

REVIEW

Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals

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Abstract

Zooecdysteroids (arthropod steroid hormones) regulate the development of arthropods and probably many other invertebrates. Phytoecdysteroids are analogues occurring in a wide range of plant species, where they contribute to the deterrence of phytophagous invertebrates. The purpose of this short review is to summarise findings on the occurrence,

metabolism and pharmacological effects of ecdysteroids in mammalian systems and to draw attention to their potential applications, particularly in gene-switch technology, where ecdysteroid analogues (steroidal and non-steroidal) can be used as effective and potent elicitors.

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Introduction

Ecdysteroids are the steroid hormones of arthropods, where they regulate moulting, metamorphosis, reproduction and diapause (Koolman 1989). They probably fulfil similar roles in many other invertebrate phyla, but these have not been so extensively investigated (Lafont 1997). Ecdysteroids are also present in 5–6% of plant species (Dinan 1995a), generally at far higher concentrations than those typically found in arthropods, where they are regarded as contributing to the deterrence of invertebrate predators (Lafont *et al.* 1991, Dinan 2001). The archetypal ecdysteroid in both arthropods and plants is 20-hydroxyecdysone (20E; Fig. 1), since it is the most commonly occurring and the most abundant, but a very wide range of structural analogues has been elucidated, especially from plant sources (Lafont *et al.* 2002). The first ecdysteroid (ecdysone; Fig. 2) was isolated in 1954 (Butenandt & Karlson 1954) and its structure was unambiguously identified only in 1965 (Huber & Hoppe 1965), with the result that research on ecdysteroids has generally lagged far behind that on the various classes of vertebrate steroid hormones, in spite of the fact that development in more than 90% of all animal species is dependent upon ecdysteroids. However, since the early reports of the occurrence of ecdysteroids in insects and plants, researchers have posed questions about the occurrence (from the diet), metabolism and possible effects of ecdysteroids in mammals. In the intervening period, a substantial body of evidence relevant to these questions has been obtained,

suggesting that ecdysteroids may have significantly positive pharmacological properties. This is consistent with the use of several ecdysteroid-containing plant species in traditional medicines. The ready availability of large amounts of 20E from certain plant sources has led to a boom in recent years in its inclusion in many commercial anabolic preparations for body-builders and sportsmen. Further, since ecdysteroids are not endogenous products of mammalian metabolism and are non-toxic to mammals, they are also finding application as elicitors of novel gene-switch systems. With the intention of bringing this area to a wider audience of endocrinologists, we shall summarise the most significant findings. Also, we shall focus on the developments in this area since our previous review (Lafont & Dinan 2003), but the reader is referred to that review for a more extensive treatment of the relevant literature and the concepts.

Occurrence of ecdysteroids in mammals

Ecdysteroids are not regarded as products of mammalian metabolism. However, they can be detected in mammalian tissues and fluids as a consequence of dietary intake of ecdysteroid-containing plants (or insects in certain population groups) or infection by invertebrate parasites. Most crop plant species do not contain phytoecdysteroids, although both spinach (*Spinacia oleracea*) and quinoa (*Chenopodium quinoa*) do contain significant levels, especially in the seeds and

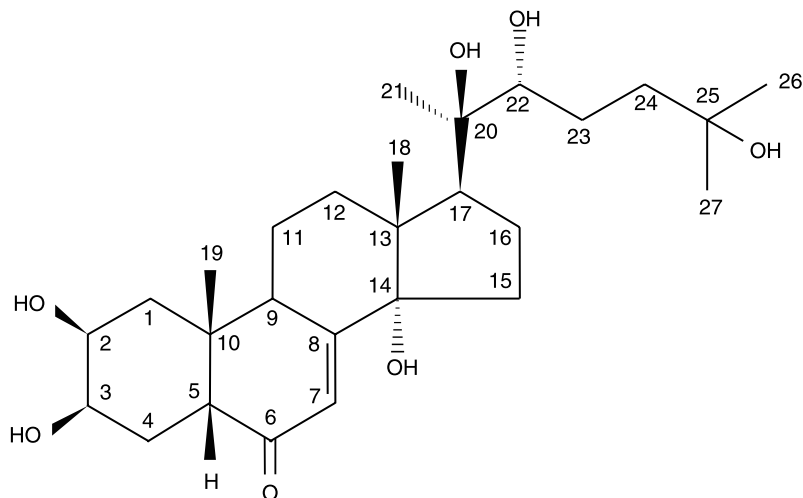


Figure 1 The structure of 20-hydroxyecdysone.

younger leaves (Dinan 1995b). Ecdysteroids differ markedly from vertebrate steroid hormones in their polarity (polyhydroxylated), bulk (C_{27} – C_{29}) and shape (A/B-*cis*-ring junction), thus one would expect there to be little interaction with the steroid-hormone receptors or steroid-metabolising enzymes in mammals. Unfortunately, this has not been systematically verified. The mechanisms, by which any pharmacological effects of ecdysteroids in mammals are mediated, are presently unknown (but see below).

Metabolism of ecdysteroids in mammals

Injected or ingested ecdysteroids are rapidly cleared from the blood. In mice, ecdysone (Fig. 2) undergoes a complex array of metabolic reactions, including dehydroxylation (removal of the 14α -OH) most probably by gut bacteria (cf. dehydroxylation of bile acids), reduction in the B-ring and epimerisation at C-3 (Lafont *et al.* 1988). In addition, when a 20,22-diol is present (as in 20E), side-chain cleavage can take place between C-20 and C-22 and the pattern of metabolites becomes very complex (Kumpun, Girault, Blais, Maria, Dauphin-Villemant, Yingyongnarongkul, Suksamrarn and Lafont, unpublished data). 14-Dehydroxylation also takes place in humans (Brandt 2003). Methods aimed at analysing 20E and its metabolites in human urine are presently being developed as anti-doping controls for humans and cattle (Tsitsimpikou *et al.* 2001, Le Bizec *et al.* 2002).

Pharmacological effects of ecdysteroids on mammals

Pharmacological tests were first performed on mammals (mice, rats) in the late 1960s to detect any harmful effects of ecdysteroids on vertebrate organisms, which could have

precluded their use as insecticides. This was the starting point for many experiments, which reported a wide array of pharmacological effects of ecdysteroids on mammals (Sláma & Lafont 1995, Syrov 2000, Kholodova 2001, Báthori 2002, Lafont & Dinan 2003, Klein 2004, Báthori & Pongrácz 2005).

Early pharmacological experiments

First of all, it must be emphasised that the acute toxicity of ecdysteroids in mammals is very low: the LD_{50} for 20E is above 6 g/kg. The first reported effect of ecdysteroids on mammals was a stimulation of protein synthesis (translation) *in vitro* by liver polysomes prepared from rats having received 20E (or any of the other then available ecdysteroids). This effect was even used as a convenient bioassay for the presence of phytoecdysteroids in plant extracts during large-scale screening programmes (Otaka *et al.* 1969). At the same time, other authors analysed the distribution and metabolic fate of injected [3 H]ecdysone (Hikino *et al.* 1972).

These pioneering studies were followed by extensive studies on possible anabolic effects of ecdysteroids in rats (and humans). The general strategy was to compare the physical performance or biochemical parameters of animals having received 20E (or another ecdysteroid) over 1–3 weeks to controls or animals treated with a ‘classical’ anabolic steroid (testosterone or dianabol), and these experiments indicated a significant anabolic effect of ecdysteroids. Several ecdysteroid preparations have been designed for sportsmen (e.g. ‘Ekdisten’ or ‘Retibol’) containing quite small amounts of 20E (5 mg/tablet). Since those early times, the market for ecdysteroid-containing preparations has developed considerably, and is now especially aimed at bodybuilders; presently, ca. 300 ecdysteroid-containing products are available on the market, some of which contain large amounts of 20E, with formulations recommending up to 1 g daily intake, usually in combination with other known anabolic chemicals and/or

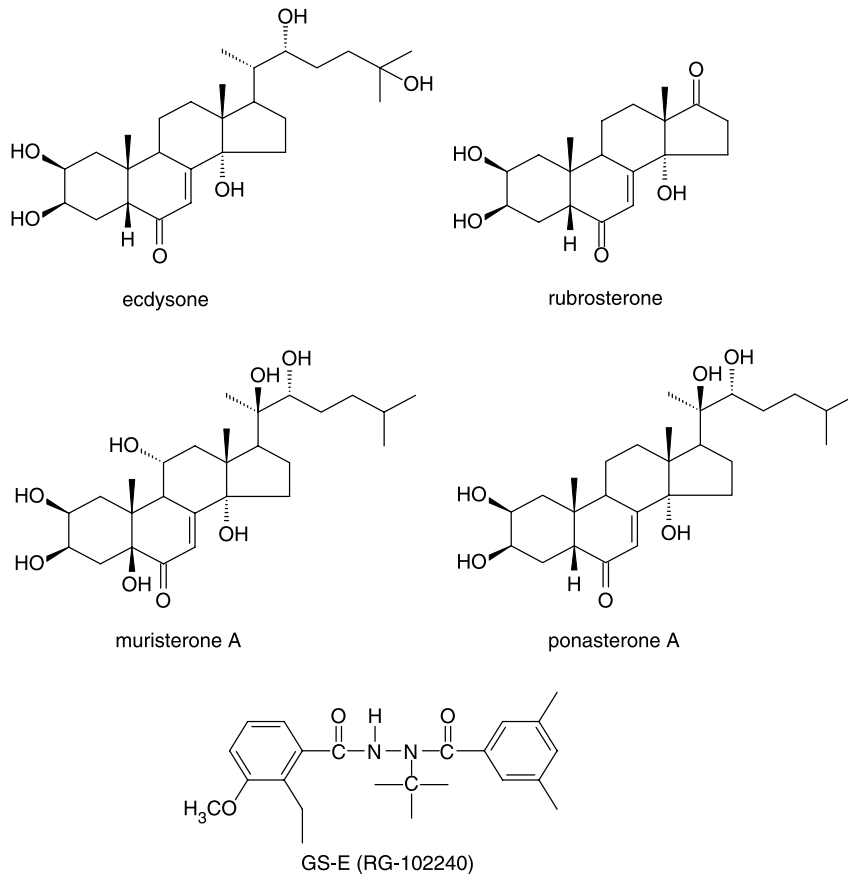


Figure 2 Structures of ecdysone, rubrosterone, muristerone A, ponasterone A and GS-E (RG-102240).

included in a high-protein diet. The basis for all the Internet claims about the effects of ecdysteroids is not clear. However, the scientific literature shows that a significant body of experiments has indeed been performed, which describe various effects of ecdysteroids on mammals/humans, and have resulted in more than 50 patents for both internal and external use. The main findings are summarised below (for more details, see Lafont & Dinan 2003).

Protein metabolism General stimulatory effects on protein synthesis have been shown for various ecdysteroids, including rubrosterone, a C-19 ecdysteroid lacking the whole side-chain (Fig. 2). These effects have been observed in mice injected with 5 mg ecdysteroid/kg and they seem to result from increased mRNA translation efficiency and not from increased mRNA synthesis (transcription) (Otake *et al.* 1968, Syrov *et al.* 1978, Khimiko *et al.* 2000).

Lipid metabolism Ecdysteroids have hypocholesterolaemic effects (Mironova *et al.* 1982, Syrov *et al.* 1983), best explained by increased conversion of cholesterol into bile acids, reminiscent of the effect of oxysterols (Schroepfer 2000). In rats, low doses (10–50 µg/kg) of injected 20E do not affect

cholesterolaemia, but reduce hepatic cholesterol levels (Lupien *et al.* 1969). In addition, dietary 20E (0.1 mg/kg per day) administered over 30 days reduces lipid peroxidation in membranes (Kuzmenko *et al.* 1997).

Carbohydrate metabolism Pretreatment with ecdysteroids (i.p. injections of 0.5 mg/kg) may reduce hyperglycaemia induced by administration of glucagon or destruction (by alloxan treatment) of pancreatic islet β -cells in mice and rats (Yoshida *et al.* 1971). Antidiabetic effects are also known for ecdysteroid-containing plants used in traditional medicine (e.g. *Ajuga iva*; Wessner *et al.* 1992) and ecdysteroid-containing preparations have been proposed for use against diabetes (Takahashi & Nishimoto 1992, Yang *et al.* 2001). Recent experiments *in vitro* with human hepatocytes have established that ecdysteroids (10^{-6} – 10^{-4} M) increase glucose consumption in an insulin-independent fashion (Chen *et al.* 2006).

Effects on specific organs/systems

The beneficial effects of ecdysteroids on the function of many organs have been reported. We will give a few examples.

Muscle Ecdysteroids increase muscle mass by enhancing protein synthesis and decreasing protein catabolism. It has been suggested that the latter effect operates indirectly through a reduction of glucocorticoid levels (i.e. a reduction of stress), but this assumption has not been directly documented.

Liver Ecdysteroids stimulate bile secretion in rats (Syrov *et al.* 1986), and they improve liver regeneration after chemically induced damage (Badal'yants *et al.* 1996).

Kidney Dietary 20E (5 mg/kg) can restore normal glomerular filtration rate and suppress albuminuria in rats treated with a nephrotoxic mixture (Syrov & Khushbaktova 2001).

Skin Ecdysteroid-containing liposomes improve skin quality by accelerating the healing of small wounds or burns (Meybeck & Bonté 1990) and have been marketed by Louis Vuitton-Moët Hennessy Company in several commercial cosmetics. 20E (2×10^{-4} M) promotes differentiation of human keratinocytes *in vitro* (Detmar *et al.* 1994) and this might explain its psoriasis-inhibiting activity (Inaoka *et al.* 1997).

Brain Several actions of ecdysteroids on the central nervous system have been described; induction of enzymes related to neurotransmitter synthesis (glutamic decarboxylase; Chaudhary *et al.* 1969) or degradation (acetylcholinesterase; Catalán *et al.* 1984) and protection of neurons against the deleterious effects of various drugs (Aikake *et al.* 1996, Xu *et al.* 1999). Neuromodulatory effects of 20E (100 μ M) on the GABA_A receptor have also been reported (Tsujiyama *et al.* 1995, Okada *et al.* 1998).

General comments

Many more (beneficial) effects have, in fact, been described for injected or ingested ecdysteroids in mammals (see Lafont & Dinan 2003, Báthori & Pongrácz 2005). The weight of the accumulated evidence leaves little doubt that ecdysteroids do have effects on mammals. However, in many individual studies, the published data need to be further substantiated by more experimental evidence to be fully convincing. When crude or semi-purified plant extracts have been used, they do not demonstrate that the observed effects result (only) from ecdysteroids themselves. Whenever pure ecdysteroids have been used, the results are not always as spectacular as claimed in publicity promoting the use of these molecules for humans (but in this case, ecdysteroids are usually part of complex cocktails). Also, the statistical significance of results is questionable when small effects are observed with a low number of replicates and, in many instances, there is no clear-cut dose-response effect. Finally, we may consider as most convincing the unexpected data obtained by users of ecdysteroid-inducible gene systems (see below), which

demonstrate ecdysteroid effects in control experiments, which means that exogenous insect ecdysteroid receptors are not required for some ecdysteroid effects to occur in mammalian/human cells (Constantino *et al.* 2001, Oehme *et al.* 2006).

Possible mechanisms of action

Besides their neuromodulatory effects on the GABA_A receptor, which can easily be explained, the other numerous effects cannot be due to a single target. Moreover, some could be due to specific metabolites rather than the administered compound. Some transcriptional effects (perhaps caused by side-chain cleavage metabolites of ecdysteroids) can be expected to take place through binding to nuclear receptors (pregnane X receptor (PXR), constitutive androgen receptor (CAR) and liver X receptor (LXR)), which can bind a wide array of ligands, including various xenobiotics (Blumberg *et al.* 1998, d'Ursi *et al.* 2005, Handschin & Meyer 2005), but to our knowledge, as yet, there is no direct evidence for such binding. Recent data (Constantino *et al.* 2001, Oehme *et al.* 2006) provide convincing evidence that ecdysteroids evoke significant changes of gene expression in mammalian cells, resulting in the activation of the phosphatidylinositol-3-kinase/protein kinase B signal transduction (PI3K/Akt) pathway responsible for anti-apoptotic effects. We may also expect some rapid actions via membrane receptors: high affinity-binding sites have already been described using 20E bound to magnetic nanoparticles (Mykhaylyk *et al.* 2001), together with rapid effects on, e.g. prostaglandin synthesis (Kotsyuruba *et al.* 1995).

Ecdysteroid receptors in arthropods

Most actions of ecdysteroids are mediated by intracellular receptor complexes, although it should not be forgotten that some rapid actions are mediated by other mechanisms (Tomaschko 1999, Schlattner *et al.* 2006). The intracellular receptors from a range of arthropod species have been characterised and the active complex consists of two proteins: the ecdysteroid-receptor (EcR) protein and an ultraspiracle (USP) or retinoid X receptor (RXR) protein (Henrich 2005). All the above are members of the nuclear receptor superfamily, and USP and RXR are homologues (Laudet & Bonneton 2005). Members of this superfamily are characterised by a domain structure, consisting of an N-terminal A/B-domain involved in transcriptional activation, a DNA-binding C-domain, a hinge D-domain, the ligand-binding E-domain and an F-domain of unknown function (which is not present in all members of the superfamily). The C-domains of EcR/USP complexes interact with specific ecdysteroid-responsive elements (EcREs) in the promoter regions of ecdysteroid-responsive genes to alter gene transcriptional activity. X-ray crystallographic structures for lepidopteran (Billas & Moras 2005) and hemipteran (Carmichael *et al.* 2005) EcR E-domains, dipteran (Clayton

et al. 2001) and lepidopteran (Billas *et al.* 2001) USP E-domains and dipteran EcR/USP C-domains (Devarakonda *et al.* 2003) have been elucidated. The ligand-binding domain (LBD) is specific for ecdysteroids and other classes of ecdysteroid agonists and antagonists (Dinan 2003, Dinan & Hormann 2005), such that the EcR/USP complex is not activated by vertebrate steroid, retinoid or thyroid hormones. USP is regarded as an orphan receptor, for which there is no definite ligand, but it has been suggested that juvenile hormones may interact with its LBD (Jones & Jones 2000, Fang *et al.* 2005). Further, when USP is replaced by RXR as EcRs partner, retinoids potentiate the activity of ecdysteroids (Saez *et al.* 2000). Since neither ecdysteroids nor EcR proteins are endogenous components of mammalian cells, this system can be used as the basis for the regulation of transfected genes possessing appropriate hormone responsive elements (HREs), by ecdysteroid receptors (also expressed from transfected genes) and the use of appropriate ecdysteroid (ant)agonists as elicitors, to generate an 'ecdysteroid-inducible gene-switch system'. While, in theory, the native arthropod components (20E, EcR and EcRE; RXR is a constitutive component of many mammalian cell types) could be used, in practice, it is necessary to modify each of the exogenous components to optimise the properties and performance of the system to improve the sensitivity, specificity and strict temporal regulation of the transfected gene (see section 'Ecdysteroid-inducible gene-expression systems').

Ecdysteroid-inducible gene expression systems

The spatial and temporal control of heterologous gene expression is an area of major current interest with application to basic and applied biological and medical research, including gene therapy and functional genomics. Such 'gene-switch' systems should provide rapid, precise and reversible induction (or suppression) of the target gene(s), but not interfere with the complex endogenous regulatory networks of the host cells/tissues. Thus, the key characteristics of the system should be (Fussenegger 2001):

- The introduced regulatory machinery (transcription factor, elicitor) should be *specific* for the target gene(s) and not interfere with endogenous regulatory networks.
- The system should be *inducible* by low concentrations of elicitor in a *dose-dependent* manner, have a very low baseline expression in the absence of elicitor and a high induction ratio.
- The elicitor should be *bio-available* and readily penetrate to the target tissues.
- The action of the inducer should be *reversible*, either through metabolism or excretion, so as to permit switching-off of the effect and repeated cycles of induction.
- The components of the gene-switch system should not be *immunogenic* in the host.

- The design of the gene-switch system should be *flexible* enough to allow for different tissue applications and circumstances.

Early ecdysteroid-related gene-switch systems (Christopher *et al.* 1992, Yao *et al.* 1993) were based on the *Drosophila melanogaster* EcR (DmEcR), in conjunction with co-transfected USP or by using endogenous RXR as the heterologous partner. Thereafter, the activation potential and specificity for specific HREs were enhanced by modifying the activation and DNA-binding domains (No *et al.* 1996). A parallel system based on the *Bombyx mori* EcR (BmEcR) has been developed, and this has the advantage that BmEcR more readily partners with RXR than does DmEcR (Swevers *et al.* 1996, Suhr *et al.* 1998). Since then several Dm/Bm EcRs have been generated (Suhr *et al.* 1998), including one (DB-EcR; Hoppe *et al.* 2000), which is independent of recombinant RXR. A lentiviral vector system has been developed for the effective delivery of ecdysteroid-regulated genes into cells or animals (Galimi *et al.* 2005). Tight transcriptional regulation of foreign gene expression in insect cells has been obtained using a further system (Dai *et al.* 2005), consisting of (i) the DEF domains of the *Choristoneura fumiferana* EcR fused to the *Saccharomyces cerevisiae* GAL4 DNA-binding domain, (ii) the EF domains of mouse RXR fused to the acidic activation domains of baculovirus transactivators IE1 and IE0 and (iii) exogenously applied GS-E (RG-102240; Fig. 2). This system shows considerable promise for the controlled expression of toxic proteins, since any expression in the absence of inducer would result in cytotoxicity of the host cells.

Most researchers' interest in ecdysteroid-inducible gene expression systems is in the functional analysis of cloned genes, which can be put under the control of ecdysteroid-regulated promoters and transfected into mammalian cells. To this end, several commercial gene-switch kits have been developed, notably those produced by Invitrogen (www.invitrogen.com) and Rheogene (www.rheogene.com). As elicitors, these systems use either the steroidal analogues muristerone A or ponasterone A, or a non-steroidal ecdysteroid agonist of the diacylhydrazine class (Nakagawa 2005), such as GS-E (Fig. 2). In the longer term, the intention is to generate systems capable of the independent and coordinated regulation of multiple genes in a tissue and, hence, gene therapy of disease states.

Ecdysteroids and diacylhydrazines possess many properties that make them suitable as gene-switch elicitors. However, EcR LBDs, when expressed in mammalian cells, do not possess the same specificity or affinity as the same LBD in an insect system. Thus, much higher concentrations of active ecdysteroids are required and only certain analogues show significant activity. Thus, the endogenous hormone in insects, 20E, is hardly active in mammalian cells, and muristerone A and ponasterone A are required at concentrations of 1–10 μM to bring about a response when the effective concentrations in insect systems are much lower (e.g. EC_{50} values in the *D. melanogaster* B_{II} bioassay are 2.2×10^{-8} and 3.1×10^{-10} M

respectively; Dinan 2003). Identification of elicitors showing the same potency as these ecdysteroids in insect systems would considerably enhance the sensitivity and reduce the possibility of side-effects during gene therapy. This is being achieved by modifying LBDs of transgenic EcRs by site-directed mutagenesis to enhance the affinity for existing ligands (Palli *et al.* 2005a), modifying the partner RXR/USP (Palli *et al.* 2005b) or by comparative quantitative structure-activity relationship (QSAR) studies on the ligand specificities of ecdysteroid receptors expressed in insect and mammalian systems to identify the differences and to design more potent ligands (Dinan & Hormann 2005).

Conclusions and prospects

Most Internet claims for 'spectacular' effects of ecdysteroids on humans and other mammals are unsubstantiated or apocryphal. However, most reports in the scientific literature have demonstrated that the pharmacological effects of ecdysteroids in mammals are positive, and it is clear that ecdysteroids may influence/improve many physiological functions. Unfortunately, no extensive, systematic trials on any mammalian species have been published. Dietary intake of ecdysteroids is possible but limited for most humans, since the crop species which contain phytoecdysteroids are not extensively eaten. Thus, there may be a future for ecdysteroids as dietary supplements to contribute to human well-being (as 'adaptogenic' substances). However, more study is required to elucidate the metabolism of exogenous ecdysteroids in mammals and the biochemical modes of action of the parent ecdysteroids and their metabolites. Such studies are also important to underpin the use of ecdysteroid-induced gene switches. These systems possess considerable potential for basic biological studies of gene function and in gene therapy.

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