Practical uses for ecdysteroids in mammals including humans: an update

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Received 3 December 2002, Accepted 3 March 2003, Published 14 March 2003

Abstract

Ecdysteroids are widely used as inducers for gene-switch systems based on insect ecdysteroid receptors and genes of interest placed under the control of ecdysteroid-response elements. We review here these systems, which are currently mainly used in vitro with cultured cells in order to analyse the role of a wide array of genes, but which are expected to represent the basis for future gene therapy strategies. Such developments raise several questions, which are addressed in detail.

First, the metabolic fate of ecdysteroids in mammals, including humans, is only poorly known, and the rapid catabolism of ecdysteroids may impede their use as in vivo inducers.

A second set of questions arose in fact much earlier with the pioneering “heterophylic” studies of Burdette in the early sixties on the pharmacological effects of ecdysteroids on mammals. These and subsequent studies showed a wide range of effects, most of them being beneficial for the organism (e.g. hypoglycaemic, hypocholesterolaemic, anabolic). These effects are reviewed and critically analysed, and some hypotheses are proposed to explain the putative mechanisms involved.

All of these pharmacological effects have led to the development of a wide array of ecdysteroid-containing preparations, which are primarily used for their anabolic and/or “adaptogenic” properties on humans (or horses or dogs). In the same way, increasing numbers of patents have been deposited concerning various beneficial effects of ecdysteroids in many medical or cosmetic domains, which make ecdysteroids very attractive candidates for several practical uses.

It may be questioned whether all these pharmacological actions are compatible with the development of ecdysteroid-inducible gene switches for gene therapy, and also if ecdysteroids should be classified among doping substances.

Abbreviation:

20E 20-hydroxyecdysone
2d20E 2-deoxy-20-hydroxyecdysone
2dE 2-deoxyecdysone
BAH bisacylhydrazine
BmEcR Bombyx mori EcR
CIEcR Choristoneura fumiferana EcR
CIUSP Choristoneura fumiferana USP
CHO Chinese hamster ovary
CMV cytomegalovirus
DBD DNA-binding domain
DmEcR Drosophila melanogaster EcR

Abbreviations continued on next page
Introduction

Ecdysteroids (zooecdysteroids) are steroid hormones that control molting and reproduction of arthropods. Whether they fulfill hormonal functions in other invertebrate groups is still a matter of debate. In 1966, the discovery of the same molecules (phytoecdysteroids) in several plant species made them easily available in large amounts, and this allowed pharmacological studies to be initiated on mammals. Such studies were at first undertaken in the hope of developing safer and more specific insecticides, and it was quickly shown that these molecules were not toxic to mammals. On the other hand, they displayed a wide array of rather beneficial pharmacological effects (e.g. against diabetes or asthenia), thus providing a plausible explanation for the properties of several plant species widely used in traditional medicine. Although they have been detected in ca. 6% of plant species analysed so far (Dinan, 2001), phytoecdysteroids are not so frequent in plant species used as human food (with the noticeable exception of spinach; Bathory et al., 1982; Grebenok et al., 1991). More than 300 different ecdysteroids have been isolated from animal and plant sources (all their structures can be found in the Ecdybase, http://ecdybase.org).

Ecdysteroids are structurally quite different from mammalian steroids, and they are not expected to bind to vertebrate steroid receptors. Soon after the isolation and cloning of *Drosophila melanogaster* ecdysone receptor proteins, it appeared very attractive to use them for designing inducible gene systems in mammalian cells. Such a system has been commercially developed by Invitrogen® and the potential use of ecdysone receptors for gene therapy is being investigated. The different ecdysteroid-based gene-switch systems will be reviewed in the first part of this article.

The *in vivo* use of ecdysteroids as inducers taken orally raises questions about their uptake, metabolism and half-life in mammals including humans, a topic which has not been extensively investigated up to now (Sláma and Lafont, 1995), and this question will be addressed in the second part of this review.

The development of ecdysteroid-regulated gene switches seems, however, to have neglected much of the previous pharmacological studies which showed the interference of ecdysteroids with many physiological processes in mammals and humans. All these effects will be summarised in the third part, paying special attention to the protocols used and the significance/limitations of the results obtained. In the light of recent data, we will present in the fourth section some working hypotheses, which could explain how ecdysteroids might act on mammalian cells.

The reported effects (mainly the anabolic effects) led initially to a (doping ?) use for high-performance sportsmen in the Eastern Bloc Countries, but nowadays a large number of ecdysteroid-based preparations are freely available on the market. Most of them are proposed as legal and non-toxic muscle-promoting substances for bodybuilders, but an extensive search on the web has led to more surprising findings (e.g. recommended use for golfers or for domestic animals). So, whether ecdysteroids should be considered as doping substances and whether their use should be controlled will be finally discussed.

Ecdysone-inducible gene expression systems

Basic requirements

Spatial and temporal control of heterologous gene expression is an area of considerable and growing interest with relevance to basic and applied biological and medical research, including gene therapy and functional genomics. However, these heterologous regulatory systems should interfere minimally with the complex endogenous regulatory networks. Ideally, heterologous modification of gene expression in host cells should give rapid, robust, precise and reversible induction (or suppression) of the target gene(s). The necessary criteria are thus (Saez et al., 1997; Bohl and Heard, 1998; DeMayo and Tsai, 2001; Fussenegger, 2001; Graham,
2002):

1. **Specificity**: the system should not interfere with endogenous regulatory networks and should be activated exclusively by exogenous non-toxic compounds.

2. **Inducibility**: the system should possess a low baseline expression and a high induction ratio.

3. **Bioavailability of the inducer**: control should be effected by a drug that readily penetrates tissue.

4. **Reversibility**: the elicitor should possess high pharmacokinetic turnover to enable reversal and permit repeated cycles of induction.

5. **Low immunogenicity**: the components of the system should not elicit immune responses in the host.

6. **Flexibility**: it should be possible to modify the system to take account of different tissue applications and to optimise the system for each of these.

7. **Dose-dependence**: the extent of the response should be dependent on the dose of elicitor applied.

**Ecdysteroid receptors in arthropods**

Ecdysteroid receptors are members of the nuclear receptor superfamily (Laudet, 1997), which are characterised by a domain structure. The N-terminal A/B-domain is highly variable and is associated with transcriptional activation. The C-domain is highly conserved and is involved in binding the receptor complex to specific response elements in the DNA. The D-domain is variable and represents a hinge region between the DNA-binding domain and the ligand-binding domain (E-domain). The E-domain is not only responsible for ligand binding, but also has been implicated in receptor dimerisation and interactions with other transcriptional activators. There may also be a C-terminal F-domain, which, if present, is highly variable between even closely related nuclear receptors (Kumar and Thompson, 1999). Nuclear receptors regulate gene expression as dimers, either as homodimers or as heterodimers with another member of the nuclear receptor superfamily. One of the most promiscuous heterodimeric partners for vertebrate nuclear receptors is RXR, of which the equivalent in insects is Ultraspiracle (Palmer et al., 1993; Vögtli et al., 1999), Choristoneura fumiferana (Kothapalli et al., 1995; Perera et al., 1999), Heliothis virescens (Martinez et al., 1999c), Locusta migratoria (Saleh et al., 1998; Hayward et al., 1999), Lucilia cuprina (Hannan and Hill, 1997; 2001), Manduca sexta (Fujiwara et al., 1995; Jindra et al., 1997), Ostrinia nubilalis (Albertsen et al., 2000), Sarcophaga crassipalpis (Rinehart et al., 2001), Tenebrio molitor (Mouillet et al., 1997; Nicolai et al., 2000), Uca pugilator (Durica et al., 2002).

The biochemical characterisation of ecdysteroid receptor complexes lags well behind that of vertebrate steroid hormone receptors and has been in a period of quiescence for the past decade, as emphasis has been placed on the characterisation and expression of the genes. The generally accepted ligand for ecdysteroid receptors in arthropods is 20E, but this does not preclude the other ecdysteroids being significant at particular stages of development or in certain tissues (Wang et al., 2000). In fact, ecdysteroid receptor complexes recognise a wide range of ecdysteroid structural analogues and sophisticated structure-activity and molecular modelling studies are now beginning to be performed (Dinan et al., 1999a; Wurtz et al., 2000; Ravi et al., 2001; Kumar et al., 2002). Owing to the importance of ecdysteroid receptors in the regulation of arthropod development, they are seen as an appropriate target for the development of new pest control agents. In the context of this review, the identification of bisacylhydrazines as non-steroidal ecdysteroid agonists (Wing, 1988; Dhadialla et al., 1998) is particularly worth mentioning as, in addition to several of these molecules being commercialised as insecticides, other analogues appear appropriate as gene switching elicitors. Antagonists for ecdysteroid receptors are also being identified (Dinan et al., 1999b).

**Ecdysteroid-responsive expression systems**

**Mammalian systems**

Ecdysteroids are apparently not endogenously generated components of mammalian systems. However,
they are normal components of the diets of many animals. The low mammalian toxicity of these compounds (Sláma and Lafont, 1995), together with the specificity of the ecdysteroid receptor complex (EcR and USP proteins), indicate that a successful gene-switching system might be developed from this system (Fig. 2). With regard to plant systems (see below), there are a significant number (ca. 6% of higher terrestrial species) of plants which accumulate phytoecdysteroids (Dinan, 2001). This may restrict the use of steroidal and non-steroidal ecdysteroid analogues as elicitors in plant systems.

Initial reports appeared in the early 1990s (Christopherson et al., 1992; Thomas et al., 1993; Yao et al., 1992; 1993). Christopherson et al. (1992) transfected a human embryonic kidney cell line (HEK293) with DmEcR and a reporter gene and assessed the ability of various ecdysteroids and vertebrate steroids (all at 1 µM) to induce reporter activity; E, 20E and polB and the vertebrate steroids were inactive, while ponA and murA were active. The domain structure of nuclear receptors allows the domains to operate autonomously (however, this should not be taken to mean that the domains operate exactly the same under all circumstances). This permitted the ligand binding domain of EcR to be fused with the DNA-binding and A/B-regions of the GR (GGEc) and the demonstration of the induction of a GRE-containing reporter gene by murA and with the same ecdysteroid specificity as for EcR in the same mammalian cells. MurA could also induce a reporter gene via a chimeric receptor recognising a consensus oestrogen response element (ERE). They also demonstrated that replacement of a portion of the GR N-terminal activation domain in GGEc with the activation domain of Herpes simplex virus VP16 resulted in 5-fold greater activity (Christopherson et al., 1992).

Yao et al. (1992) showed that USP could substitute for RXR as a heterodimeric partner for RAR, TR, VDR and PPAR and showed that, for many mammalian cell types, cotransfection of USP with EcR was necessary to make the cells ecdysteroid-responsive, demonstrating that USP is an essential part of the ecdysteroid receptor complex. Thomas et al. (1993) found that certain mammalian cell lines (e.g. HeLa) could support ecdysteroid-responsive transactivation while others (e.g. CV-1) could not. They demonstrated that the factor responsible for this was RXR. RXR could not be replaced by RAR-α, TR-α or COUP-TF, but USP was an effective partner for EcR. MurA was effective at enhancing the DNA-binding activity (as assessed by gel-shift assays) of EcR:RXR, but not EcR:USP. Interestingly, the ligand of RXR 9-cis-retinoic acid, also enhanced the DNA-binding activity of EcR:RXR complexes.

The system has been further developed (No et al., 1996). The final form of this development (VgEcR) was more specific and gave a lower basal activity than tetracycline-responsive systems. The starting point for the developments of No et al. (1996) was the observation that mammalian cells cotransfected with EcR and USP only produce a 3-fold induction on treatment with murA (1 µM). To improve the induction ratio they carried out a number of modifications. Replacement of USP by RXR gave 34-fold induction. Creation of a fusion protein consisting of an N-terminal truncation of EcR attached to the VP16 activation domain (generating VpEcR) gave 212-fold induction. Inclusion of binding sites for the transcription factor Sp1 into the reporter vector between minimal promoter and the EcREs enhanced the induction by a further 5-fold.

Since the ecdysteroid response element might be weakly activated by endogenous farnesoid X receptors FXR, the EcRE (to give 2 different half-sites with a 1-nucleotide spacer, AGGTCA-AGAACA, generating E/GRE) and DBD of EcR (by mutating 3 amino acids in the P-box of the DNA-binding domain, generating VgEcR) have been modified to ensure that the response element will only bind the modified EcR. The transcription-regulatory potential of EcR has been enhanced by replacing the endogenous activation domain by the Herpes simplex virus VP16 activation domain (DeMayo and Tsai, 2001). The final system gives a 1200-fold induction with 1 µM murA, without interference from glucocorticoid or farnesoid.

No et al. (1996) also generated transgenic mice harbouring an ecdysteroid-inducible promoter or a T-cell-specific expression construct of VpEcR and RXR. Crossing of these two strains of mice gave double transgenic offspring, which were induced to generate the reporter gene transcript specifically in the thymus by injection of murA (10 mg/mouse). Mice expressing VpEcR and RXR were healthy, fertile and apparently normal.

Yang et al. (1995) produced a Chinese hamster ovary (CHO) cell line stably transfected with EcR isoform B1 and showed that the cells produce functional receptor of the correct Mr (105 kDa), which is recognised by specific antibodies, binds to EcRE in gel-shift assays and mediates reporter gene expression in a ligand-dependent manner (ponA; 4 - 100 µM). The authors suggest that CHO cells produce high levels of RXR, which can heterodimerise with
The commercially available Invitrogen system (http://www.invitrogen.com: Fig. 3) has been used to regulate the expression of a wide range of transfected genes in mammalian cells (Sawicki et al., 1998; Chen et al., 2000; Lüers et al., 2000; Niikura et al., 2000; Rampazzo et al., 2000; Abeyssinghe et al., 2001; Baba et al., 2001; Cole et al., 2001; Gill et al., 2001; Hennigan and Stambrook, 2001; Iwata et al., 2001; Jana et al., 2001; Kondo et al., 2001; Patrick et al., 2001; Schmidt and Fan, 2001; Shi et al., 2001; Sparacio et al., 2001; Stauffer et al., 2001; Stolarov et al., 2001; Wang et al., 2001; Xu et al., 2001; Yam et al., 2001; Yarovoi and Pederson, 2001; Zhu et al., 2001; Chen et al., 2002; Coulthard et al., 2002; Davis et al., 2002; Hashimoto et al., 2002; Kuate et al., 2002; Kudo et al., 2002; Meents et al., 2002; Mellon et al., 2002; Odero-Marah et al., 2002; Plows et al., 2002; Vickers and Sharrocks, 2002; Wang et al., 2002; Wolter et al., 2002; Xiao et al., 2003; Xu and Mellgren, 2002; Zhang et al., 2002) and in a tissue-specific manner in mice (No et al., 1996; Albanese et al., 2000). The literature on the application of ecdysteroid-regulated transgenic systems is currently growing exponentially.

Wyborski et al. (2001) developed a bicistronic expression vector from which VgEcR and RXR can be co-expressed. They used the general cytomegalovirus (CMV) promoter, but this can be replaced with a cell-type specific promoter.

Albanese et al. (2000) developed a system for mammory gland-specific expression of an ecdysteroid-regulatable gene in mice and have examined the pharmacokinetics of injected ponA in the animals. Serum clearance was rapid (activity half-life = 48 min).

Karns et al. (2001) have developed an alternative to the Invitrogen system. The basic system (Figure 4) consists of the (i) plasmid pGAL4-EcR, encoding a fusion protein of the yeast GAL4 DNA-binding domain and the ligand-binding domain of the ecdysteroid receptor from Choristoneura fumiferana, (ii) plasmid pVP16-mRXR, encoding a fusion protein of the Herpes simplex transcriptional transactivator VP16 and mouse RXR protein, and which, in the presence of ecdysteroid-type ligands heterodimerises with GAL4-EcR, (iii) an indicator and selection plasmid, either pGAL4-EGFP-SV40-neo (consisting of 5 copies of the GAL4 response element, followed by the minimal promoter region of the major late promoter from adenovirus, the coding region of enhanced green fluorescent protein [EGFP], the SV40 promoter and the neomycin resistance locus) or pGAL4-SEAP (for stable transformation, containing 5 x the GAL4 response element and the coding region of the SEAP protein as reporter gene) iv) the BAH GS-E (1 - 15 µM). This system forms the basis of RHeoGene’s RHeoswitch Technology. As RHeoGene

- Figure 3. The Invitrogen system for mammalian cells; the elicitor is muristerone A (murA) or ponasterone A (ponA). See text for further details (www.invitrogen.com).

- Figure 4. The RHeoGene system for mammalian cells; the elicitor depicted is the bisacylhydrazine GS-E (1-[3-methoxy-2-ethylbenzoyl]-2-[3,5-dimethylbenzoyl]-2-tert-butylhydrazine). See text for further details (Karns et al., 2001).
have access to a large number of BAH analogues (RHeoChem Ligands) and EcR genes from a wide variety of insect species (RHeocept Receptors), they are able to identify BAH analogues which are specific for particular EcR LBDs and, thus, have the possibility to regulate multiple genes in a coordinated manner, and this is being developed under the title of RHeoPlex Systems (www.rheogene.com). Karns et al. (2001) also considered the suitability of GS-E as an in vivo inducer in mice and obtained maximal induction of reporter protein in 6-12 hrs and return to basal expression levels by 12-24 hrs.

**Plant systems**

Most of the published research in this area has been conducted by the industrial research labs at Zeneca Agrochemicals (now Syngenta) and has been based on the ecdysteroid receptor protein from *Heliotis virescens* (HvEcR), which was cloned and characterised (Martinez et al., 1999c). This protein has most similarity to EcRs from other lepidopteran species and is closely related to the B1 isoform from *D. melanogaster* (DmEcRB1). Transfection of mammalian HEK293 cells (RXR-containing) with HvEcR and a reporter gene resulted in induction of the reporter gene by murA (50% response at ca. 5 μM), but not by 20E (Martinez et al., 1999a). For the development of the plant system (Fig. 5), a chimeric receptor consisting of the hinge and ligand-binding domains of HvEcR was fused to the transactivation domain of the glucocorticoid receptor and transfected into tobacco protoplasts. The use of the GR DNA binding domain circumvents the need to incorporate USP/RXR into the system, since glucocorticoid receptors bind to their response elements as homodimers. The second component of the gene regulatory system consisted of 6 copies of the glucocorticoid response element fused to the minimal 35S cauliflower mosaic virus (35SCaMV) promoter (conferring expression in all tissues and throughout development) and a β-glucuronidase gene. Although induction was observed with murA (100 μM), its steroidal nature precludes its use under field conditions. Consequently, the non-steroidal BAH RH5992 (1 - 10 μM) was used as an elicitor. In addition to being ecdysteroid agonists, these compounds are not phytotoxic. Incorporating the regulatory and reporter components via *Agrobacterium tumefaciens* transformation generated transgenic lines of tobacco plants. Germination of the transformed seeds in the presence of murA or RH5992 resulted in induction of the reporter gene activity (up to 420-fold). RH5992 is 100-fold more potent than murA in this system, giving maximal activation at 12.5 μM and 50% activation at ca. 1 μM (Martinez et al., 1999a). Parallel studies using maize protoplasts compared chimeric receptors involving ligand and hinge regions of either the *D. melanogaster* or *H. virescens* EcR fused to the A/B/C-domains of the glucocorticoid receptor, showed that RH5992 activates in the presence of GRH, but not GRD (Martinez et al., 1999b). On the other hand, murA (100 μM) activates in the presence of GRD, but not GRH. The preferential activation of GRH by RH5992 is in accord with the higher affinity of this BAH for lepidopteran EcR/USP complexes than for dipteran complexes (Dhadialla et al., 1998), but the lack of activation of GRH by murA is not readily explained and seems to indicate that the conformation of the LBD of the chimeric receptor is significantly altered.

Unger et al. (2002) have developed a BAH-regulated system for the control of male fertility in maize. Ms45 is a nuclear male fertility gene, which is expressed in anthers. Homozygous recessive mutants are male sterile. The aim was to create a Ms45 construct which would allow male fertility to be restored after application of an elicitor. The hinge and ligand-binding domains (domains D-F) of the *Ostrinia nubilalis* EcR gene were linked to the VP16-GAL4 or C1-GAL4 transcription activators, under the regulation of the Ubiquitin 1 promoter, which gave constitutive expression of the receptor construct. The Ms45 promoter region was replaced by 5 copies of the yeast 17 bp US₃. Regulatory proteins containing the GAL4 DNA-binding domain bind to UAS₃. The authors demonstrate that treatment of transformed maize callus with methoxyfenozide (10 μM) induces Ms45 expression. Further, when incorporated into plants, the plants were male sterile in the absence of methoxyfenozide, but fertility was restored by treating plants with methoxyfenozide.

Padidam et al. (2002) have recently developed an ecdysteroid receptor-based gene expression system based on a modified *Choristoneura fumiferana* EcR and the BAH methoxyfenozide and demonstrated its effectiveness in transgenic *Arabidopsis thaliana* and *Nicotiana tabacum*.

In addition to ecdysteroid/BAH controllable systems, other chemically inducible gene expression systems for plants are also being investigated (reviewed in Gatz and Lenk, 1998; Jepson et al., 1998). These systems have been recently compared and reviewed (Padidam, 2003).

**Figure 5.** The Syngenta system for plant cells. See text for further details (Martinez et al., 1999a&b).
**Fungi**

When transfected into *Saccharomyces cerevisiae*, DmEcR is able to transactivate a reporter gene in the absence of USP/RXR or ecdysteroid (ponA or murA) (Delacruz and Mak, 1997). Activation is EcR-dependent, but, unexpectedly, ecdysteroid- and heterodimerisation partner-independent. Interestingly, high affinity specific binding of $[^{3}H]$ponA ($K_d = 1.8 \text{ nM}$) by yeast extracts was dependent on coexpression of EcR and USP (or RXR). Radiolabelled hormone displacement assays for yeast-expressed EcR/USP with ponA, murA, 20E and RH5849 (Delacruz and Mak, 1997) indicate similar specificity and affinity to *D. melanogaster* ecdysteroid receptor complexes in insect systems (Bidmon and Sliter, 1990). Thus, the situation prevailing when ecdysteroid receptors are expressed in yeast cells is apparently very different to that for mammalian or plant cells.

Using the ecdysteroid receptor genes from *Choristoneura fumiferana* (CfEcR and CfUSP) coexpressed in yeast with a reporter gene containing EcREs, Tran et al. (2001) showed that EcR and USP together (but not individually) induced reporter gene expression in the absence of ligand, with RH5992 (10 $\mu$M) only providing a small enhancement in reporter gene expression. Deletion of the A/B-regions of CfEcR, in conjunction with CfUSP, still gave ligand-independent transactivation with some enhancement on addition of RH5992. However, deletion of the A/B-regions of CfUSP (generating CfΔUSP) abolished reporter gene expression, regardless of whether coexpression was with CfEcR or CfΔEcR and in the presence or absence of RH5992. Together, these data showed that EcR:USP is not suitable for a ligand-dependent transactivation assay in yeast. Replacement of USP with RXR$\alpha$, RXR$\beta$ or RXR$\gamma$, when co-expressed with EcR, resulted in no induction of the reporter gene in the presence or absence of RH5992. However, co-expression of GRIP1 (a member of the p160 family of coactivators) and CfΔEcR:RXR or CfΔEcR:CFΔUSP resulted in significant ligand-dependent transactivation of the reporter gene activity. The system with RXR$\beta$ appeared to have a low sensitivity to RH5992 and other BAHs and was not pursued further. Comparison of three yeast systems ($\Delta$EcR:USP:GRIP1, $\Delta$EcR:RXR$\alpha$:GRIP1 and $\Delta$EcR:RXR$\gamma$:GRIP1) with an insect cell CfEcR:USP-containing system (L57; DmEcR-negative Kc cells transfected with CfEcR and $\beta$-galactosidase reporter controlled by 6 x EcRE and a minimal promoter) using a range of BAHs and murA and ponA showed induction in all systems by active compounds, but i) the degree of induction was far lower in the yeast systems and ii) the ecdysteroids were very much poorer inducers in the yeast $\Delta$EcR:USP system than in the insect cells and did not induce the $\Delta$EcR:RXR systems. Further, 9-cis-retinoic acid (a natural ligand of RXR receptors) induced the $\Delta$EcR:RXR$\alpha$:GRIP1 and $\Delta$EcR:RXR$\gamma$:GRIP1 systems, complicating interpretation of results from these systems if they were used in screening processes. In part these problems may derive from poor access of test compounds through the thick yeast cell wall or rapid export from the cells. Tran et al. (2001) provide evidence that use of yeast strains with mutations in certain ABC transporter pathway loci results in improved sensitivity (100-fold for RH5992). Tran et al. (2001) propose their transactivation assay as a screen to identify potential insecticides with ecdysteroid agonist activity.

**Commercially available systems**

The system devised by No et al. (1996) has been developed and commercialised by Invitrogen (http://order.invitrogen.com/). The company provides kits consisting of mammalian cells (CV-1, HEK293 and CHO) stably expressing a functional ecdysteroid receptor from pVgRXR, the inducing agent (now ponA) and a vector by which the gene of choice can be introduced into the cells, after introduction of the gene into the vector by simple recombination using Cre recombinase. The components are also available individually. The pVgRXR expression vector includes VgEcR, RXR and a gene for Zeocin resistance, which allows for selection of stable cells expressing the heterodimeric receptor (VgEcR:RXR).

Stable ecdysteroid-inducible mammalian cell lines can be difficult to establish because of either high basal expression of the target gene or poor induction of gene expression, because of low expression of the receptors (VgEcR and RXR) or the transgene. Ideally, stable cell lines expressing the receptors should be established first and the cells should be screened by transient expression of an ecdysteroid-regulatable transgene to identify those expressing the receptor proteins effectively. An improvement on Invitrogen’s pNDSlacZ reporter system (which generates $\beta$-galactosidase activity has been reported (Wakita et al., 2001), which uses a firefly luciferase reporter system. This considerably reduces analysis time (15 s, rather than 2 h) and obviates background interference from endogenous $\beta$-galactosidase activity. A similar advance has been suggested by Lüers et al. (2000) who prepared an expression plasmid for green fluorescent protein (EGFP) and the reporter protein of interest. The co-inducible production of EGFP permits the visual verification of target gene expression and the selection of expressing cells by flow-cytometry.

As described above (Section 2.3.1), RHeoGene LLC are commercialising their ecdysteroid receptor-based gene switching system (www.rheogene.com) and are identifying specific receptors/ligand pairs which allow the simultaneous, but independent, regulation of transfected genes (Kumar et al., 2002). Further, hybrid receptors are being optimised to give very low basal activity and high induction on addition of ligand (Palli et al., 2003). A two-hybrid format switch where the GAL4 DNA-binding domain was fused to CfEcR domains D, E and F and the VP16 activation domain was fused to mouse RXR domains E and F, transactivating a reporter gene under the control of GAL4 response elements and a synthetic TATAA
promoter was found to give the best combination with rapid turn-on and turn-off responses on the addition and removal of RG-102240 (GS-E), respectively.

**Ecdysteroid systems vs. other systems**

Fussenegger (2001) provides a comprehensive description of the heterologous molecular switching systems currently under consideration. Each has its own advantages, but none fulfills all the desirable criteria perfectly. From the time of early studies, transgenic ecdysteroid-inducible gene expression systems in mammalian cells have appeared to possess lower basal activity and higher inducibility than tetracycline-based systems (No et al., 1996). Senner et al. (2001) compared 3 inducer systems (tetracycline, dimerizer and ecdysteroid) in one system (rat C6 glioma cells) under identical conditions. Each system required transient transfection with two plasmids (a regulator plasmid and a reporter plasmid) and treatment with an elicitor (inducers for the ecdysteroid and dimerizer systems and a repressor for the tetracycline system). The ecdysteroid system provided the highest induced activity, but the authors conclude that each of the systems may be beneficial, depending on what the experimental goals are. Van Craenenbroeck et al. (2001) compared the tetracycline and ecdysteroid systems to regulate the expression of neurotransmitter receptors in mouse fibrosarcoma L929sA and HEK293 cells. The tetracycline system resulted in higher levels of the neurotransmitter receptors being expressed, but the ecdysteroid system gave more tightly regulated expression. Moreover, this study underlined the importance of the genetic background of the cells being used.

Morgan et al. (1999) compare several exogenously regulatable promoter systems for their suitability for the study of the functions of genes implicated in aging.

**Ecdysteroid specificity**

Gene expression systems in mammalian and plants cells possess markedly different ecdysteroid specificities to ecdysteroid receptors in insect systems. Both the affinity and specificity seem to be affected. Thus, the generally accepted endogenous hormone in insects, 20E, is inactive in transgenic systems. Two phytoecdysteroids, murA and ponA, are normally used to activate the transgenic systems, but even these are required at least 100-fold lower concentrations than in insect systems; e.g. EC50 values for the transgenic systems, but even these are required at least 100-fold lower concentrations than in insect systems; e.g. EC50 = ca. 5 x 10^{-7}M, with ponasterone C being moderately active (EC50 = 2 x 10^{-8}M), poI B being weakly active and 20E, 14-deoxymuristerone A, E, 2-deoxyecdysone, 20E 22-acetate and 2-deoxy-20-hydroxyecdysone being inactive or only very weakly active at 10^{-4}M. This study also showed that the presence of a natural (9-cis-retinoic acid) or synthetic (LG268 or LG1069) RXR ligands, while inactive in itself, potentiated the activity of ponA by 3- to 5-fold.

**Availability of ligands**

**Ecdysteroids**

MurA has only been isolated once in large amounts (Canonica et al., 1972), and then from a Himalayan plant (*Ipomoea calonyction*). Consequently, world supplies of this phytoecdysteroid became very restricted and did not suffice for full-scale trials of ecdysteroid-induced transgenic systems. However, Sequoia Sciences (http://www.sequoiasciences.com) report on their website that they have recently re-isolated murA. PonA, which has been isolated from several named plant species and which can be chemically generated from 20E (Heinrich, 1970), is also active. Examination of the ecdysteroid specificity in more detail could result in the identification of more active inducers. While *in vitro* work may not suffer unduly from the poor activity of currently used ecdysteroid inducers, other than having to use much larger amounts of expensive chemicals, the *in vivo* prospects for transgenic systems using ecdysteroids would be enormously enhanced if they were as active as in insect systems.

**Bisacylhydrazines (BAHs)**

Bisacylhydrazines were identified as non-steroidal agonists of ecdysteroid receptors in 1988 (Wing, 1988). Their chemical simplicity, low mammalian toxicity and selectivity for certain Orders of insects has led to several being developed as insecticides (Dhadialla et al., 1998). They could also be used for the induction of transgenic systems (Carlson et al., 2001) and, as is apparent above, RH-5992 has found application in this context. Further analogues (e.g. GS-E; Fig. 1) have been identified which appear to be more potent for use with mammalian systems (Carlson, 2000). However, the very limited water solubility of these compounds may limit their application *in vivo*. Modified receptors

A further approach to overcoming the current lack of really potent ligands for transgenic induction would be to modify the ligand-binding domain of the transgenic EcR to either enhance the affinity for a particular analogue, or to alter the specificity, so that a readily available analogue (e.g. 20E) or a non-diaryl ecdysteroid is recognised. Cloning and sequence data for ecdysteroid receptor proteins (EcR and USP) from a range of arthropod species provide the basis for site-directed mutagenesis to modify specific amino-acid residues. Both this and the previous approach require a more thorough understanding of ecdysteroid receptor recognition, not only in *D. melanogaster* (Dinan et al., 1999a; Ravi et al., 2001), but also in other arthropod species and in transgenic systems. The ultimate goal of such studies is to engineer a range of EcR proteins, some of which respond to non-steroidal inducers, but not to ecdysteroids,
while others respond to selected ecdysteroids, but not to other classes of agonists (Graham, 2002). Strategies are being developed for the synthesis of further non-steroidal ligands for selective activation of ecdysone receptor (Tice et al., 2003) and for the targeted modification of ligand specificity of ecdysteroid receptors (Kumar et al., 2002).

Registration problems
Development of ecdysteroid systems for human therapeutic use may be hampered by the steroidal nature of ecdysteroids and the insecticidal origin of BAHs, which may prejudice their use as elicitors, this being in spite of the fact that both ecdysteroids and BAHs have low mammalian toxicities and ecdysteroids are a normal elicitors, this being in spite of the fact that both ecdysteroids and BAHs have low mammalian toxicities and ecdysteroids are a normal

Biochemical problems
Although the systems developed to date are effective for use in in vitro expression systems, the requirements for an effective in vivo system are much more stringent. In this context, one can identify the following aspects of ecdysteroid-regulatable systems which would need to be improved in order to generate a medically viable system:

- Integration of heterologous DNA into host cells is not site-specific and is unpredictable with regard to copy number.
- The current systems are genetically complex, requiring both VgEcR and RXR.
- The artificial transactivator is potentially immunogenic.
- RXR is a reluctant dimerization partner for EcR and, therefore, very high cellular RXR concentrations are required. Overexpression of RXR may result in pleiotropic effects in mammals.
- The maximal expression levels achieved are modest.
- Most ecdysteroids are not very active. Only muristerone A and ponasterone A are effective.
- Ecdysteroids are not orally available
- Ecdysteroids or ecdysteroid analogues are not likely to get approval for human therapeutic use.

Prospects
There is little doubt that ecdysteroid-regulated transgenic systems have considerable potential for in vitro work. The applied potential is somewhat more questionable at present, owing to the following current limitations: i) genetic complexity, ii) altered affinity and selectivity of VgEcR for ligands and iii) potential problems in the registration of ecdysteroids and BAHs for human therapeutic use or with plant transgenic systems. However, significant progress is being made in designing chimaeric receptors which would allow only one trans-acting factor to be transfected. It is only a matter of time until the reasons for the altered affinity and selectivity of ecdysone receptors in mammalian and plant cells are elucidated and more efficient systems are developed either by identifying more effective ligands or site-directed mutagenesis of EcR to enhance affinity for currently used ligands. Although ecdysteroids and BAHs are nontoxic to humans, general public resistance to steroids and insecticides may hamper their registration.

Ecdysteroid metabolism in mammals, including humans
Although the question of mammalian metabolism is certainly of importance for the practicability of the in vivo use of ecdysteroid-inducible gene expression systems (with the aim of using them for gene therapy), it is not well documented at the present time. Ecdysteroids have a very low toxicity in mammals: in the mouse, the LD50 of 20E is 6.4 g/kg (for intra-peritoneal injection) and it is 29 g/kg when given orally (Matsuda et al., 1970; Ogawa et al., 1974). Up to now, studies have concerned mice, rats, lambs and humans, and all have shown that these molecules are short-lived in mammals. Several strategies have been used to analyse the metabolic fate of ecdysteroids.

Ecdysteroids are rapidly eliminated
In the case of humans, two different studies have been performed. Simon and Koolman (1989) analysed the pharmacokinetics of E and 20E (given orally, 0.2 mg/kg b.w.) to a male volunteer, by monitoring with a radioimmunoassay the subsequent plasma and urine titres. This gave an effective half-time (EHT) of elimination of 4 hours for E and 9 hours for 20E. In lambs, EHT for 20E was shown to depend strongly on the mode of administration, with values of 0.4, 0.2 and 2 hours after oral, intravenous and intramuscular administration, respectively (Simon and Koolman, 1989). The method used did not allow the detection of metabolites, if present. The half-life seems shorter in smaller mammals, with reported values of 8.15 min for 20E in mice (Dzukharova et al., 1987). More recently, Albanese et al. (2000) found a plasma half-life of 48 min for ponA in mice after intraperitoneal injection of 750 µg of this compound.

Both urinary and faecal routes seem to be used for the elimination of the administered molecules. In mice, the faecal route was found to be the major one by Hikino et al. (1972a,b) and Lafont et al. (1988), although Dzukharova et al. (1987) found that faecal and urinary routes were equally important. Such a question can be easily assessed only by the use of radiolabelled molecules, but no data are available for humans. Kinetic studies in mice showed that ecdysteroids were taken up by the liver and then excreted into the gut via the bile (Hikino et al., 1972a,b; Lafont et al., 1988).

Metabolic conversions
Another question concerns whether ecdysteroids undergo metabolic conversions in mammals. The presently available data are not fully consistent. In mice, Girault et al. (1988) analysed the faecal metabolites of injected E and isolated unchanged E, a major metabolite identified by MS and proton NMR as 14-deoxyecdysone together with molecules with a fully reduced B-ring and, additionally, epimerized in position 3 (Figure 6A). Such a metabolism is reminiscent of the hepatic reduction of the 4-en-3-one on ring-A of vertebrate steroid hormones, whereas dehydroxylation resembles that of bile acids and could result from the actions of anaerobic intestinal bacteria.
More recent studies were performed on ingested 20E in rats (Ramazanov et al., 1996) and humans (Tsitsimpikou et al., 2001). In these cases only urine was analyzed. Ramazanov et al. administered 20E to 40 rats (50 mg/kg) directly in stomach with a special probe, and they collected urine (3.5 L) over the following 10 days. After several chromatographic steps, they isolated unchanged 20E and three new metabolites, which were analyzed by IR and mass spectrometry. The IR spectra showed the disappearance of the signal at 650 cm⁻¹ (7-en-6-one) and the structures were deduced from MS data (Figure 6B).

Tsitsimpikou et al. (2001) analysed the urine of a volunteer having ingested 20 mg of “Ecdysten™” (a commercial preparation containing 20E – see section 6); they collected urine over 5 days and analysed ecdysteroids by GC-MS after derivatization. They found, together with 20E, two less hydroxylated metabolites, which they tentatively identified as 2d20E and 2dE by comparison with available reference molecules.

Mass spectrometry does not provide sufficient information, and only NMR can allow an unambiguous determination of structures. Anyway, it seems reasonable to assume that modification of the B-ring and dehydroxylation are general features of ecdysteroid metabolism in mammals.

**Figure 6.** Major E and 20E metabolites in Mammals (see text for details). A: E metabolites (2-4) isolated from murine faeces (Girault et al., 1988); B: 20E metabolites (6-8) isolated from rat urine (Ramazanov et al., 1996).

**Conclusions/prospects**

There is rapid catabolism/elimination of ecdysteroids, which means that large amounts would have to be used in order to maintain circulating levels above the concentration required for gene switches systems to be activated. Alternatively, slow-delivery systems like subcutaneous implants represent another way to maintain sustained ecdysteroid levels for several days (Albanese et al., 2000). Another remaining question concerns the metabolism in peripheral tissues. As we have seen with mice, the observed conversions are most probably performed by hepatocytes and intestinal bacteria. It would be of interest to determine whether other mammalian tissues are able to metabolise ecdysteroids, and the nature of the reactions they can perform.

Whether side-chain cleavage between C-20 and C-22 (and possibly also between C-17 and C-20) can take place is a very important question which remains to be investigated by using ecdysteroids labelled on the steroid nucleus, as labelling on the side-chain would be lost if such a reaction would occur. This question seems particularly important for several reasons: (1) cleavage between C-20 and C-22 would result in the formation of 21C steroids that would share some resemblance with vertebrate neurosteroids (Lafont and Sláma, 1995), and (2) in some pharmacological studies rubrosterone (2β,3β,14α-trihydroxy-β-androst-7-ene-6,17-dione) was as active as 20E (Otaka et al., 1968).

**Pharmacological effects of ecdysteroids on vertebrates**

The pharmacological actions of ecdysteroids on vertebrates have been reviewed in several previous articles (Burdette, 1962, 1972; Ogawa et al., 1974; Syrov, 1984, 1994; Sláma and Lafont, 1995; Xu et al., 1997; Syrov, 2000; Kholodova, 2001; Báthori, 2002). We will therefore focus on some aspects only, especially on those where recent developments have occurred. The most important data are summarised in Table 1.

**Ecdysteroids and growth (Table 2)**

The anabolic effects of several phytoecdysteroids (20E, cyasterone, turkesterone, viticosterone E – see structures on Ecdybase) on mice or rats were reported long ago (see e.g. Okui et al., 1968; Syrov and Kurnukov, 1975a&b; 1976a-c, Syrov et al., 1978, 1981a; Stopka et al., 1999). Growth-promoting effects have also been more recently reported for pigs (Kratky et al., 1997) and Japanese quails (Koudela et al., 1995; Sláma et al., 1996). In many instances however, these effects are not spectacular when considering the growth (weight) curves as they are observed during certain phases of growth or for one sex only and, in many cases, adequate statistical analyses are lacking. Nevertheless, even small effects (i.e. <5 % increase) on growth could be of economical interest for nutritionists, but their firm establishment requires the use of large numbers of animals, which is hardly feasible with large mammals. The addition of E to sheep food increases body growth rate and also wool growth (Purser and Baker, 1994). Surprisingly, these effects were obtained with minute amounts of ecdysone (0.02 µg/kg per day!), and were more evident when animals were fed on a poor quality diet, which indicates that E improves food utilization. In this case, it has been suggested that the effect results from the toxicity of E towards rumen protozoa, but this has not been fully
Table 1. Pharmacological effects of ecdysteroids on mammals or humans (see also Sláma and Lafont, 1995 for additional references – in red : references to patents)

| Biological area       | Ecdysteroid Effects                                                                 | References                                                                                     |
|-----------------------|-------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|
| Growth                | Slight stimulation of growth in mice (by dietary 20E or cyasterone)                 | Hikino et al., 1969                                                                            |
|                       | No effect of dietary ponA , 20E and inokosterone on rat growth                      | Matsuda et al., 1970                                                                           |
|                       | Stimulation of growth in rats (by dietary viticosterone E, 20E, turkesterone and turkesterone tetraacetate) | Syrov and Kurmukov, 1975a, 1976a, b                                                           |
|                       | Stimulation of growth in shee p by E                                               | Purser and Baker, 1994                                                                         |
|                       | Simulation of growth in quails by dietary 20E                                      | Koudela et al., 1995, Sláma et al., 1996                                                       |
|                       | Stimulation of growth in pigs (by y 20E in sections)                                | Krátký et al., 1997                                                                           |
|                       | Stimulation of growth in mice (by y 20E)                                            | Stopka et al., 1999                                                                           |
| Cell proliferation and differentiation | Dietary 20E (5mg/kg) a ccelerates bone fracture healing process in rats | Syrov et al., 1986a                                                                           |
|                       | 26E possesses wound healing and skin regenerating properties                      | Lin and Lin, 1989; Meybeck and Bonté, 1990, 1993; Meybeck et al., 1994                         |
|                       | 20E (100 µg/ml) promotes keratinocyte differentiation in vitro                    | Detmar et al., 1994                                                                            |
|                       | E induces breast and lung neoplastic lesions in mice                               | El-Mofty et al., 1994                                                                          |
|                       | 20E as skin metabolism-activating and anti-wrinkling agent                         | Tsuji et al., 1995a                                                                            |
|                       | PonA in a hair tonic preparation                                                    | Tsuji et al., 1995b                                                                            |
|                       | Dietary ecdysteroids (20E, cyasterone, turkesterone, 5 mg/kg) accelerate wound healing in rats | Syrov and Khushbaktova, 1996                                                                  |
|                       | 20E and some analogues inhibit psoriasis                                          | Inaoka et al., 1997                                                                            |
|                       | 20E (200 µg/ml) stimulates proliferation of human umbilical vein endothelial cells | Lin et al., 1997                                                                               |
|                       | Dietary ecdysteroids (20E, turkesterone,...) enhance erythro-poiesis in rats        | Syrov et al., 1997b                                                                            |
|                       | Phytoecdysteroids use for the treatment of burns and wounds                        | Darmograi et al., 1998                                                                         |
|                       | 20E promotes proliferation of osteoblast-like cells                                | Gao et al., 2000                                                                               |
| Reproduction, development | 20E and polydopine B show embryotoxicity                                           | Kosar et al., 1997                                                                             |
|                       | Ecdysteroids might increase milk production in mammals                             | Khalitova and Syrov, 1998                                                                      |
|                       | Dietary 20E (5-10 mg/kg) enhances sexual function in rats                          | Mirzaev et al., 1992, 2000                                                                     |
| Protein metabolism    | Injected ecdysteroids (20E , 5 mg/kg) stimulate protein synthesis in mouse liver; this effect corresponds to a stimulation of translation | Okui et al., 1968; Otaka et al., 1968, 1969 a,b;                                                |
|                       | 20E stimulates protein synthesis in mouse or gans                                   | Todorov et al., 2000                                                                           |
| Carbohydrate metabolism | Hypoglycaemic formulation containing 20E                                           | Uchiyama and O gawa, 1970                                                                      |
|                       | Injection of 20E (0.1–10 mg/kg) in mice reduces hyperglycaemia provoked by alloxan or glucagon , and increases glucose incorporation into glycogen and proteins | Yoshida et al., 1971                                                                           |
|                       | Dietary 20E (5 mg/kg) for 25 days increases glycogen content in heart , liver and muscles of rats | Syrov et al., 1975a, Aizikov et al., 1978                                                      |
|                       | Antidiabetic agents containing 20E or inokosterone                                 | Takahashi and Nishimoto, 1992                                                                   |
|                       | Dietary administration of 20E (5 mg/kg) or other ecdysteroids reduces alloxan-induced hyperglycaemia in rats | Syrov et al., 1997a                                                                            |
|                       | 20E in oral antidiabetic preparations                                               | Yang et al., 2001                                                                              |
| Lipid metabolism      | Injections of E (10-50 µg/kg) reduce de novo cholesterol biosynthesis in rats      | Lupien et al., 1969                                                                             |
|                       | Dietary 20E (0.1 µg/kg/day) for 30 days prevents (= has antiradical properties) free-radical lipid peroxidation of membranes in tissues from vitamin D-deficient rats | Kuzmenko et al., 1997                                                                          |
|                       | 20E in vitro prevents lipid peroxidation in liposomes micelles                      | Osynskaya et al., 1992, Kuzmenko et al., 2001                                                   |
| Nervous system        | Analgesics containing 20E                                                           | Takemoto et al., 1988                                                                          |
|                       | 20E exerts a neuromodulatory action on GABA A receptor of rat cortical neurons      | Tsujiyama et al., 1995, Sasa et al., 1996, Okada et al., 1998                                  |
|                       | Cerebral neuron protective effects of 20E                                          | Akaike et al., 1996                                                                            |
|                       | 20E has antiepileptic effects on rats                                              | Hanaya et al., 1997                                                                            |
|                       | 20E has protective effects on amnesia induced by diazepam and alcohol              | Xu et al., 1999                                                                                 |

Table 1. Continued on next page
established. In fact, through a stimulation of protein synthesis (and/or a reduction of protein catabolism), ecdysteroids would increase the lean body mass. In pigs, doses of 0.2-0.4 mg/kg/day resulted in better nitrogen retention and a body weight increase of 112-116% in the lean body mass. In pigs, doses of 0.2-0.4 mg/kg/day resulted in better nitrogen retention and a body weight increase of 112-116% in the lean body mass.

**Ecdysteroids and physical performance**

20E is claimed to have tonic properties (Abubakirov et al., 1988). Indeed it stimulates muscle growth, provided that protein supply is adequate. Such anabolic effects result in increased physical performance without training (Chermnykh et al., 1988). This was for instance demonstrated using the forced swimming test with rats: animals given ecdysteroids for one week were able to swim for significantly longer times (Azizov and Seifulla, 1998). These effects are similar to those of anabolic steroids. 20E is also able to increase animals given ecdysteroids for one week were able to swim for significantly longer times (Azizov and Seifulla, 1998). These effects are similar to those of anabolic steroids. 20E is also able to increase animals given ecdysteroids for one week were able to swim for significantly longer times (Azizov and Seifulla, 1998). These effects are similar to those of anabolic steroids.

**Table 1. Continued from previous page**

| Heart and circulatory system | Defensive systems | Liver function and detoxification mechanisms | Antibiotic activity |
|-----------------------------|-------------------|---------------------------------------------|---------------------|
| 20E can eliminate arrhythmia induced by occlusion of the left coronary descending branch or by aconitine or calcium chloride | Dietary 20E (10-20 mg/kg/day) has anti-inflammatory properties in mice and rats | Dietary 20E improves liver regeneration after chemically induced damage | 20E can be used for the treatment of herpes zoster |
| 20E can eliminate arrhythmia induced by aconitine | Dietary 20E protects gastric mucosa against ulcerogenic chemicals | Dietary 20E or oysterone (5-50 mg/kg) stimulate bile secretion in rats | Administration of 20E to rabbits reduces infection by protozoa (**Lamblia duodenalis**, **Fla gallinae**) |
| 20E (0.25-2.5 mg/kg) can restore normal rheologic indices (fibrinogen concentration and viscosity) of blood from rats with a “high blood viscosity syndrome” induced by cerebral ischemia. | Dietary 20E stimulates primary immune reaction | Dietary 20E or oysterone (5-50 mg/kg) stimulate bile secretion in rats | 20E (200 μg/ml) possesses antibacterial and antifungal activities |
| 20E can counteract the damages induced by TNF α on human umbilical vein endothelial cells. | 20E (1 μM) and other ecdysteroids enhance DNA synthesis in mitogen-stimulated lymphocytes in vitro | Dietary 20E (5 mg/kg) restores normal glomerular filtration rate and suppresses albuminuria in rats treated with a nephrotoxic mixture | Vargas Gonzalez, 1986 |
| 20E (10⁻⁸ – 10⁻⁵ M) inhibits histamine release by mast cells | 20E (10⁻⁸ – 10⁻⁵ M) inhibits histamine release by mast cells | Dietary 20E (5 mg/kg) has no effect on experimentally induced inflammatory processes (pleuresia, arthritis) in rats | Syrov and Klushbatkova, 2001 |
| Dietary 20E (0.2-0.4 mg/kg/day) results in a significant longer times (Azizov and Seifulla, 1998). These effects are similar to those of anabolic steroids. 20E is also able to increase animals given ecdysteroids for one week were able to swim for significantly longer times (Azizov and Seifulla, 1998). These effects are similar to those of anabolic steroids. 20E is also able to increase animals given ecdysteroids for one week were able to swim for significantly longer times (Azizov and Seifulla, 1998). These effects are similar to those of anabolic steroids. | 20E (7.5.10⁻¹³ – 7.5.10⁻⁸ M) activate human lymphocytes in vitro (E-rosette formation, agar migration test) | Dietary 20E (10–9 – 10–5 M) inhibits histamine release by mast cells | Ahmad et al., 1996 |
| Dietary 20E (5 mg/kg) protects gastric mucosa against ulcerogenic chemicals | Dietary 20E protects gastric mucosa against ulcerogenic chemicals | Dietary 20E (5 mg/kg) has no effect on experimentally induced inflammatory processes (pleuresia, arthritis) in rats | Vargas Gonzalez, 1986 |
| 20E (7.5.10⁻¹³ – 7.5.10⁻⁸ M) activate human lymphocytes in vitro (E-rosette formation, agar migration test) | Dietary 20E (5 mg/kg) has no effect on experimentally induced inflammatory processes (pleuresia, arthritis) in rats | Dietary 20E (5 mg/kg) has no effect on experimentally induced inflammatory processes (pleuresia, arthritis) in rats | Syrov et al., 1983 |
| Dietary 20E (5 mg/kg) stimulates T-cell CD2 presentation in vitro | Dietary 20E (5 mg/kg) has no effect on experimentally induced inflammatory processes (pleuresia, arthritis) in rats | Dietary 20E (5 mg/kg) has no effect on experimentally induced inflammatory processes (pleuresia, arthritis) in rats | Syrov et al., 1992 |
| Dietary 20E improves liver regeneration after chemically induced damage | Dietary 20E (5 mg/kg) has no effect on experimentally induced inflammatory processes (pleuresia, arthritis) in rats | Dietary 20E (5 mg/kg) has no effect on experimentally induced inflammatory processes (pleuresia, arthritis) in rats | Syrov et al., 1983 |
| Dietary 20E (5 mg/kg) restores normal glomerular filtration rate and suppresses albuminuria in rats treated with a nephrotoxic mixture | Dietary 20E improves liver regeneration after chemically induced damage | Dietary 20E (5 mg/kg) has no effect on experimentally induced inflammatory processes (pleuresia, arthritis) in rats | Syrov et al., 1983 |

**Ecdysteroids: effects on cellular proliferation and differentiation**

Wound-healing effects of ecdysteroids have been described (Syrov and Khushbatkova, 1996; Darmograi et al., 1998). 20E (applied at 0.1% w/w in liposomes) shortens the duration of skin repair after superficial wounding and 20E (2 x 10⁻⁴M) stimulates keratinocyte differentiation in vitro (Detmar et al., 1994), an effect measured by the increase of the activity of transglutaminase (an enzyme involved in protein connection through isopeptidic bond...
Regarding the use of ecdysteroids in cosmetics (Lin and Lin, 1989; Meybeck and Bonté, 1990, 1993; Meybeck et al., 1994; Tsuji et al., 1995a&b; Darmograi et al. 1998; Meybeck 1999a&b). In this context, the incorporation of 20E or its acyl ester (2,3,22-tripalmitate) into liposomes has been tested as a slow-release form (Politova et al., 2001).

20E administered orally to rats (5 mg/kg) accelerates the healing process after an experimental bone fracture (Syrov et al., 1986a), and the same molecule (10-100 ng/ml) can stimulate the in vitro proliferation of rat osteosarcoma UMR106 cells (osteoblasts) by 41% (Gao and Wang, 2000). Similarly, 20E stimulates proliferation of human umbilical vein endothelial cells (Lin et al., 1997; Wu et al., 1998b), and several phytoecdysteroids can stimulate erythropoiesis in rats (Syrov et al., 1997b).

The effects of ecdysteroids on tumors cells proliferation are somewhat conflicting: Lagova and Valueva (1981) reported that 20E (0.1-300 mg/kg, subcutaneous injections for 5 days) was mainly ineffective on tumour growth in mice, but it stimulated the growth of mammary gland carcinomas in mice and rats. El-Mofî (1987, 1994) reported that E was able to induce neoplastic lesions in toads and mice; other authors reported inhibitory effects on tumor cell proliferation (Hirono et al., 1969; Burdette, 1974; Shibatani et al., 1996). More recently, Konovalova et al. (2002) showed that injected 20E had a synergistic effect with low doses of an antitumour drug (cis-platin). Most probably, the results may differ according with the cell types, the nature and concentration of ecdysteroids used, and this clearly requires more extensive studies. In addition, genoprotective effects of ecdysteroids have been reported (Gubskii et al., 1993; Levitskii et al., 1993a&b, 1996; Chabanny et al., 1994); ecdysteroids can prevent chromatin damages induced by various chemicals.

### Ecdysteroids and protein synthesis

Stimulatory effects of ecdysone on protein synthesis were reported as early as 1963 (Burdette and Coda, 1963), and the discovery of phytoecdysteroids made these molecules available in large amounts for pharmacological assays. It was rapidly shown that ecdysteroids were able to stimulate protein synthesis in mouse liver (Okui et al., 1968; Otaka et al., 1968, 1969a&b). In fact, it was shown that 20E stimulates the incorporation of [14C]leucine in a cell-free translation system (rat liver polysomes), i.e. it increases the efficiency of the translational machinery (Syrov et al., 1978). Such conclusions have been confirmed and extended to other tissues, especially heart and muscles (Syrov et al., 1975a; Aizikov et al., 1978; Khimiko et al., 2000). Recent structure-activity studies (Syrov et al., 2001) as measured by a stimulation of [14C] aminoacid incorporation into proteins showed that among the compounds tested turkesterone was the most active, followed by cyasterone and 20E.

### Table 2. Effects of ingested or injected ecdysteroids on growth of various vertebrate species (in red, reference to patents).

| Species                  | Ecdysteroids | Daily dose | Mode of administration | Duration of treatments (days) | Effects                                                                 | References                      |
|--------------------------|--------------|------------|------------------------|------------------------------|------------------------------------------------------------------------|---------------------------------|
| Mice                     | 20E, Cyas    | 0.005-0.1 mg | per os                | 90                           | Increased protein synthesis in liver and kidney                         | Hikino et al., 1969             |
| Mice                     | Turk         | 5 mg/kg    | i.p.                   |                              | Increased liver protein synthesis in vivo and in vitro                 | Syrov et al., 1978             |
| Mice (juveniles and adults) | 20E          | 0.1, 0.5, 1 mg | i.p.                   | 30                           | Increased growth of juvenile females and adults of both sexes           | Stopka et al., 1999             |
| Rats (29-day old)        | E, 20E, PonA | 2, 10, 50 mg | per os                | 5                            | No effect on growth rate                                               | Matsuda et al., 1970            |
| Rats (castrated juveniles) | VitE, Turk   | 0.5-10 mg/kg | i.p.                   | 10                           | Increased muscle and liver weight                                      | Syrov and Kurnukov, 1975a,b     |
| Rats (juveniles and adults) | 20E          | 5 mg/kg    | per os                | 7                            | Increased muscle and liver weight                                      | Syrov and Kurnukov, 1975b       |
| Rats (juveniles, adults and ovariecto-mized juveniles) | 20E | 5 mg/kg | i.p.                   | 7                            | Increased body weight and growth of liver, kidneys, muscles             | Syrov et al., 1981a             |
| Sheep                    | E            | 0.02 µg/kg | per os, or i.v.        | 35-150                       | Increased growth rate of body and of wool                              | Purser and Baker, 1994          |
| Pigs                     | 20E          | 0.2-0.4 mg/kg | per os                | 30                           | Increased growth and higher nitrogen retention                           | Krätky et al., 1997             |
| Japanese quails          | 20E          | 20, 100, 500 mg/kg of food | per os | 28                           | Increased growth                                                       | Sláma et al., 1996             |

Cyas: cyasterone; E: ecdysone; 20E: 20-hydroxyecdysone; PonA: ponasterone A; Turk: turkesterone; VitE: viticosterone E

i.p. : intraperitoneal injection ; i.v.: intravenous injection.
Ecdysteroids and glucose metabolism

It was shown early on (Table 3) that 20E given per os to rats reduces hyperglycaemia induced either by glucagon or by alloxan treatment (Matsuda et al., 1970; Uchiyama and Ogawa, 1970; Yoshida et al., 1971, Uchiyama and Yoshida, 1974). In fact, 20E stimulates the incorporation of glucose into glycogen and protein in mouse liver (Yoshida et al., 1971) and more generally it enhances glucose utilization by tissues (Syrov et al., 1997a). The mechanism involved seems to be an increase of tissue sensitivity to insulin (Kosovsky et al., 1989) and preparations containing phytoecdysteroids have been proposed as oral antidiabetics (Takahashi and Nishimoto, 1992; Yang et al., 2001). Depending on the extent of hyperglycaemia, phytoecdysteroid effects may be more or less pronounced that those of manilil, a widely used pharmacological molecule (Kutepova et al., 2001).

Ecdysteroids and lipid metabolism

Ecdysteroids display hypocholesterolaemic effects (Lupien et al., 1969; Mironova et al., 1982; Syrov et al., 1983), through a reduction of cholesterol biosynthesis and an increase of its catabolism (Uchiyama and Yoshida, 1974). 20E (5 mg/kg per os) stimulates the conversion of cholesterol into bile acids in rats (Syrov et al., 1986b), and such an effect is reminiscent of some oxysterols (Schroepfer, 2000). In connection with these effects, ecdysteroids may also have antiatherosclerotic actions (Matsuda et al., 1974; Syrov et al., 1983). Intraperitoneally injected 20E (0.5 mg/ kg in rats) also enhances [14C]acetate incorporation into liver triglycerides and reduces triglyceride lipase activity (Catalán et al., 1985).

Ecdysteroids: a “universal medicine”?

An impressive number of papers dealing with ecdysteroid effects are available in the literature. They concern almost every physiological function, and we will give below a brief insight of the published data. It must be noted, however, that in many instances that, in addition to the difficulties caused by language barriers, the published data. It must  be noted, however, that in many instances

Table 3. Effects of ingested or injected ecdysteroids on carbohydrate metabolism (in red, reference to patents).

| Species | Ecdysteroids | Dose | Mode of administration | Duration of treatments (days) | Effects | References |
|---------|--------------|------|------------------------|-----------------------------|---------|------------|
| Rats or Mice | 20E | 0.5 mg/kg | p.o. | Hypoglycaemic | Yoshida et al., 1971; Uchiyama and Yoshida, 1974 |
| Rats | 20E | 5 mg/kg | per os | Recovery of liver mitochondrial function after alloxan treatment | Tadmukhamedova et al., 1985 Syrov et al., 1992 |
| Rats | 20E | | | Increase sensitivity to insulin treatment in experimental diabetes | Kosovsky et al., 1989 |
| Humans | 20E, Ino | per os | Antidiabetic effects | Takahashi and Nishimoto, 1992 |
| Rats | 20E, Cyas, Turk | per os | Decrease of glycaemia in diabetic animals | Syrov et al., 1997a |
| Humans | 20E + 20E2Ac | per os | | Antidiabetic effects | Yang et al., 2001 |

Cyas: cyasterone; E: ec dysone; 20E: 20-hydroxyec dysone; Ino : inokosterone A; Turk: turk esterone.
2d20E, polB, turkesterone, 1-5 mg/kg) increase the concentration of antibody-forming cells in the spleen of mice immunised with sheep red blood cells (Sakhibov et al., 1989). Low (7.5x10^{-12}-7.5x10^{-8} M) concentrations of 20E induce the activation (E-rosette formation test) of human lymphocytes (Trenin et al., 1996; Trenin and Volodin, 1999). Low to moderate (10^{-12}-10^{-5} M) concentrations of 20E or other ecdysteroids stimulate, whereas higher (10^{-4} M) concentrations eventually inhibit, DNA synthesis in concanavalin A – activated lymphocytes (Kuzmitsky et al., 1990; Fomovska et al., 1992; Chiang et al., 1992).

20E (10-20 mg/kg/day per os) has antiinflammatory properties similar to cortisone acetate in rats and mice (Kurumukov and Syrov, 1988; Fomovska et al., 1992) and turkesterone improves lung defence mechanisms in diabetic rats (Najmutdinova and Saatov, 1999). 20E was shown to inhibit in a dose-dependent fashion (10^{-4}-10^{-4} M) histamine release from rat peritoneal mast cells induced by anti-IgE or concanavalain A (Takei et al., 1991). Taniguchi et al. (1997), however, could not observe any antiinflammatory effect of 20E given orally to rats (5 mg/kg/day for 7 days).

**Ecdysteroids have antioxidant properties:** 20E has antioxidative and anti-free radical properties (Osynska et al., 1992) and it can thus reduce lipid peroxidation (Kuzmenko et al., 1997, 2001). Several models were used in these studies, as the chemiluminescence of blood serum induced by H2O2 using rats receiving a vitamin D-deficient diet eventually supplemented with 0.1 mg 20E/kg per day, or the uptake of oxygen by methyl linolate micelles in the presence or absence of 20E.

**Are ecdysteroids toxic to microorganisms?:** There are a few reports about antimicrobial activity of ecdysteroids. However, Ahmad et al. (1996) reported antifungal and antibacterial activity of 20E at rather high concentrations (between 100 and 400 µg/ml, i.e. 2-8 x 10^{-4} M). An antimicrobial activity of 20E and its acetates was also observed by Volodin et al. (1999). Toxic effects on protozoa have also been reported; rabbits receiving 20E per os (5 mg/l/day for 3 months) showed a reduced infection with *Lambitia duodenalis* (Syrov et al., 1990), and the improvement of ruminant productivity by ecdysone was also interpreted by its toxicity towards rumen protozoa (Purser and Baker, 1994).

**Ecdysteroids are not toxic to vertebrates:** Ecdysteroids have a very low toxicity (LD50 > 6g/kg), they are not hypertensive and, in spite of their anabolic action, they would have neither androgenic nor oestrogenic (or anti-oestrogenic) effects; they induce no virilisation and they do not induce significant changes in castrated animals (e.g. Prabhu and Nayar, 1974). All together this suggests that ecdysteroids are attractive compounds for a wide array of uses, which have been proposed, and of course it would be of particular interest to understand more precisely their mode(s) of action in mammals.

**Genomic and/or non-genomic effects of ecdysteroids?**

**Do ecdysteroids have genomic effects on vertebrates?**

In insects, ecdysteroids have well-known genomic effects which involve nuclear receptors (see Section 2). When considering the molecules in 3-dimensions, it is clear that they show striking differences to vertebrate sex or adrenal steroids, and their full cholesterol side-chain most probably prevents any binding to the receptors of these vertebrate hormones. Recently, however, it has been found that some previously “orphan” nuclear receptors (e.g. LXR, PXR) bind endogenously produced oxysterols (Janowski et al., 1999; Schroeppfer, 2000) or have a broad specificity and may bind a wide array of xenobiotics including several steroids (Jones et al., 2000). Given this broad ligand specificity, these proteins might rather function as “endocrine sensors” rather than “true receptors” (Evans, 2002). So, until ecdysteroids are directly tested for binding to such receptors, it remains conceivable that they may have transcriptional effects through binding to some nuclear receptor(s); indeed early studies showed a rapid in vivo stimulation by 20E of the incorporation of [14C]orotic acid into RNA in mouse liver (Uchiyama and Otaka, 1974).

**Ecdysteroids: membrane effects?**

Membrane effects of steroids are nowadays well documented and they may proceed through three different pathways (Figure 7). According to Brann et al. (1995), these effects may either involve: (1) the dissolution of ecdysteroids in the membrane bilayer and a change in the environment of some membrane proteins (and hence of their activity), (2) their interaction with a specific membrane receptor, which will activate some transduction mechanism, or (3) their binding to a modulatory site of the receptor for another molecule. These different effects are not mutually exclusive. Very recently, a membrane progestin receptor involved in fish oocyte meiotic reinitiation was cloned and its physiological relevance was fully established (Zhu et al., 2003).

**Dissolution of ecdysteroids in the membrane lipid bilayer**

In order to test for the first hypothesis, Tuganova and Kotsyuruba (1996) developed experiments designed to analyse the dissolution of ecdysteroids in human erythrocyte membranes. They did not perform direct experiments, i.e. by measuring the incorporation of radiolabelled ecdysteroids. In a first set of experiments, erythrocytes were first incubated with various steroids (10^{-6} M) and then with [3H]cholesterol; both 2d20E and 20E pretreatments reduced the radioactivity associated with membrane fractions. In a second set of experiments, the authors first incubated erythrocytes with various concentrations of 20E (10^{-14} to 10^{-10} M) then with either radiolabelled cholesterol, cholecalciferol or calcitriol; 20E reduced mainly calcitriol incorporation. Such experiments support the idea that ecdysteroids can be incorporated into membrane bilayers, although they do not constitute an absolute proof. It is tempting to make a relation between

![Figure 7. Three possible ways for a membrane effect of ecdysteroids (adapted from Brann et al., 1995).](image-url)
these results and the rapid effect of 20E on Na+/K+ ATPase activity in *D. melanogaster* salivary gland cells (Schneider et al., 1996).

**Rapid membrane effects**

A rapid increase of cGMP and a decrease of cAMP levels in mouse plasma, together with a decrease of PKA activity in liver were described 40 min after an intraperitoneal injection of 10 µg 20E (Catalán et al., 1979a&b, 1982). More recently, it was shown that 20E evokes rapid (1-2 min) and transitory effects on membranes (Kotsyuruba et al., 1995a-c, 1998a&b, 1999); 20E increases the pool of free arachidonic acid and the synthesis of leukotrienes and prostaglandins. Such responses were observed with different cell types (hepatocytes, erythocytes, lymphocytes, macrophages etc.). In many instances the effects of 20E resemble those evoked by calcitriol (1,25OHD3), a molecule often used for comparison by those working on ecdysteroid pharmacology (Barsony and Marx, 1988). The same effects were also produced by 20E bound to magnetite nanoparticles (Mykhaylyk et al., 1999; 2001), a formulation which should prevent 20E diffusion into target cells, and thus restricting its possible action(s) to the plasma membrane level.

We should emphasise here that ecdysteroids can be recognised by membrane receptors in arthropods: ecdysteroids can be detected by taste cell receptors both in Crustacea (Tomashko, 1999) and insects (Tanaka et al., 1994; Descoinds and Marion-Poll, 1999), and in vertebrates too steroids can work as pheromones (see e.g. Sorensen et al., 1990). Thus, such a mode of action is conceivable.

**Neuromodulatory actions**

Such effects are well documented for vertebrate neurosteroids, which may modulate the response of neurotransmitter receptors to their cognate ligands. The binding of the steroid alone has no apparent effect. Thus, the GABAa receptor possesses (in a domain separate for the neurotransmitter binding site) a binding site for steroids, which may therefore modify the response to GABA. In a similar way, 20-hydroxyecdysone showed a neuromodulatory effect on GABAa receptor of rat cortical neurons (Tsuijyama et al., 1995; Sasa et al., 1996), although this was observed for rather high concentrations (10-100 µM). In connection with this effect, 20E showed an antiepileptic activity in rats (Hanaya et al., 1997); when 20E was given orally (100-200 mg/kg) to spontaneous epileptic rats, it was able to reduce tonic convulsions.

Some recent data

Recently, Constantino et al. (2001) fortuitously made a crucial observation. Using the Invitrogen® ecdysteroid-inducible expression system to analyse the transduction mechanisms of interleukin-3 (IL-3) in a pro-B lymphocyte cell line, they found in control experiments that murA and ponA were able to potentiate the IL-3-dependent activation of PI 3-kinase/Akt pathway in non-transformed cells.

Given the central role of the Akt/PKB pathway in mammalian cell metabolism (e.g. Brazil and Hemmings, 2001; Whiteman et al., 2002), such results provide an interesting basis for explaining in a single way many effects of ecdysteroids on mammals, as concerns their hypoglycaemic, antiapoptotic and anabolic actions (Figure 8). The available data do not allow to decide whether ecdysteroids act on the IL-3 receptor itself or on a downstream step.

Where is 20-hydroxyecdysone found?

Phytoecdysteroids are found in many plant species, where they can reach concentrations above 1-2% of the plant dry weight (e.g. Lafont, 1998, Dinan, 2001). Ecdysteroid-rich species are found among ferns and angiosperms and some of these species are either very common (e.g. the fern *Polysporium vulgarare*) or they are cultivated on a large scale for their pharmacological properties (e.g. *Leuza*, *Pfaffia*, *Cyanotis*). Given the still limited market at the moment, a few plant species only (Table 4) are currently used as a source of phytoecdysteroids: (1) *Leuza* (= *Rhaponticum*) carthamoides (Asteraceae) from Eastern Europe countries, where it is cultivated as a remedy in traditional medicine, (2) *Pfaffia* (in fact a group of related species) = Brazilian ginseng (= Suma), again a plant used in traditional medicine and (3) *Cyanotis vaga* or *C. arachnoides*, a monocotyledonous plant, extracts of which are used on a large scale also for the synchronization of spinning in silkworm larvae (Guo, 1989; Chandrakala et al., 1998).

Over 140 different preparations containing ecdysteroids for oral use can be found on the market (Table 5). We may distinguish several categories among them: (1) those containing crude or semi-purified plant extracts (plant powders, or alcoholic extracts - elixirs) and (2) those containing “pure” 20E or a defined ecdysteroid mixture. Most of them are proposed for use by bodybuilders, but some have been designed for more specific users (e.g. golfers), or for animals (dogs, horses). In addition, ecdysteroids are also present...
Lafont R., Dinan L. 2003. Practical uses for ecdysteroids in mammals including humans: an update. 30pp. *Journal of Insect Science*, 3:7, Available online: insectscience.org/3.7

The impressive development of preparations containing ecdysteroids suggests that this class of molecule has indeed at least some of the claimed effects. The scientific justification for such commercial developments relies, however, on just a few references (ca. 10), often with the same ones being cited to support quite different effects.

| Species Family | Local name | Part of the plant used |
|----------------|------------|------------------------|
| *Ajuga turkestanica* Labiatae | Whole plants | |
| *Cynotis vaga* Commelinaceae | Whole plants and/or roots | |
| *Cynathula capitata* Commelinaceae | Chuan Niu Hsi | Roots |
| *Leuzea (=Rhaponticum) carthamoides* Asteraceae | Maral | Roots, seeds |
| *Pfaffia treinoides* Amaranthaceae | Sama, Para Toda | Roots |
| *Pfaffia paniculata* Amaranthaceae | Sama | Roots |
| *Pfaffia stenophylla* Amaranthaceae | Sama | Roots |
| *Polypodium aureum* Ferns | Calaguala | Rhizomes |
| *Polypodium lepidopteris* Ferns | Samambaia | Rhizomes, leaves |
| *Polypodium vulgare* Ferns | common polypody | Rhizomes |

| Species Family | Local name | Part of the plant used |
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| *Pfaffia treinoides* Amaranthaceae | Sama, Para Toda | Roots |
| *Pfaffia paniculata* Amaranthaceae | Sama | Roots |
| *Pfaffia stenophylla* Amaranthaceae | Sama | Roots |
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| *Pfaffia paniculata* Amaranthaceae | Sama | Roots |
| *Pfaffia stenophylla* Amaranthaceae | Sama | Roots |
| *Polypodium aureum* Ferns | Calaguala | Rhizomes |
| *Polypodium lepidopteris* Ferns | Samambaia | Rhizomes, leaves |
| *Polypodium vulgare* Ferns | common polypody | Rhizomes |

in at least two cosmetic preparations (Hydrastar and Phenomen A from Christian Dior).

Table 4. Plants currently used to obtain the ecdysteroids used for various preparations

| Nr | Product | Supplier | Web address | Plant source(s) |
|----|---------|----------|-------------|-----------------|
| 1  | Active  | Velocity | http://www.fastathlete.com/velocity/active.html | Leuzea + Cynotis |
| 2  | ACT-SUM™ | 7th Millenium Nutrition | http://www.diabetestea.com/actsum.html | Pfaffia |
| 3  | Adaptogen N | Muscle And Sport Science (MASS) | http://www.musclemass.com/prohorseome.html | Pfaffia |
| 4  | Adrena+ | Greens+™ | http://www.greenspluscanada.com/fr/infonp.htm | Pfaffia |
| 5  | Advanced Compound 2 | Advanced Labs | http://www.reach4life.com/details.asp?ProdID=4534 | Pfaffia |
| 6  | ALL-iN1 | Sports Nutrition Net | http://www.sports-supplements.co.uk/reviews/sportsnutrition/allin1.shtml | Leuzea + Cynotis |
| 7  | Amazonia Immuno Forte | Nutrisana | http://www.nutrisana.com/html/amazonia_fr.htm | Pfaffia |
| 8  | Animal Methoxy Stak | Universal Nutrition | http://www.fitnessfirstusa.com/details.asp?item=1781 | ? |
| 9  | Atomic Muscle | Maximus Nutrition | http://www.maximusnutrition.com/products/MN012.html | ? |
| 10 | Beta-Builder | Ultimate Nutrition | http://www.onlineprotocols.com/product/beta_product.htm | ? |
| 11 | B-Ecdysone | NuTrex Nutrition | http://www.nutrex.com/load_frames.html?anabolic-products/b-ecdysone | ? |
| 12 | Beta-Ecdysterone | Gernapharm | http://www.gernapharm.com/BetaEcdysteroneFrame.asp | ? |
| 13 | Beta-Ecdysterone Stack | PEAK Nutrition | http://peaknutrition.com/betaestk6lab.html | Pfaffia |
| 14 | Beta-Mass | 7th Millenium Nutrition | http://www.diabetestea.com/beta1.html | Pfaffia |
| 15 | Betoxytol | BSN | http://www.bodybuilding.com/store/bsn/beta.html | Leuzea |
| 16 | Beta-X capsules/liquid Nutrition | Flexstar Sports Nutrition | http://primenutrition.com/betbet180cap.html | Cynotis |
| 17 | Bicreatol | BSN | http://www.oxygenliving.net/bsnbc12.html | ? |
| 18 | Bio-Pro Plus | Body Ammo | http://www.nutritionblvd.com/284132.html | Pfaffia |
| 19 | BPS | Syntax | http://store.yahoo.com/xsportsnutrition/syntaxbhs.html | Leuzea |
| 20 | Brazilian Ginseng | Paradise Herbs | http://www.herbs.com/braziliangin.html | Pfaffia |
| 21 | Bug Juice | ? | http://www.supernita.com/top12/bugjuice.htm | ? |
| 22 | Changing Times™ | Nature’s Plus | http://www.natureplus.com/products/ | Pfaffia |
| 23 | Clear Energy | Garden of Life | http://www.herb.com/clearenergy.html | Leuzea |
| 24 | Creatolic fuzz | Maximum Human Performance | http://www.fitnessfirstusa.com/details.asp?item=7138 | Pfaffia |
| 25 | Cyclone | Maximuscle | http://gymrats.co.uk/bodybuilding-supplements/item44.htm | ? |
| 26 | Desire-X | MasoN natural | http://www.italiannutrition.com/exsemenhancer/hornyygoeke/dgoeke.html | Polypondium |

Conclusion

Ecdysteroids are probably the most abundant steroids in nature because they are produced not only by arthropods, but also by many plant species. They seem to display a wide array of pharmacological effects on vertebrates, many of which are beneficial. However, these claims require more thorough validation and clinical testing. Ecdysteroids are used by an increasing number of preparations based on purified ecdysteroids or on ecdysteroid-containing plant powders/extracts.
| Product                          | Source                                                                 | Link                                                                 |
|---------------------------------|------------------------------------------------------------------------|----------------------------------------------------------------------|
| EcdyMax HP                      | EAS                                                                    | [http://www.bodybuilding.com/store/cas/ecdymax.html](http://www.bodybuilding.com/store/cas/ecdymax.html) |
| Ecdy-20                         | MRM                                                                   | [http://www.metabolicresponse.com/detail.asp?id=86](http://www.metabolicresponse.com/detail.asp?id=86) |
| EcdyBol                         | BodySincs Pinnacle                                                     | [http://www.bodysincs.com/ecdybol.html](http://www.bodysincs.com/ecdybol.html) |
| Ecdy-Bolin                      | ?                                                                     | [http://www.trulyhuge.com/ecdy-bolin.htm](http://www.trulyhuge.com/ecdy-bolin.htm) |
| Ecdy-Force                      | PEAK Nutrition                                                        | [http://www.peaknutrition.com/ec180tab.html](http://www.peaknutrition.com/ec180tab.html) |
| Ecdylean                        | NuTrex Nutrition                                                      | [http://www.nutremen/products/ecdylane.html](http://www.nutremen/products/ecdylane.html) |
| Ecdy-Mass                       | Gasparr Nutrition                                                     | [http://www.richgasparr.com/products/ecdymass.html](http://www.richgasparr.com/products/ecdymass.html) |
| EcdymaxTM                       | A R Nutrition                                                         | [http://www.bodybuilding.com/store/ar/ecdymax.html](http://www.bodybuilding.com/store/ar/ecdymax.html) |
| Ecdyzone                        | Reflex Nutrition                                                      | [http://www.createstore.co.uk/productsinfo/Ecdyzone_x_90_Capsules.asp](http://www.createstore.co.uk/productsinfo/Ecdyzone_x_90_Capsules.asp) |
| Ecdysten                        | Thermo Life International                                            | [http://www.thermofolic.com/Products-Ecdysten.html](http://www.thermofolic.com/Products-Ecdysten.html) |
| Ecdyosterone                    | ZOE Labs                                                              | ?                                                                    |
| EcdyVone                        | Prolab                                                                | [http://www.fitnessfirstusa.com/details.asp?item=7701](http://www.fitnessfirstusa.com/details.asp?item=7701) |
| EcdyVone Plasma                 | Prolab                                                                | [http://www.bodyworks-nutrition.com/ecdyvone-plasma.html](http://www.bodyworks-nutrition.com/ecdyvone-plasma.html) |
| Ecdisten                        | BodybuildinG Estonia                                                 | [http://www.bodybuilding.ee/est/texts/tast/eckisten.htm](http://www.bodybuilding.ee/est/texts/tast/eckisten.htm) |
| 20-H                            | TKE (The Kutting Edge)                                                | [http://www.tkefitness.com/20h.asp](http://www.tkefitness.com/20h.asp) |
| Horny Goat Weed                 | Bodyonics Pinnacle                                                    | [http://www.marigarden.com/hg/index.html](http://www.marigarden.com/hg/index.html) |
| Horny Goat Weed Nature’s A      | Ecdy-2 CapsTM 7th Millenium Nutrition                                 | [http://www.diabetescare.com/erolyzy.html](http://www.diabetescare.com/erolyzy.html) |
| Especially Yours                | Nature’s Plus                                                          | [http://www.naturesplus.com/products/](http://www.naturesplus.com/products/) |
| Excite                          | Dynamicize Nutrition                                                 | [http://www.affordablesupplements.com/excite.as](http://www.affordablesupplements.com/excite.as) |
| Fem Actin                       | Herbal Actives                                                        | [http://www.healthclub.ru/natures/femactin.php](http://www.healthclub.ru/natures/femactin.php) |
| FarmEase                        | Gero Vita International                                               | [http://www.gvstore.com/gbd/fixa.html](http://www.gvstore.com/gbd/fixa.html) |
| Fitnesky                        | Slovakofarma/Intercaps                                                | [http://www.ishop.purus.cz/start.php?menu=E%7C3%20as%20soubor%20podkat.php](http://www.ishop.purus.cz/start.php?menu=E%7C3%20as%20soubor%20podkat.php) |
| 20-H                            | TKE (The Kutting Edge)                                                | [http://www.tkefitness.com/20h.asp](http://www.tkefitness.com/20h.asp) |
| Horny Goat Weed                 | Bodyonics Pinnacle                                                    | [http://www.marigarden.com/hg/index.html](http://www.marigarden.com/hg/index.html) |
| Horny Goat Weed Nature’s A      | [http://www.horny-goat-weed-maca-pure.com/ingredients.htm](http://www.horny-goat-weed-maca-pure.com/ingredients.htm) |
| Horny Goat Weed Herbal Remedies USA™ |                                                            | [http://www.herbalremedies.com/horgoatweed6.html](http://www.herbalremedies.com/horgoatweed6.html) |
| Horny Goat Weed Plus Natural Men’s Health |                                                             | [http://www.onlynaturals.com/store/hornygoat.html](http://www.onlynaturals.com/store/hornygoat.html) |
| HumanoPro GEN® Human Performance Nutrition |                                                                 | [http://www.pacific-nutrition.com/whey-humanoapro.htm](http://www.pacific-nutrition.com/whey-humanoapro.htm) |
| Hydra-Star                      | Christian Dior                                                        | [http://www.auravita.com/factfile.asp?Type=5](http://www.auravita.com/factfile.asp?Type=5) |
| IMH1-Ecdysterone                | Supplements Research & Advancements (SRA)                              | [http://www.healthnutwarehouse.com/imhec90cap.html](http://www.healthnutwarehouse.com/imhec90cap.html) |
| Immunocear®                     | Nature’s Plus                                                          | [http://www.natureplus.com/products/](http://www.natureplus.com/products/) |
| IsoStak AFC                     | Universal Nutrition                                                   | [http://www.sportscoaching.nl/wostak.html](http://www.sportscoaching.nl/wostak.html) |
| IT-Ideal Transformation Formula | Miada Sports Nutrition (NZ)                                          | [http://www.miadasport.com/](http://www.miadasport.com/) |
| Lean 65 MRP                     | Iron-Tek                                                              | [http://www.fitnessone.com/lean_65_mrp](http://www.fitnessone.com/lean_65_mrp) |
| Leuzea drops                    | Slovakofarma                                                         | [http://www.aha.ru/~slovakof/products/leuzea.html](http://www.aha.ru/~slovakof/products/leuzea.html) |
| Liquid Dynamite                 | Beast Sports                                                          | [http://www.buyjustnatural.com/liquiddynamite.html](http://www.buyjustnatural.com/liquiddynamite.html) |
| Maralan                        | Firma Kr’en                                                           | [http://www.kren.cz/maralan.htm](http://www.kren.cz/maralan.htm) |
| Maralan Super                   | Firma Kr’en                                                           | [http://www.kren.cz/maralan.htm](http://www.kren.cz/maralan.htm) |
| Maxabol II                     | Maxam Nutraceutics                                                   | [http://ssl.ipms.com/maxam/Maxam_ASP_ViewProduct_LongDescription.asp?ProductID C0X-35](http://ssl.ipms.com/maxam/Maxam_ASP_ViewProduct_LongDescription.asp?ProductID C0X-35) |
| Medicinal Cyathula Root         | Smoking                                                               | [http://sinoherbgmk.com/sk/s41052.html](http://sinoherbgmk.com/sk/s41052.html) |
| Methoxerone Plus                | MaxMuscle                                                            | [http://www.maxmuscle.com/pressrelease/methoxerone/methoxerone.htm](http://www.maxmuscle.com/pressrelease/methoxerone/methoxerone.htm) |
| Methoxy ECD™ Xtreme             | EAS                                                                    | [http://www.usadiamondnutrition.com/MethoxyECDxtreme.html](http://www.usadiamondnutrition.com/MethoxyECDxtreme.html) |
| Methoxy Factor HP               | EAS                                                                   | ?                                                                    |
| Methoxy Grn™ Nutritional Products |                                                                | [http://myvitamet.com/vitamin/3met100mg60c.html](http://myvitamet.com/vitamin/3met100mg60c.html) |
| Methoxy Pure                    | Sports Science Research                                              | [http://www.sportscience.com/pages/mythoxy_pure.asp](http://www.sportscience.com/pages/mythoxy_pure.asp) |
| Methoxynal® Ecdysterone Sublingual Stack | Supplements Research & Research &                                      | [http://www.gotsupplements.com/methoxyal Sterone-sublingual-stack.html](http://www.gotsupplements.com/methoxyal Sterone-sublingual-stack.html) |
| Methoxybol-7                   | Scientific Advanced Nutrition (SAN)                                   | [http://store.yahoo.com/ampharma/methbsyn.html](http://store.yahoo.com/ampharma/methbsyn.html) |
| Rank | Product Name                  | Company/Brand                     | Web Link                                                                 | Country |
|------|------------------------------|-----------------------------------|--------------------------------------------------------------------------|---------|
| 75   | Methoxy-bolic X               | Total Nutritional Technologies (TNT) | [http://www11.nitrition.com/tat_methoxybolic_x_page.html](http://www11.nitrition.com/tat_methoxybolic_x_page.html) |         |
| 76   | Methoxy-sterone               | Muscle Science                     | [http://www.jidcu.co.za/JKDU_new/jkd_supp/methoxy.htm](http://www.jidcu.co.za/JKDU_new/jkd_supp/methoxy.htm) |         |
| 77   | Methoxy-Tek™                  | Iron-Tek                          | [http://www.advantagesupplements.com/bmrmet60cap.html](http://www.advantagesupplements.com/bmrmet60cap.html) |         |
| 78   | Methoxy Whey                   | Ultimate Nutrition                | [http://www.fitnessfestussia.com/retifas.asp?sttm=770d](http://www.fitnessfestussia.com/retifas.asp?sttm=770d) |         |
| 79   | MSB                           | Synax                             | [http://www.totalhealthsource.com/07013.asp](http://www.totalhealthsource.com/07013.asp) |         |
| 80   | Muscle Drive HP                | EAS                               | [http://www.sportssuppl ementsource.com/sportsource/easmusdrivehp.html](http://www.sportssuppl ementsource.com/sportsource/easmusdrivehp.html) |         |
| 81   | Muscle Drive HP Bars           | EAS                               | [http://www10.nitrition.com/eas_muscle_drive_bars_page.html](http://www10.nitrition.com/eas_muscle_drive_bars_page.html) |         |
| 82   | MX-7 Extreme                  | GEN® Human Performance Nutrition  | [http://www11.nitrition.com/gen_mx7_extreme_page.html](http://www11.nitrition.com/gen_mx7_extreme_page.html) |         |
| 83   | MyoBlast II                   | BodyLife Science                  | [http://www.pinsonsfitness.com/body_life_sciences.html](http://www.pinsonsfitness.com/body_life_sciences.html) |         |
| 84   | No-Nitro-S™                   | Nutec                             | [http://www.nutecperformance.com/productinfo.asp](http://www.nutecperformance.com/productinfo.asp) |         |
| 85   | Nutrejuva                      | Microlight                        | [http://www.microlight.net/nutrejuva2.asp](http://www.microlight.net/nutrejuva2.asp) |         |
| 86   | NutriZAC                       | Nature’s Plus                     | [http://www.natureplus.com/products/natureplus.html](http://www.natureplus.com/products/natureplus.html) |         |
| 87   | Natural Sterol Extreme        | Universal Nutrition               | [http://www.b-fit.com/natsterex90.html](http://www.b-fit.com/natsterex90.html) |         |
| 88   | New Power                      | Anew?                             | [http://www.anew.com.br/02_new_power.asp](http://www.anew.com.br/02_new_power.asp) |         |
| 89   | Norateen II                    | LA™ Muscle                        | [http://www.norateen.com/docs/norateen2.php3](http://www.norateen.com/docs/norateen2.php3) |         |
| 90   | Nu-Piste®                      | Stenandiol                       | [http://www.pinsonsfitness.com/stenandiol.html](http://www.pinsonsfitness.com/stenandiol.html) |         |
| 91   | Nutteh                        | Christian Dior                    | [http://www.hyperbelle.com/tests/phenomena.html](http://www.hyperbelle.com/tests/phenomena.html) |         |
| 92   | Organic Germanium              | Nature Most                       | [http://www.totalisdcountaminos.com/merchant/germaniumframe.htm](http://www.totalisdcountaminos.com/merchant/germaniumframe.htm) |         |
| 93   | Peak Performance-2             | K-9 Power Products LLC            | [http://www.dogzone.com/apt/ingredie.htm](http://www.dogzone.com/apt/ingredie.htm) |         |
| 94   | Pfaffia paniculata?            | ?                                 | [http://www.demratamedicus.se/produc.html](http://www.demratamedicus.se/produc.html) |         |
| 95   | Pfaffia paniculata             | ?                                 | [http://www.newdeal.ch/pfaffia.htm](http://www.newdeal.ch/pfaffia.htm) |         |
| 96   | Pfaffia paniculata             | ?                                 | [http://www.anew.com.br/02_pfaffianco.asp](http://www.anew.com.br/02_pfaffianco.asp) |         |
| 97   | Phenomen-A                     | Christian Dior                    | [http://www.hyperbelle.com/tests/phenomena.html](http://www.hyperbelle.com/tests/phenomena.html) |         |
| 98   | Phenomen-A                     | Christian Dior                    | [http://www.hyperbelle.com/tests/phenomena.html](http://www.hyperbelle.com/tests/phenomena.html) |         |
| 99   | Phytobol                       | ASN                               | [http://www.musclesurf.com/phytobol.html](http://www.musclesurf.com/phytobol.html) |         |
| 100  | Power Meal                     | Advanced Pharmaceutical Research  | [http://shop.store.yahoo.com/sports-nutrition/powermeal35cho1.html](http://shop.store.yahoo.com/sports-nutrition/powermeal35cho1.html) |         |
| 101  | Primavar Xtreme                | American Research Labs            | [http://www.pinsonsfitness.com/primavar_xtreme.html](http://www.pinsonsfitness.com/primavar_xtreme.html) |         |
| 102  | Prime One                      | Prime Quest™ International        | [http://www.whatskillingyou.com/ingredients.html](http://www.whatskillingyou.com/ingredients.html) |         |
| 103  | Prime Plus                     | Prime Quest™ International        | [http://www.primequest.co.za/primeplus.htm](http://www.primequest.co.za/primeplus.htm) |         |
| 104  | Pro-Complex PM                 | Optimum Nutrition                 | [http://shop.store.yahoo.com/sports-nutrition/procompmchoc.html](http://shop.store.yahoo.com/sports-nutrition/procompmchoc.html) |         |
| 105  | Pro-Ereto™                     | 7th Millennium Nutrition          | [http://www.diabetestea.com/eretopy.html](http://www.diabetestea.com/eretopy.html) |         |
| 106  | Prenax Extreme                 | Maximuscle                       | [http://affordablesupplements.co.uk/ProductDetail.asp?ProductID=443](http://affordablesupplements.co.uk/ProductDetail.asp?ProductID=443) |     |
| 107  | Protein P™                     | Interactive Nutrition            | [http://www.antirectivenutrition.com/products/proteinpm.php](http://www.antirectivenutrition.com/products/proteinpm.php) |     |
| 108  | REAP                          | Phyto-longevity, Inc              | [http://www.phytolongevity.com/PRODUCTS/PERFORMANCE/performance.html](http://www.phytolongevity.com/PRODUCTS/PERFORMANCE/performance.html) |         |
| 109  | Retabol®                      | Eissel Research                   | [http://www.eissel.se/produkt/Retabol.htm](http://www.eissel.se/produkt/Retabol.htm) |         |
| 110  | Robofil tinktura               | Bio System BT                     | [http://www.vardanet.hu/bosystem/r.html](http://www.vardanet.hu/bosystem/r.html) |         |
| 111  | Rus-Olympic                    | Nutri-Tech International AS       | [http://www.adaptogeno.com/rus_olympic.htm](http://www.adaptogeno.com/rus_olympic.htm) |         |
| 112  | Russian Secret                 | Power Health, Inc.                | [http://powerhealth.com/detail.cfm?id=365](http://powerhealth.com/detail.cfm?id=365) |     |
| 113  | SMP                           | Syntrax                           | [http://www.bodybuilding.com/store/syn/smp.html](http://www.bodybuilding.com/store/syn/smp.html) |     |
| 114  | Stenandol                     | German American Technologies      | [http://www.pinsonsfitness.com/stenandiol.html](http://www.pinsonsfitness.com/stenandiol.html) |     |
| 115  | Stinaral™                     | Virobrky?                         | [http://www.energy.sk/vyrobky/stinaral.htm](http://www.energy.sk/vyrobky/stinaral.htm) |         |
| 116  | Suma                          | Nature’s Way                      | [http://www.n101.com/Static/Products/suma_root_1740051275.html](http://www.n101.com/Static/Products/suma_root_1740051275.html) |         |
| 117  | SuMaFlex™ Plus                | Native Essence Herb Company       | [http://www.norx.com/smxc.html](http://www.norx.com/smxc.html) |         |
| 118  | SuMaca™ Plus                  | Native Essence Herb Company       | [http://www.norx.com/smxc.html](http://www.norx.com/smxc.html) |         |
| 119  | Sumacazon™                    | Rainforest Bio-energetics         | [http://www.pipeline.com/~dan_glick/Rainforest/maca.html](http://www.pipeline.com/~dan_glick/Rainforest/maca.html) |         |
| 120  | SumaCaps™ Plus                | Native Essence Herb Company       | [http://www.norx.com/smxc.html](http://www.norx.com/smxc.html) |         |
| 121  | SumaFlex™ Plus                | Native Essence Herb Company       | [http://www.norx.com/smxc.html](http://www.norx.com/smxc.html) |         |
| 122  | Sumaforme                     | Laboratoire Holonomn             | [http://www.clherbs.com/SUMAFORM.htm](http://www.clherbs.com/SUMAFORM.htm) |         |
| 123  | Sumah-5                       | The Millenium Nutrition          | [http://www.diabetestea.com/suhma.html](http://www.diabetestea.com/suhma.html) |         |
| 124  | Sumax                         | Ultimate Nutrition               | [http://www.bodybuilding.com/store/sumax.html](http://www.bodybuilding.com/store/sumax.html) |         |
| 125  | Syn-R-Gy                      | Amerinden                         | [http://www.netriceuticals.com/listing.asp?id=70](http://www.netriceuticals.com/listing.asp?id=70) |     |
| 126  | Syntrabol                     | Syntral                           | [http://www.syntrabol.com](http://www.syntrabol.com) |     |
| 127  | Ten Lives                     | Higher Ideals                     | [http://www.touchalific.com/tenlives.htm](http://www.touchalific.com/tenlives.htm) |         |
| 128  | Testo Intelligence Stack       | Ripfast                           | [http://www11.nitrition.com/tat_methoxybolic_x_page.html](http://www11.nitrition.com/tat_methoxybolic_x_page.html) |         |

Table 5. Continued on next page
of humans as anabolic compounds, and it may well be that in the near future they will also be used on domesticated animals. This is the reason why new methods of detection and quantification have been recently proposed (Tsitsimpikou, 2001; Le Bizec, 2002) and further developments in this area are required. Whether ecdysteroid use will become controlled (e.g. for high-performance sportsmen or domestic animals [e.g. race horses]) is still open.

Ecdysteroids have also been successfully developed as effective inducers for gene switch control systems, several of which are presently in use. Ecdysteroids and/or bisacylhydrazines fulfil many of the required criteria, but not all. There are still problems which need to be overcome (e.g. the need for highly potent ligands for modified ecdysteroid receptors in transformed mammalian or plant cells). However, there is clearly great potential in this area. The future of ecdysteroid-regulated gene switches as an experimental tool is assured, but the prospects as \textit{in vivo} systems is more debatable; the numerous pharmacological effects of ecdysteroids may preclude the development of their use in humans for gene therapy systems. This can only be resolved if more effort is invested into examining the biochemical fate and pharmacological consequences of ecdysteroids in mammals, especially humans.

Acknowledgements

The authors wish to thank Dr. Juraj Harmatha (Prague, Czech Republic) and Dr. Maria Bátori (Szeged, Hungary) for their help in collecting the data of Table 5.

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