophages [21]. Although the specific laminarin used in this PPP could be safe by itself, the action of β-glucans greatly depends on their molecular weight and conformation [22]. Furthermore, it is possible that excipients contained in Vacciplant® could be the cause of the observed effects on PBMC metabolic activity and IL-1β production. When tested on zebra fish larvae, this product began to disturb development at 2 μg/mL and was lethal at 20 μg/mL. These concentrations are much lower than the EC50 described for the rainbow trout assay (Table 1, 88 μg/mL). Furthermore, data of toxicity on zebra fish larvae using the same range of concentration as used for PBMC treatment clearly demonstrate that Vacciplant appears to be the most toxic of the 4 compounds tested.

5. Conclusions

Since IL-1β is a key inflammatory cytokine, measuring its level is relevant to assess the potential toxicity of PPP. We found that formulated compounds can have inflammatory effects that were not described for molecules corresponding to their active compounds, probably because they were not investigated. Furthermore, it appears that toxicological tests must be conducted on the commercially available products, and not only on the active compounds, in order to avoid possible undesirable effects of the adjuvants [23]. These tests, fitting with the goal of reducing studies on animal models, could be routinely done to screen the potential adverse effects of compounds that can potentially cross-react with human innate immunity.

Declaration of Competing Interest

There are no conflicts of interest to declare.

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