Identification of ligands and coligands for the ecdysone-regulated gene switch

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The ecdysone-inducible gene switch is a useful tool for modulating gene expression in mammalian cells and transgenic animals. We have identified inducers derived from plants as well as certain classes of insecticides that increase the versatility of this gene regulation system. Phytoecdysteroids share the favorable kinetics of steroids, but are inert in mammals. The gene regulation properties of one of these ecdysteroids have been examined in cell culture and in newly developed strains of ecdysone-system transgenic mice. Ponasterone A is a potent regulator of gene expression in cells and transgenic animals, enabling reporter genes to be turned on and off rapidly. A number of nonsteroidal insecticides have been identified that also activate the ecdysone system. Because the gene-controlling properties of the ecdysone switch are based on a heterodimer composed of a modified ecdysone receptor (VgEcR) and the retinoid X receptor (RXR), we have tested the effect of RXR ligands on the VgEcR/RXR complex. Used alone, RXR ligands display no activity on the ecdysone switch. However, when used in combination with a VgEcR ligand, RXR ligands dramatically enhance the absolute levels of induction. This property of the heterodimer has allowed the development of superinducer combinations that increase the dynamic range of the system.

The ability to manipulate gene function will hasten progress in many areas of biology and medicine. Several inducible systems have been developed to regulate gene expression at the transcriptional level in a temporal and quantitative manner (1–4). The more efficient systems of inducible gene regulation are based on components of the tetracycline resistance operon, on synthetic and nonmammalian steroids, and on compounds that bind altered immunophilins. Each of these schemes relies on the existence of a small molecule (the inducer) that modifies the activity of a synthetic transcription factor, which in turn regulates the expression of a target gene placed under the control of a heterologous promoter.

Methods devised to control gene expression must allow for rapid, robust, precise, and reversible induction of gene activity. Because steroids are small lipophilic molecules that penetrate all tissues and are quickly metabolized and cleared, they appear to be ideal inducers for developing gene regulation systems that fulfill these criteria. To exploit the pharmacokinetics of steroids while eluding the potential complications of the use of a mammalian hormone as the inducer, our laboratory developed an inducible system based on the insect steroid ecdysone and the nuclear receptor that mediates its effects (5). In its advanced format, a chimeric protein composed of the VP16 activation domain fused to an ecdysone receptor with altered DNA-binding specificity heterodimerizes with the retinoid X receptor (RXR) and binds a unique synthetic response element not recognized by natural nuclear hormone receptors. Upon exposure to the ecdysone analog muristerone A (murA), the VgEcR/RXR complex efficiently induces the expression of reporter genes in mammalian cells and transgenic animals.

The popularity of the ecdysone-inducible gene switch led to the unexpected rapid depletion of murA stocks, the primary inducer used in the system. MurA was isolated in the early 1970s from the seeds of kaladana (*Ipomoea calonyction*), a plant native to the southern slopes of the Himalayas (6, 7). The difficulty in obtaining kaladana seeds prompted us to seek alternative inducers derived from more common plants. There are an estimated 27 million to 29 million species of insects in the world, more than half of which are herbivores (8). To deter insect feeding, plants protect themselves from this entomological onslaught by producing an astonishing array of chemicals that are toxic to insects. Ecdysteroids are among the weapons that plants bear in this chemical warfare: as insect larvae eat the leaves of their hosts, they ingest ecdysteroids that mimic the action of the endogenous insect hormone and bring about abnormal molting and premature demise (9). The concentration of these defensive agents in plants can be extraordinary, sometimes representing over 1% of total dry weight (10).

During the 1960s and 1970s, interest in the purported antitumor action of ecdysteroids led to the isolation of these compounds from various plant species (10–13). The presence of ecdysone-like molecules was typically assessed by testing an extract from the plant in question in an *in vivo* assay in which the molting/pupation of larvae depended on the solution possessing ecdysteroid activity (14). Although no effects on mammalian cell growth were ever documented, these efforts resulted in the unearthing of over 100 plant compounds with insect-molting activity.

To broaden the utility of the ecdysone-based gene switch, we have evaluated the ability of many phytoecdysones to serve as inducers. We have also found several small nonsteroidal molecules that behave as activators in the ecdysone-inducible gene regulation system. The ability of new inducers to regulate gene expression *in vivo* has been examined using a set of transgenic mice in whose skin a luciferase reporter gene is under the control of the ecdysone system. In addition to the identification of VgEcR activators, we have discovered that simultaneous administration of VgEcR and RXR ligands dramatically enhances the ability of the VgEcR/RXR heterodimer to generate robust inductions. Synthetic RXR ligands increase by 3- to 5-fold the magnitude of the induction generated by the VgEcR ligand alone.

Materials and Methods

Chemicals. MurA was obtained from Zambon and Invitrogen. Many phytoecdysteroids tested were a gift of K. Nakanishi (Columbia University). Ponasterone A (ponA) was procured from Dr. Nakanishi, produced in our laboratory, and obtained from Invitrogen and Alexis. Before acquiring ponA from commercial sources, we isolated it from *Podocarpus macrophyllus* (15–18). An HPLC purification step was added to ensure the purity of the final compound (19). Makisterone A was obtained from Alexis. 20-Hydroxyecdysone, 2-deoxyecdysone,

Abbreviations: murA, muristerone A; ponA, ponasterone A; EcR, ecdysone receptor; RXR, retinoid X receptor.

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20-hydroxyecdysone-22-acetate, 2-deoxy-20-hydroxyecdysone, α-ecdysone, and polypodine B were purchased from Sigma. Pesticides were acquired from Chem Service, Supelco, and Sigma. Nonsteroidal insecticides were obtained from Rohm and Haas. RXR ligands were provided by Ligand Pharmaceuticals.

Identification of Inducers. Compounds were dissolved in ethanol or DMSO. Transfections were performed in triplicate, multiple times. To identify inducers, CV-1 cells were transiently transfected with plasmids encoding the components of the ecdysone-inducible system. The E/GRE$_4$-ΔMTV-luciferase plasmid was used as a reporter (5). A CMX-β-galactosidase reporter plasmid was included to control for transfection efficiency. Approximately 12 h after transfection, the medium was replaced with medium containing putative inducers. Cells were exposed to candidate activators for 20–48 h and then assayed for luciferase and β-galactosidase activity. Luciferase values were normalized using β-galactosidase activity levels.

Transgenic Animals. Transactivator mice expressing VgEcR and RXR in the skin were generated by separately placing these genes under the control of the human keratin 5 promoter (from Dr. E. Fuchs, University of Chicago) and coinjecting the two transgenes. VgEcR/RXR expression in the skin of founders was analyzed, and lines were established from promising animals. Luciferase reporter mice were generated by injecting an E/GRE$_4$-ΔMTV-luciferase transgene. Transactivator and reporter mice were crossed to obtain bigenic animals. Skin and tail biopsies from these mice were obtained before drug treatment, and at several time points after administration of inducer. Inducers were dissolved in a minimal amount of DMSO (30–50 μl) and mixed with 100–150 μl of olive or sesame oil; inducer suspensions were injected intraperitoneally. Ecdysone-regulated reporter gene expression was assessed by preparing RNA from the biopsies or by analyzing luciferase activity normalized to the protein concentration of these samples (20–22).

Results

Identification of Ecdysteroid Inducers. As a first step in the search for new inducers for the ecdysone-inducible gene switch, a review of the literature was conducted to identify those phytoecdysteroids that had shown the greatest molting activity. Promising ecdysteroids were evaluated by treating cells transiently transfected with the components of the inducible system (a plasmid to constitutively express the VgEcR/RXR heterodimer, and a second one encoding the luciferase gene under the control of a VgEcR/RXR-responsive promoter). Increasing concentrations of potential VgEcR/RXR ligands were added 24 h after transfection, and the relative inducing activity of each ecdysteroid was determined by comparing it with vehicle.

A typical dose–response curve is shown in Fig. 1*A*. Of the phytoecdysteroids screened, only ponA, ponC, and polypodine B showed significant gene induction activity. Despite their reported molting effects *in vivo* or *in vitro* (in assays such as the morphological transformation of the *Drosophila* Kc cell line), inokosterone, makisterone A, and the natural insect hormone 20-hydroxyecdysone (β-ecdysone) were very poor activators of the VgEcR/RXR heterodimer, even at high concentrations (100 μM). Ecdysone (α-ecdysone), 2-deoxyecdysone, 20-hydroxyecdysone-22-acetate, and 2-deoxy-20-hydroxyecdysone were inactive (not shown). We also tested 14-deoxymuristerone, a compound with such an exceptional molting hormone activity that it has been dubbed the "superhormone" (23). Despite having 5- to 8-fold greater affinity for the ecdysteroid receptor of the *Drosophila* Kc cell line than muristerone or ponA, 14-deoxymuristerone was not a better VgEcR/RXR inducer than murA itself (Fig. 1*B*).

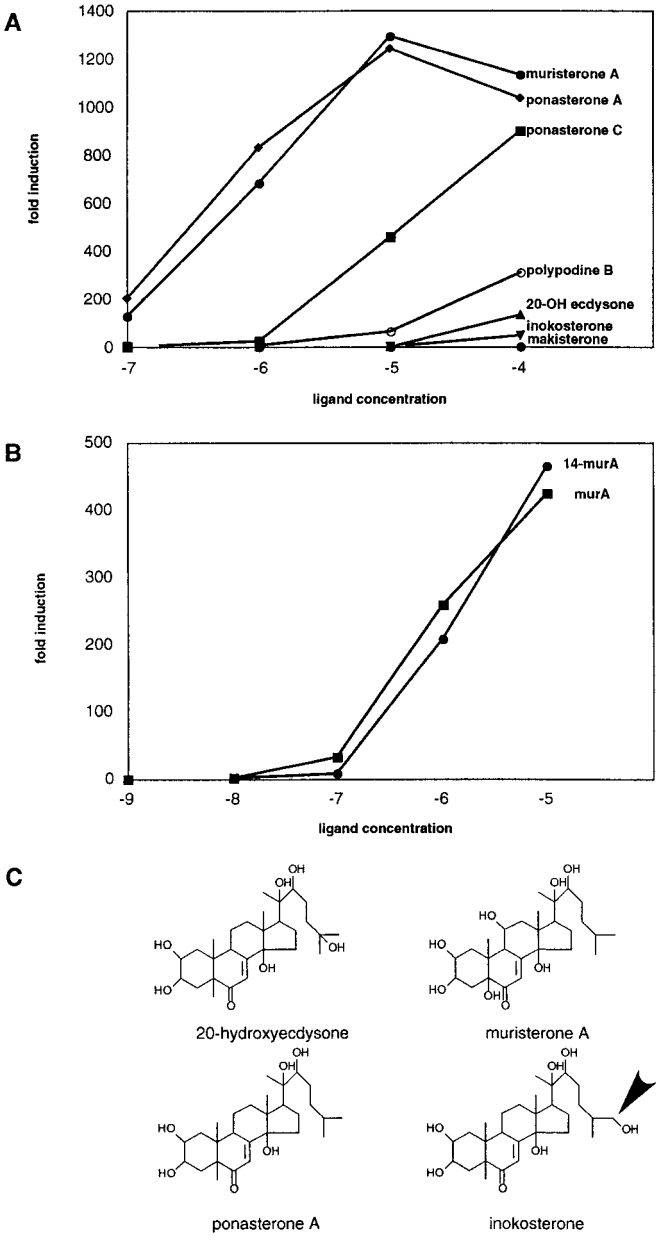

Fig. 1. Identification of phytoecdysteroid ecdysone-system inducers. (*A*) Dose–response curve for several ecdysteroids. (*B*) Potency comparison between murA and 14-deoxymuristerone A. (*C*) Chemical structure of some ecdysteroids tested. MurA and ponA are good inducers, whereas 20-hydroxyecdysone and inokosterone are very poor activators.

The structures of some good and poor phytoecdysteroid inducers are shown in Fig. 1*C*. Because active and inactive ecdysteroids share many of the same features, without crystal structures it is unclear what constitutes potent versus ineffective ligands. The most informative characteristic may be the absence of a hydroxyl group at position C-25 in the three most potent inducers: murA, ponA, and ponC. These ecdysteroids do possess hydroxyl groups at C-20 and C-22, features that have been shown to be important for molting activity (24). Because the main difference between ponA and inokosterone is the presence in the latter of a hydroxyl group at C-26 (see arrow), this addition may be responsible for the inability of inokosterone to activate the VgEcR/RXR heterodimer.

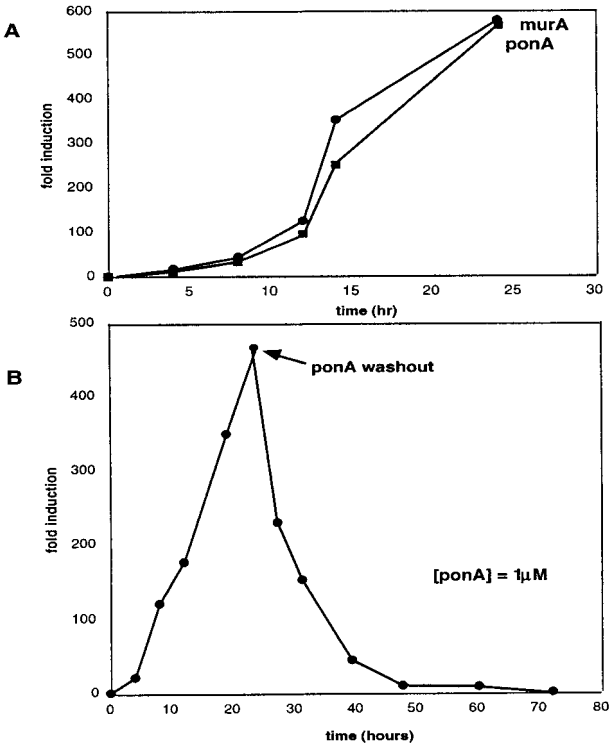

Fig. 2. *In vitro* characterization of ponA. (*A*) Comparison of the potency and kinetics of induction of ponA and murA. (*B*) Kinetics of induction and shutoff of the ecdysone system with 1 μM ponA as the inducer.

Characterization of ponA. Our phytosteroid screen indicated that ponA behaved like a potent inducer, matching the efficacy of murA. The ponasterones were the first ecdysteroids to be isolated from plants in 1966 (25). In contrast to murA, extracted from the rare kaladana seeds, ponA can be purified from the leaves of many plants, including those of the *Podocarpacea* family, a widespread conifer species (15). Although *Podocarpus nakaii*, the family member with the greatest content of ponA, is indigenous to Southeast Asia, *Podocarpus macrophyllus* also contains large amounts of ponA and can be found in many western gardens. We used described methods to extract ponA from the leaves of *P. macrophyllus* obtained at a local nursery (15–17).

Purified ponA exhibits a potency similar to that of murA when tested on cells (Fig. 2*A*). Furthermore, the kinetics of the *in vitro* response of the ecdysone system are virtually identical, with an induction detectable within 4 h of exposure to inducer. The magnitude of the induction reaches 250- to 400-fold within 14 h and continues to increase before stabilizing at approximately 600-fold between 24 and 36 h after ligand addition. To examine how quickly the ecdysone system shuts off, ponA medium was replaced with vehicle-containing medium, and luciferase activity was measured at various time points after inducer removal. Even when cells are exposed to high concentrations of ponA, once the inducer is removed the system promptly returns to the basal state (Fig. 2*B*). A 50% reduction in luciferase levels is observed 6 h after inducer removal. By 20 h, the amount of luciferase protein produced by the ecdysone system approaches uninduced levels.

The potency and favorable kinetics of ponA as an ecdysone-system inducer *in vitro* spurred us to evaluate its performance *in vivo*. To measure the pharmacokinetics of ponA-mediated gene induction in animals, two new lines of ecdysone-system transgenic mice were engineered (Fig. 3*A* and *Materials and Methods*). The human keratin 5 promoter was used to create a transactivator strain coexpressing VgEcR and RXR in the basal keratinocytes of stratified epithelia (mainly skin and tail). A universal reporter strain was generated using the luciferase reporter plasmid from our *in vitro* analysis. In principle, once these strains are bred together, expression of the ecdysone system in the skin should permit the inducible regulation of the reporter transgene selectively in this tissue. This combination of transgenes was chosen primarily because the luciferase protein has a short half-life that allows precise kinetic studies (26). Moreover, because a small biopsy of skin or tail suffices to establish luciferase levels, the temporal effects of putative inducers can be measured in the same mouse.

To assess the ability of ponA to regulate genes *in vivo*, transactivator and reporter strains were crossed and skin and tail biopsies were taken from bigenic offspring before and at various times after ecdysteroid administration (Fig. 3*A*). Drug delivery was confirmed by collecting blood from treated mice and measuring serum levels of inducer with a cell-based assay (5). In the absence of inducer, luciferase activity in the skin or tail of bigenic animals was barely detectable. In fact, basal luciferase levels in transactivator-reporter bigenic animals were lower than those in mice carrying the reporter transgene alone, suggesting that in the absence of ligand the VgEcR/RXR complex may actually act as a repressor (not shown). MurA and ponA regulated gene expression in bigenic mice with similar kinetics and potency (Fig. 3*B*). A single injection of either phytoecdysteroid produced an induction that was easily detectable at the protein level 4–6 h after treatment. Luciferase activity peaked 9–12 h after inducer injection, dropped significantly by 24 h, and returned toward basal by 48 h. This pattern of induction was reproducible across mice (Fig. 3*C*). The two ecdysteroids generated inductions of similar magnitude, on the order of 20- to 200-fold, depending on the amount of inducer used. Doses of 3, 5, and 10 mg of ponA produced robust inductions (Fig. 3*D* and not shown). Inductions in the skin begin to trail off around 1 mg, but the inducing ability of ponA may differ between tissues, as full inductions in mammary gland are achieved with only 750 μg, with effective inductions observed at even lower doses (27).

Nonsteroidal Ecdysone-System Inducers. Having characterized ponA as an effective inducer for the ecdysone gene switch, we sought to identify inducers with different chemistries and potentially differing regulatory properties *in vitro* and *in vivo*. In one approach, over 100 pesticides selected on the basis of their LD_{50} and solubility were tested using the transient transfection method described earlier. Despite their diverse chemistry, none of these compounds proved to be an effective inducer, which indicates that their pest control properties are probably not due to stimulation of insect ecdysone receptors. In a second approach, we screened members of a family of nonsteroidal insect growth regulators that have been reported to behave as ecdysone mimics (28, 29). These compounds can compete with ecdysteroids for binding to ecdysone receptor. Nine of 21 of these chemicals showed some ability to activate the VgEcR/RXR heterodimer. The gene induction activity of these compounds does not compare with that of the ecdysteroids unless they are used at high concentrations, but several compounds show promising properties (Fig. 4*A*). Unfortunately, these nonsteroidal inducers share a common diacylhydrazine structure (Fig. 4*B*) that renders them rather insoluble and to date has precluded extensive *in vivo* analysis.

RXR Ligands as Ecdysone Synergizers. Complexes of nuclear receptors that heterodimerize with RXR are described as being permissive if the ligand for either partner is sufficient to activate the transcriptional function of the heterodimer (30). Alternatively, they are termed nonpermissive if RXR ligands do not enhance the transcriptional activity of the complex unless the

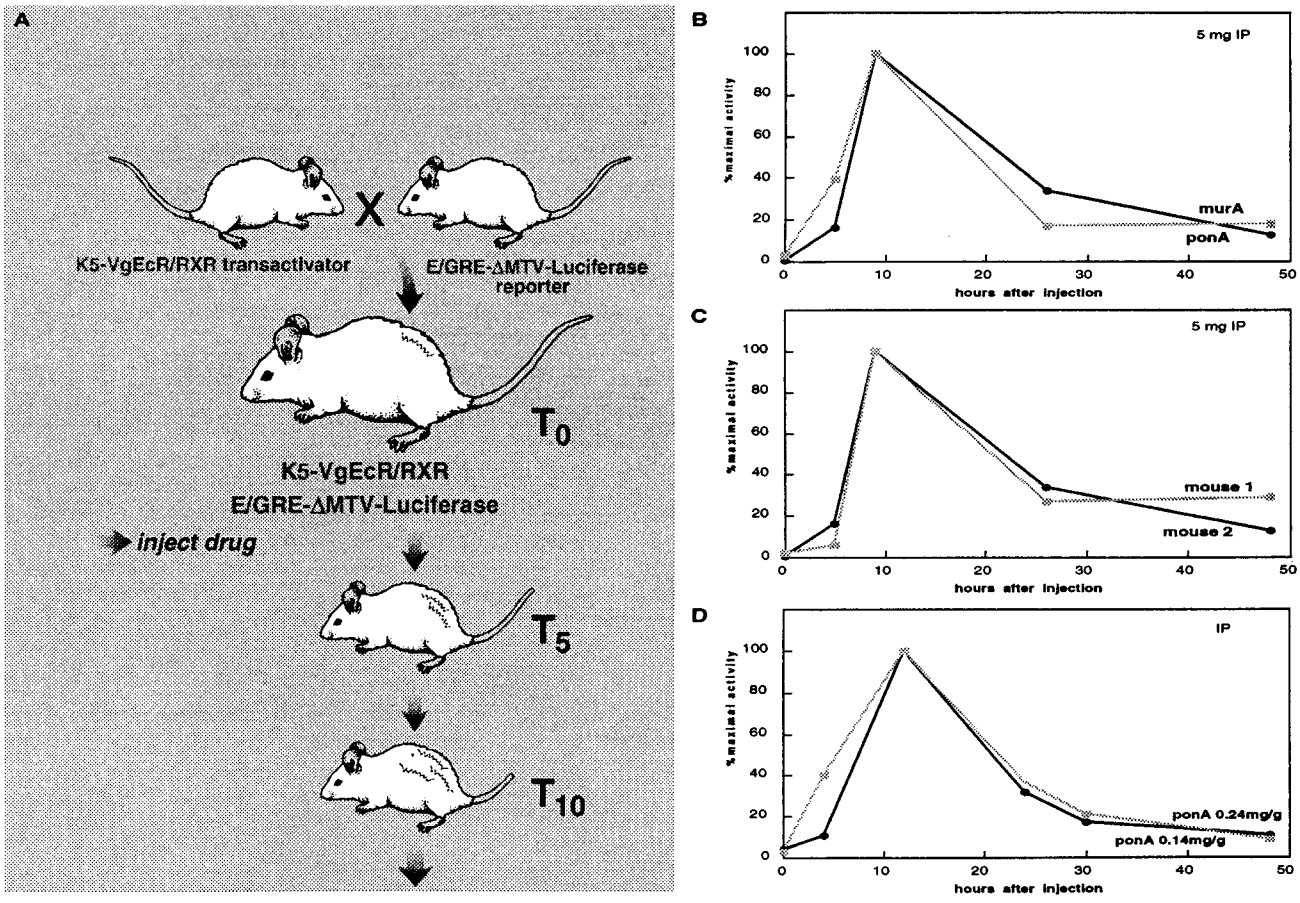

Fig. 3. *In vivo* characterization of ponA. (*A*) Schematic of the transgenic approach used to test the ability of ponA to regulate gene expression in animals. Transactivator (K5-VgEcR/RXR) and reporter (E/GRE_4-ΔMTV-luciferase) mice were bred, and samples of blood, dorsal skin, and tail were obtained from bigenic offspring before and at various times after inducer administration. (*B*) Comparison of ponA and murA *in vivo*. Luciferase levels in skin and tail biopsies from bigenic animals injected with ponA or murA were measured and normalized to protein content. Shown is the analysis of tail samples from mice injected with 5 mg of inducer. (*C*) Reproducibility of ponA-inducible gene regulation: pattern of gene induction in tail samples from two different mice injected with 5 mg of ponA. (*D*) Inductions generated by 3 or 5 mg of ponA. Tail samples are shown.

ligand for the heterodimeric partner is also present. Because the ecdysone-inducible system is based on the VgEcR/RXR heterodimer, we examined the effect of RXR ligands on the gene-control properties of this complex (31). As defined above, the VgEcR/RXR heterodimer is nonpermissive, because the addition of an RXR ligand does not promote the expression of ecdysone target genes (Fig. 5*A*). However, simultaneous administration of VgEcR and RXR ligands dramatically enhances the ability of the heterodimer to generate powerful inductions. Synthetic RXR ligands present at nanomolar concentrations can increase by 3- to 5-fold the absolute levels of induction brought about by the VgEcR/RXR heterodimer. The unique response of the EcR heterodimer to RXR ligands eludes the standard definitions. Accordingly, we refer to this behavior as "semipermissive" and suggest that it may be prototypical of a new class of hormone responsiveness.

The synergizing effect of RXR ligands is independent of the VgEcR activator or RXR ligand used, although the natural RXR ligand 9-*cis* retinoic acid seems to be weaker than the synthetic RXR ligands (Fig. 5*B*). Moreover, by combining an inferior nonsteroidal VgEcR agonist with a synthetic RXR ligand, inducing mixtures can be created that are superior to murA alone (Fig. 5*C*). It has yet to be determined whether the stronger induction brought about by the combination of VgEcR and RXR ligands is reflective of increased protein production per cell or, rather, of a greater number of cells making the regulated protein.

Discussion

The advent of the postgenomic era has accentuated the need to develop methods to determine gene function. Precise control of gene expression is an important means of establishing the role of gene products during development, normal physiology, and disease. Deliberate manipulation of gene activity is also an indispensable tool for clinical applications such as gene therapy. The ecdysone-inducible system has proved useful for studying a multitude of processes, such as apoptosis (32–36), cancer and cell-cycle regulation (37–40), embryonic development (41, 42), signal transduction (43–45), lipid metabolism (46), and neuronal function (47–50). The strengths of the system appear to be its tight regulation, its dose responsiveness, and the favorable uptake and clearance kinetics of the steroid inducer, which results in rapid gene switching.

To facilitate the use of the ecdysone-inducible system, we searched for activators of the VgEcR/RXR complex. Incidentally, the presence of ecdysone-like molecules in the plant kingdom is nearly universal. Plants use ecdysteroids as natural insecticides, yet phytoecdysteroids are completely innocuous in mammals. Despite their steroidal skeleton, they have no teratogenic, sex-hormonal, anabolic, or analgesic properties. In rats,

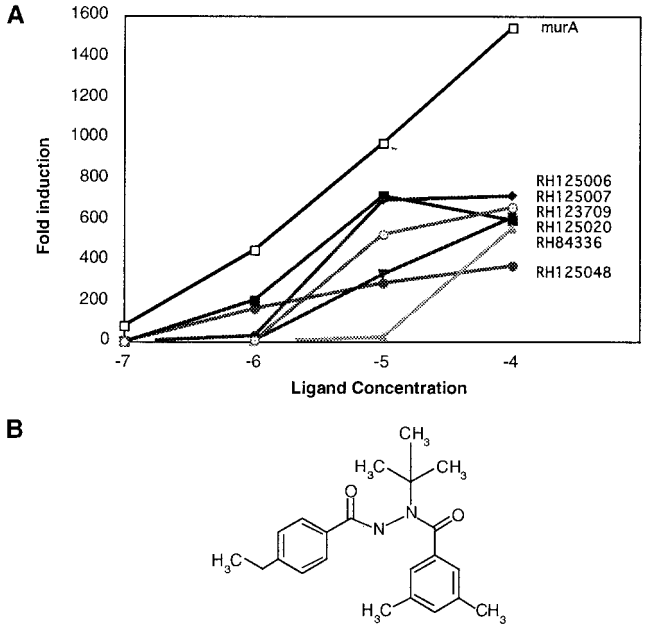

Fig. 4. Nonsteroidal ecdysone-system inducers. (*A*) Dose–response curve with selected nonsteroidal agonists. (*B*) Structure of RH 5992 (Tebufenozide), showing the basic features of this family of ecdysone receptor agonists.

daily administration of 2 g/kg of ecdysteroid for 35 days has no effect on serum chemistry or organ weight (51). A maximum permissible oral dose of 9 g/kg of ecdysteroid does not have any lethal effects in mice (51). Moreover, humans consume large amounts of dietary ecdysteroids without detrimental effects. For example, fresh spinach contains over 100 μg/g of polypodine B and 20-hydroxyecdysone (52). Ingested ecdysteroids survive the acidic conditions of the stomach, are quickly taken up, are promptly removed from the bloodstream, and are disposed of primarily through biliary excretion into the intestine (53). Ecdysteroid processing is fast; serum levels in humans peak 30 min to 2 h after administration of an oral dose (54). Their safety and swift kinetics makes phytoecdysteroids particularly well suited as inducers for gene regulation *in vivo*.

PonA is a potent and effective inducer of the ecdysone system *in vitro* and in transgenic animals. The ease with which ponA can be isolated from common plants ensures a plentiful supply of this inducer. PonA can also be chemically derived in large quantities by removal of a hydroxyl group from 20-hydroxyecdysone (55). Our work with transgenic animals substantiates the safety and rapid turnover of phytoecdysteroids *in vivo*. More prolonged inductions can be achieved by the implantation of timed-release pellets. A recent report demonstrates that pellets with as little as 200 μg of ponA are sufficient to sustain transgene expression in the mammary gland for at least 10 days (27). The smaller dose required to regulate genes in the breast compared with our studies in the skin may be a reflection of the lower sensitivity of our transgene combination.

Several nonsteroidal compounds originally developed as rational pest control agents can also activate the VgEcR/RXR heterodimer. These chemicals are not as potent as ponA, but they are manufactured in industrial quantities. Their poor solubility may hinder their use *in vivo*, but animal experiments suggest that they are cleared slowly, so they could be useful in scenarios where sustained induction of gene expression is necessary (56). Other nonsteroidal compounds, such as the irioid glycoside 8-*O*-acetylharpagide, found in the bugleweed *Ajuga reptans*, can also bind the *Drosophila* ecdysone receptor (57, 58).

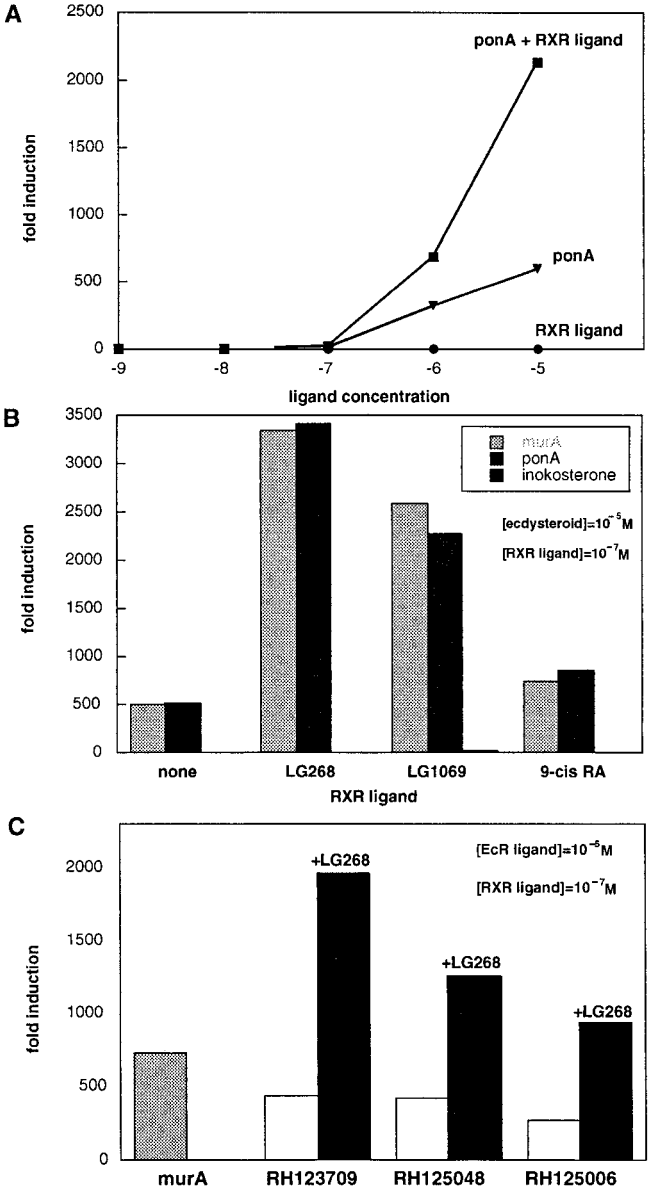

Fig. 5. Effect of RXR ligands on the transcriptional activity VgEcR/RXR complex. (*A*) Dose–response for ponA, an RXR ligand, or ponA plus a constant concentration of RXR ligand (100 nM). Increasing concentrations of the RXR ligand LG268 had no effect on the VgEcR/RXR heterodimer. Combination of 100 nM LG268 with increasing amounts of ponA resulted in considerably greater inductions than when ponA was used alone. (*B*) Combined effect of ecdysteroids and RXR ligands on VgEcR/RXR activation. Cells were treated with 10 μM murA, ponA, or inokosterone, alone or in combination with one of three RXR ligands present at 100 nM concentration. (*C*) LG268 potentiates the inducing properties of nonsteroidal VgEcR agonists. Cells were treated with 10 μM nonsteroidal ecdysone receptor ligand, alone or in combination with LG268 at 100 nM concentration. These mixtures induced higher levels of reporter activity than 10 μM murA.

The ability of these molecules to serve as inducers remains to be established.

In its present form, the ecdysone-inducible system relies on the gene-controlling capacity of the VgEcR/RXR heterodimer. Overexpression of exogenous RXR does not have deleterious effects in any *in vitro* or *in vivo* situation tested thus far. By exploiting the potential of both partners, researchers can use

RXR ligands to significantly potentiate the utility of the system. The semipermissive nature of the VgEcR/RXR heterodimer allows the development of inducer mixtures composed of ecdysone receptor and RXR ligands that are superior to ecdysteroid inducers. Because RXR ligands do influence some physiological parameters, their use may not be appropriate in all cases (59). Nonetheless, mixtures of VgEcR and RXR ligands will be useful in applications where only transient exposure to the inducer mix is required.

Plants also contain insect-molting inhibitors of varied chemistry. For example, the phytosteroid ajugalactone from *Ajuga decumbens* and the triterpene cucurbitacins that can be isolated from the candytuft (*Iberis umbellata*) are powerful antagonists of 20-hydroxyecdysone in molting assays, inhibiting the action of the hormone by competing for binding to its receptor (60, 61). The development of these molecules as ecdysone-system antagonists could make the system more pulsatile.

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