Comparative effects of a non-steroidal ecdysone agonist RH-5992 and 20-hydroxyecdysone in a lepidopteran cell line (IAL-PID2)

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Abstract

The non-steroidal ecdysone agonist, RH-5992, exhibits ecdysteroid activities in vivo as well as in vitro more effectively than 20-hydroxyecdysone (20E). Using the IAL-PID2 cells derived from imaginal wing discs of last larval instar of Plodia interpunctella, we investigated the action of RH-5992 in the control of cell growth. Its effects on the proliferative activity of IAL-PID2 cells, the induction level in G2/M arrest and on the expression rate of Plodia B cyclin (PcycB), ecdysone B1-isoform (PIEcR-B1) and Ultraspiracle-2 isoform (PIUSP-2) were examined. From these cellular and molecular assays, our results brought evidence that RH-5992, like 20E, induced an inhibition on cell proliferation by blocking IAL-PID2 cells in G2/M phase. Moreover, this G2/M arrest was preceded by a decrease in the expression level of PcycB and a high induction of PIEcR-B1, PIUSP-2 mRNAs. Dose–response experiments revealed that RH-5992 was even more potent than 20E. On these parameters, we therefore suggest that the differential observed in the expression level of USP and EcR by RH-5992 and 20E could contribute to the difference observed for the biological potency of these two compounds.

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1. Introduction

In insects, the postembryonic development is achieved by successive molts that lead progressively to the imaginal stage. The renewal of the old exoskeleton and the metamorphic process are controlled by the steroid hormone 20-hydroxyecdysone (20E) and the sesquiterpenoid juvenile hormone (JH). The morphological and biochemical changes that occur during the metamorphosis are associated with hemolymphatic peaks of 20E in the absence of JH. Most of these 20E-induced responses are mediated by a nuclear heterodimeric complex of ecdysone receptor (EcR)/Ultraspiracle (USP) (Koelle et al., 1991; Yao et al., 1992) which, under 20E activation, induces the sequential transcription of genes encoding proteins identified as transcription factors such as E75 (Segraves and Hogness, 1990) and HR3 (Koelle et al., 1992).

During the past two decades, the discovery of non-steroidal ecdysteroid agonists belonging to the bisacylhydrazine class, provided a promising alternative to conventional insecticides for the pest control (Wing et al., 1988; Aller and Ramsay, 1988; Retnakaran et al., 2003). Among the bisacylhydrazines, the tebufenozide (coded as RH-5992) exhibited an insecticidal activity by inducing precocious lethal molts in all lepidopteran larvae tested, including the tobacco hornworm, Manduca sexta (Retnakaran et al., 1995), the spruce budworm, Choristoneura fumiferana (Retnakaran et al., 1997), the silkworm, Bombyx mori (Kumar et al., 2000) and the cotton leaf-worm, Spodoptera littoralis (Mourad et al., 2004). From imaginal discs development assays, it has...
been reported that RH-5992 initiates and sustains the evagination of imaginal wing discs of lepidopteran cultured in vitro with a higher potency than 20E itself (Smagghe et al., 1996, 2002). Cellular and biochemical effects of RH-5992 were also studied on various cell lines derived from Malacosoma disstria, Choristoneura fumiferana and Chironomus tentans. RH-5992 inhibited cell proliferation and induced chitin synthesis as well as morphological changes by producing clumps and cytoplasmic extensions (Spindler-Barth et al., 1991; Quack et al., 1995; Sohi et al., 1995; Zheng et al., 2003; Hu et al., 2004). Moreover, RH-5992 was more potent than 20E in inducing these various responses.

To investigate the molecular action of RH-5992, recent studies have examined the binding of this compound to the ecdysteroid receptor complex and its effects on the induction of ecdysteroid responsive genes expression. Like 20E, RH-5992 manifested its biological effects by binding to the EcR/USP complex with a higher affinity than 20E (Dhadialla et al., 1998; Nakagawa et al., 2000; Smagghe et al., 2002). Additional reports have shown that RH-5992 was able to act by stimulating the expression of Mandaica HR3 (MHR3), Plodia HR3 (PHR3), Choristoneura HR3 and E75 (CHR3, CTE75) genes (Retnakaran et al., 1995, 2001; Palli et al., 1992, 1996, 1997; Debernard et al., 2001; Sundaram et al., 1998). RH-5992 was about 10 times more effective than 20E in the induction of MHR3, PHR3 and CHR3 mRNAs.

The Indian meal-moth cell line, IAL-PID2, has been established from pupally committed imaginal wing discs of Plodia interpunctella (Lynn and Oberlander, 1983). The IAL-PID2 cells retain a sensitivity to 20E and RH-5992 and respond by inhibiting cell proliferation and long-term morphological differentiation as marked by the formation of pseudoepithelial aggregates structures (Cassier et al., 1991; Porcheron et al., 1991). Recent papers have demonstrated that the 20E-induced growth inhibition resulted from a blockade of IAL-PID2 cells in the G2/M phase of their cell cycle (Mottier et al., 2004; Siaussat et al., 2004b). It has been reported the cloning of several sequences encoding Plodia B cyclin (PcycB) (Mottier et al., 2004), ecdysone receptor B1-isoform (PIEcR-B1) (Siaussat et al., 2004a) and Ultraspiracle 2-isoform (PIUSP-2) (Siaussat et al., 2005). Some experiments indicated that the G2/M arrest was preceded by a decrease in the expression level of PcycB (Mottier et al., 2004) and a high induction of PIEcR-B1 and PIUSP-2 mRNAs (Siaussat et al., 2005). Therefore, the IAL-PID2 cell line seemed to be a potent system both to specify the mode of action of RH-5992 and to identify some of the factors responsible for the differential effectiveness of RH-5992 and 20E. We thus investigated the action of RH-5992 in the control of cell growth by examining its effects on the proliferative activity of IAL-PID2 cells, the induction level to G2/M arrest and on the expression rate of PIUSP-2, PIEcR-B1 and PcycB as compared to 20E. From these cytological and molecular assays, our results suggest that RH-5992 could control the proliferation of imaginal cells by modulating the level of PIUSP-2, PIEcR-B1 and PcycB transcripts in a manner similar to 20E but with a higher potency than 20E. We also suggest that the differential in the induction level of USP and EcR mRNAs by RH-5992 and 20E could contribute to the difference in the biological potency of these compounds.

2. Material and methods

2.1. Cell culture

The IAL-PID2 cell line was established from imaginal wing discs of last larval instar of P. interpunctella, the Indian meal-moth (Lynn and Oberlander, 1983) and routinely maintained at 27°C in the laboratory. Cells grow as a loosely attached monolayer in a 25 cm² tissue culture flask containing 4 ml of antibiotic-free Grace’s medium (Life Technologies Cergy Pontoise, France) supplemented with 10% foetal bovine serum (FBS, Roche, Molecular Diagnostics, Meylan, France) and 1% bovine serum albumin (BSA, Fraction V, Sigma, Saint Quentin Fallavier, France). Cells were rinsed off the bottom of the flask in a gentle stream of culture medium and resuspended. Cell density was estimated by counting the cells in an aliquot of the suspension in a Mallassez haemocytometer. All the cultures were initiated by seeding flasks with 5 × 10⁵ cells and cultured under normal growth conditions.

2.2. Ecdysteroids and non-steroidal agonists

The 20-hydroxyecdysone was kindly provided by Dr. René Lafont (Paris, France) and RH-5992 was a gift from Dr. Guy Smagghe (Gent, Belgium). Stock solutions of 20E and RH-5992 were prepared, respectively, in ethanol and DMSO, then stored at −20°C. For use in culture, stock solutions were diluted in Grace’s medium and added to wells or flasks at the final concentrations of 2 × 10⁻⁹, 5 × 10⁻⁹, 10⁻⁸, 2 × 10⁻⁸, 4 × 10⁻⁸, 8 × 10⁻⁸, 2 × 10⁻⁷, 4 × 10⁻⁷ and 8 × 10⁻⁷ M which are in the range of physiological titers in Lepidoptera (Lynn and Oberlander, 1983). Final ethanol and DMSO concentrations in all treatments and control cultures were always maintained under 0.1% in order to prevent any possible toxic effect of the solvent.

2.3. Cell proliferation assay

Proliferative activity of the cells was measured by the XTT assay (Roche Laboratory, France) based on the reduction of a tetrazolium salt (XTT) into yellow
formazan salt by active mitochondria (Mosmann, 1983). For this assay, cells were transferred into a 96-well microtiter plate, and the cell density adjusted to 20,000 cells/well. Each assay was repeated at least five times using two sets of eight wells per concentration tested. The effects of 20E and RH-5992 on cell proliferation were measured by the XTT reduction assay 48 h after the compound addition: cells were then incubated 4 h with a mixture of XTT-PMS at 27 °C, and each well absorbance was evaluated in a microtiter plate spectrophotometer (Multiskan, Labsystem, France).

2.4. Flow cytometry analysis

Cell DNA content was determined by staining cells with propidium iodide and measuring their fluorescence (FACScan, Becton Dickinson, Institut Jacques Monod, Paris, France). The IAL-PID2 cells were resuspended and fixed at −20 °C with 70% ethanol/PBS (10 mM Na₂HPO₄, 138 mM NaCl, 2.7 mM KCl, pH 7.4). The fixed cells were incubated for 30 min at 37 °C in a solution containing 100 μg/ml RNase and 40 μg/ml propidium iodide. For each cell population, 10,000 cells were analysed by FACS and the percentage of cells in a specific stage of the cell cycle was determined with the propidium iodide DNA staining technique. Cells were classified in G0/G1, S and G2/M phases according to the intensity of the fluorescence peaks (Crissman et al., 1975).

2.5. Isolation of RNA

Total RNAs from cells were extracted with TRIzol reagent (Gibco, BRL) and quantified by spectrophotometry at 260 nm. The quality of RNA was checked by electrophoresis on a formaldehyde-agarose gel (1%).

2.6. Northern blotting

Northern blot hybridization analysis was performed according to the manufacturer’s instructions. RNA samples (15 μg) were denatured with formamide (50%) and formaldehyde (2.2 M), separated on 1% denaturing agarose gel and transferred to a Boehringer Mannheim positively charged nylon membrane. Blotted RNAs were hybridized overnight at 55 °C with the PIEcR-B1 or PcycB probe and at 45 °C with the PIUSP-2 probe. A DIG-labelled fragment of the cDNA encoding the RpL8 ribosomal protein of P. interpunctella was used as a control probe. An immunological detection signal was performed as described in Roche’s DIG system User’s Guide for filter hybridization. A molecular RNA marker ladder DIG-labelled (Roche, Meylan, France) was run in parallel on Northern blots to determine the molecular weight of hybridizing RNAs.

3. Results

3.1. Effect on the proliferative activity of IAL-PID2 cells

The IAL-PID2 cells were cultured under normal growth conditions for 72 h then treated for 36 h with 20E or RH-5992 at various concentrations ranging from $2 \times 10^{-9}$ to $8 \times 10^{-7}$ M. At the end of treatment, the proliferative activity of the cells was evaluated by the XTT assay. Fig. 1 indicates that like 20E, RH-5992 induced a striking decrease in the proliferative activity of the cells. EC₅₀’s determined from concentration dependent–response curves were 13 and 54 nM for RH-5992 and 20E, respectively (Table 1). These values express higher inhibitory effect of RH-5992 on cell proliferation as compared to that of 20E.

3.2. Effect on cyclic activity of IAL-PID2 cells

Recent experiments have shown that the 20E-induced proliferation inhibition was due to an arrest of cells in G2/M phase (Mottier et al., 2004). To compare the action of RH-5992 with that of 20E in the control of cell cycle, we followed within cycle phases, the distribution of treated cells either by RH-5992 or 20E at $4 \times 10^{-8}$ M for 36 h. This period of time corresponds to the population doubling time in our model (Hatt et al., 1997; Auzoux-Bordenave et al., 2002). The flow cytometry analysis showed that the untreated cells were...
distributed in the different phases of the cell cycle as follows: 49% ± 3%, 23% ± 2% and 28% ± 4% in G0/G1, S, and G2/M stages, respectively (Fig. 2). We have experienced that this distribution remains unchanged with time in normally growing cells (data not shown). By contrast, in presence of RH-5992 or 20E at 4 × 10⁻⁸ M, the cells progressively accumulated in G2/M phase (Fig. 3A). A consistent increase in the number of cells in G2/M occurred after 18 h exposure to RH-5992 whereas this accumulation was only detected after 24 h treatment with 20E (Fig. 3A). The histograms of fluorescence represented in Figs. 3B and 3C illustrate the G2/M peaks of treated cells with RH-5992 (68 ± 5%) or 20E (49 ± 3%) at the end of the experiment.

To determine the relative potencies of 20E and RH-5992 in the induction of G2/M arrest, the IAL-PID2 cells were exposed to RH-5992 or 20E at various concentrations for 36 h, time required for the maximal induction of G2/M arrest. At the end of the treatment, the percent of IAL-PID2 cells in G2/M phase was determined by fluorescent-activated flow cytometry. Concentration-dependent–response curves show that RH-5992 induces a level of G2/M arrest identical to that observed with 20E.
at higher concentrations (Fig. 4). EC<sub>50</sub>'s for RH-5992 and 20E were 8.2 nM and 55 nM respectively (Table 1). The potency of RH-5992 to induce the G2/M arrest was therefore about 6.7-fold greater than that of 20E.

### 3.3. Effect on molecular events associated with G2/M arrest

The *Plodia* B cyclin, ecdysone receptor B1-isoform and Ultraspiracle 2-isoform have been recently isolated and it has been demonstrated that the 20E-induced G2/M arrest of IAL-PID2 cells was associated to a decrease in the expression level of PcycB (Mottier et al., 2004) and a high induction of PIEcR-B1 and PIUSP-2 mRNAs (Siaussat et al., 2004a, 2005). We therefore decided to examine the RH-5992 action at a molecular level by reporting its effects on PcycB, PIEcR-B1 and PIUSP-2 expression in correlation to G2/M arrest.

#### 3.3.1. Effect on PcycB expression

To analyse the respective effects of RH-5992 and 20E on PcycB expression, the IAL-PID2 cells were treated with RH-5992 or 20E at various concentrations and the level of PcycB mRNA assessed after 18 h exposure, time required for the maximum decline in the PcycB expression. As shown in the Fig. 5B, the inhibition level of PcycB mRNA increased in parallel with the concentration of 20E or RH-5992 up to 4 x 10<sup>-7</sup>M. EC<sub>50</sub>'s demonstrated that RH-5992 caused 50% inhibition at 8.2 nM, whereas to induce the same effect with 20E, a concentration as high as 49 nM was required. Thus, the suppressive activity of RH-5992 on the Pcyc B expression was about 6-fold greater than that of 20E.

#### 3.3.2. Effect on PIEcR-B1/PIUSP-2 complex expression

PIEcR-B1 and PIUSP-2 induction patterns by RH-5992 and 20E treatment were also studied. The IAL-PID2 cells were cultured in medium containing either 20E or RH-5992 at 4 x 10<sup>-7</sup>M and the level of PIUSP-2 and PIEcR-B1 mRNAs were determined at different exposure times. In the absence of RH-5992 or 20E, PIEcR-B1 and PIUSP-2 mRNAs were never detected (data not shown). We remarked that a maximum induction of PIUSP-2 mRNA was observed at 12 h for RH-5992 (Fig. 6A) whereas the induction profile of PIEcR-B1 mRNA was characterized by a biphasic response with two peaks, respectively, at 2 and 20–24 h (Fig. 7A). Nevertheless, the induction level for both transcripts was higher with RH-5992 than with 20E.

To evaluate the relative potencies of RH-5992 and 20E in inducing the expression of PIUSP-2 and PIEcR-B1 genes, the IAL-PID2 cells were treated with 20E or RH-5992 at various concentrations and the level of PIUSP-2 and PIEcR-B1 mRNAs assessed after 12 and 20 h exposure, respectively (times required for the maximum induction of mRNAs). The Figs. 6B and 7B show that the amount of PIUSP-2 and PIEcR-B1 mRNAs increased in a concentration dependent manner with both 20E and RH-5992. From 2 x 10<sup>-9</sup> to 8 x 10<sup>-7</sup>M, RH-5992 exhibited a higher potency in inducing PIEcR-B1 and PIUSP-2 mRNAs than 20E. The concentration required for obtaining 50% relative induction of PIUSP-2 mRNA was 8.3 nM for RH-5992 and 24 nM for 20E (Table 1). With regard to the PIEcR-B1 mRNA induction, the EC<sub>50</sub> for RH-5992 was 10 nM and 38 nM for 20E (Table 1). These results indicate that RH-5992 is 3 and 3.8-fold more effective than 20E in inducing PIUSP-2 and PIEcR-B1 mRNAs, respectively.

### 4. Discussion

The dibenzoyl hydrazine, RH-5992, was the first commercial non-steroidal agonist of the 20E, the natural
insect molting hormone. RH-5992 exhibits ecdysteroid activities in vivo as well as in vitro more effectively than 20E. Using the IAL-PID2 cell line established from imaginal wing discs of the last larval instar of *P. interpunctella*, we have therefore carried out molecular and cellular assays in order to identify some of the mechanisms by which RH-5992 mimics the 20E-induced responses and thus define some of the factors that contribute to the differential effectiveness observed between RH-5992 and 20E.

To study the action of RH-5992 in the control of cell growth and test its effects on the proliferative and cyclic activities of IAL-PID2 imaginal cells as compared to 20E, several techniques were tested. Using the formazan assay (XTT) we confirmed that RH-5992 was able to inhibit the proliferation of imaginal cells as reported in chironomus tentas epithelial cells (Quack et al., 1995). In addition, use of flow cytometry analysis brought evidence that like 20E, RH-5992-induced proliferative arrest resulted from an accumulation of IAL-PID2 imaginal cells in the G2/M phase of their cell cycle.

From dose response experiments, we evaluated the relative potencies of RH-5992 and 20E in inducing the cellular responses described previously. The EC₅₀ values showed that the activity of RH-5992 was higher than that of 20E. These data are in concordance with the ability of these compounds to stimulate the evagination of lepidopteran imaginal discs cultured in vitro (Smagghe et al., 1996, 2002) and their toxicity for the larvae of *Chironomus tentas* and *Choristoneura fumiferana* (Palli et al., 1995; Sundaram et al., 1998; Smagghe et al., 2002).

The *Plodia* B cyclin (PycB), ecdysone receptor B1-isoform (PIEcR-B1) and Ultraspiracle 2-isoform (PIUSP-2) have been recently identified as putative components of a 20E-signalling pathway responsible for the proliferative arrest and long-term morphological differentiation of IAL-PID2 imaginal cells (Siaussat et al., 2004a, 2005; Mottier et al., 2004). To verify
whether the RH-5992-induced G2/M arrest was mediated by an ecdysteroid-molecular cascade, we investigated the effects of RH-5992 on the expression of PycB, PIEcR-1 and PIUSP-2.

Our results support the role of RH-5992 in inducing a decrease in PycB mRNA levels. This decline in PycB mRNA precedes the beginning of the accumulation of cells in G2/M phase, which occurs after 18 h of RH-5992 exposure. Therefore, this inhibitory effect of RH-5992 on B cyclin expression could be partly responsible for the blockade of IAL-PID2 imaginal cells in G2/M phase. Taken together, these data suggest that RH-5992 could regulate the cyclic activity of cells in a similar fashion to that of 20E by acting on cell cycle regulators such as B cyclin, which is known to be involved in the control of G2/M transition. From IAL-PID2 cells, recent works have showed that the 20E-induced G2/M arrest was due to a simultaneously inhibition of B and A cyclin expression (Mottier et al., 2004). Therefore, further experiments, to investigate the effect of RH-5992 on A cyclin could be of interest.

As concerns PIUSP-2 and PIEcR-B1, RH-5992 increased the expression level of these two nuclear receptors and their induction patterns were identical to those obtained in the presence of 20E. The RH-5992 inducibility of PIEcR-B1 and PIUSP-2 was in agreement with the stimulation of other ecdysteroid induced proteins such as HR3 and E75 transcription factors in *M. sexta* (MHR3, Retnakaran et al., 1995; Palli et al., 1992) and *Choristoneura fumiferana* lepidopteran (CHR3, CFE75; Palli et al., 1996, 1997).

It was noted that the induction of PIUSP-2 and PIEcR-B1 mRNAs by RH-5992 was enhanced as early as 2–4 h since exposure and thus, prior to the inhibition of PycB expression. These data suggest that like 20E, RH-5992 could initiate a genetic cascade involving
EcR-B1, USP-2 to regulate the expression of B cyclin and ultimately the G2/M transition. Some RNA interference experiments are therefore in progress to identify the sequence of these molecular events that could link the 20E or RH-5992 action with the proliferative arrest of imaginal cells.

The molecular assays based on the expression of PcycB, PIUSP-2 and PIEcR-B1 clearly showed a significant difference of potency between 20E and RH-5992. In these three assays, our results indicated that RH-5992 was more active than 20E. Therefore, there was a positive correlation between the cellular assays based on G2/M arrest induction and the molecular assays. This observation represents an additional argument for the existence of a link between 20E signalling pathway involving EcR-B1, USP-2, B cyclin and the proliferative arrest.

It has been demonstrated that RH-5992 manifests its effects via an interaction with the EcR/USP complex (Dhadialla et al., 1998). Thus, the differential potency of RH-5992 and 20E in the induction level for PIUSP-2 and PIEcR-B1 mRNAs could contribute to the difference in their biological activities. However, others factors such as a better binding affinity of RH-5992 to the ecdysteroid receptor complex (Smagghe et al., 2002) and lower metabolism in cells (Sundaram et al., 1998) could also play a role in the more potent biological activity of RH-5992 as compared to that of 20E.

Finally, our findings provide the first evidence for the role of RH-5992 in the control of the cellular proliferation and suggest that RH-5992 could act by modulating the expression of EcR, USP and cell cycle regulators such as cyclins. In addition, we also provide data sustaining a correlation between the induction level of the EcR/USP complex and the ecdysteroids biological activities which could be useful in developing a rapid assay system for screening new ecdysteroids agonists as potential candidates for pest control. This assay would be more sensitive than the mortality bioassay in that it would require very little of the test compound and...
could be faster, cheaper, and simpler when performed in ecdysone-responsive cell lines.

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