Recent Progress in Ecdysteroid Pharmacology

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Фармакологические эффекты экдистероидов на млекопитающих исследуются более 40 лет. Эти исследования не учитывались западными учеными, но за последние годы ситуация значительно изменилась. Экдистероиды показывают перспективность использования в медицине и сейчас их потенциал активно изучается. В данный момент фармакологический интерес касается в основном снижения жировой массы, защиты от увеличения массы и предупреждения остеопороза. Также ведутся эксперименты по идентификации молекулярных мишеней экдистероидов у млекопитающих.

The pharmacological effects of ecdysteroids on mammals have been investigated for more than 40 years. These studies have been largely ignored by Western scientists, but over recent years the situation has dramatically changed. Ecdysteroids show great promise for human medicine, and their potential is now being actively investigated. Current pharmacological interests concern in particular the reduction of fat mass, the protection of lean mass and the prevention of osteoporosis. In addition, experiments are in progress in order to identify the molecular targets of ecdysteroids in mammals.

Ключевые слова: экдистероиды, фармакология, млекопитающие, мышцы, жир, гликемия

Keywords: edysteroid, pharmacology, mammal, muscle, fat, glycemia

Introduction

Phytoecdysteroids are plant secondary metabolites structurally related to insect moulting hormones. Soon after their discovery, their use as potential insect control substances (as endocrine disruptors) was considered, and this led to toxicological studies in order to assess if these molecules were devoid of toxicity on mammals. However, although unrelated to vertebrate steroid hormones, phytoecdysteroids are able to evoke a wide range of pharmacological effects when ingested by mammals/humans. On the other hand, they are devoid of acute toxicity (LD50 > 9 g/kg per os in rats). The first studies were performed in Japan and showed stimulatory effects on protein synthesis by rat hepatocytes [1]. Soon thereafter, their interference with glucose metabolism and their hypoglycemic effects were demonstrated [2]. Further experiments showed a broad range of metabolic effects [3] among which the anabolic effects were particularly highlighted [4] and this led to the inclusion of phytoecdysteroids amongst the «adaptogenic» substances used by high-level sportsmen and bodybuilders for performance improvement [5, 6]. Most of these experiments were undertaken in Uzbekistan, Russia and Ukraine and published in the Russian language, which generated a strong barrier to their accessibility. Over recent years, several review articles have appeared [5 – 11], and active research has started in western countries, which may sometimes duplicate former studies, but which also extends the earlier pharmacological studies by the use of modern molecular methods. The aim of the present review is to describe and discuss these recent studies in three different areas: protein, carbohydrate and lipid metabolism. The second part will be devoted to a discussion of the possible mechanisms involved.

1-Effects on glucose metabolism

Most of the experiments have used 20-hydroxyecdysone (20E). The effect of 20E on glycemia was first tested by *in vivo* experiments using three mice/rat models: (1) hyperglycemia induced by glucagon injection, (2) hyperglycemia induced by alloxan injection (which is toxic for pancreatic beta-cells (the source of insulin) and mimicking type 1 diabetes), and (3) hyperglycemia induced by injection of an anti-insulin antiserum [2]. It was observed that a single injection of 20E (0,1–10 mg/kg) significantly reduced hyperglycemia, and modified several enzyme activities in the liver (which explains this hypoglycemic effect): it stimulated glycogen synthase, reduced glucose 6-phosphatase (hence the ability of liver to release glucose) and stimulated glucose 6-phosphate dehydrogenase, the first step of the pentose pathway (which suggests a higher conversion of glucose into lipids as is also the case upon insulin injection). The

hypoglycemic effects of 20E were then confirmed by many authors, and it was also observed that several plants traditionally used by diabetic people contain significant amounts of ecdysteroids, e.g. *Ajuga iva* [12], *A. turkestanica* [13] or *Achyranthes bidentata* in Japan [14] .

More recently, several experiments performed with in vitro hepatocyte cell cultures allowed more extensive experiments to be performed [15 – 21] [1]. Chen et al. [16] showed that 20E (1-100 μM) increased in a dose- (and time-) -dependent manner glucose consumption by insulin-resistant HepG2 cells, with a maximal effect already observed with 5 μM 20E which is independent of insulin (Fig. 1). This effect is connected with an increase of the glucose transporter Glut-4 activity on hepatocyte membrane, probably resulting from both new enzyme production and enhanced exocytosis of the enzyme bound to endomembranes. In H4IIE hepatoma cells, Kizelsztein et al. [21] have shown that 20E action is mediated by the PI3K/Akt system: 20E stimulates PI3K, which controls the phosphorylation (activation) of Akt, then phospho-Akt stimulates the translocation of Glut-4 to cell membranes, thus enhancing glucose uptake. Simultaneously, 20E reduces the transcription of genes encoding glucose 6-phosphatase and PEPCK (phosphoenolpyruvate carboxykinase), the latter representing a key enzyme of gluconeogenesis. Finally, 20E reduced the release of glucose by hepatocytes stimulated by dexamethasone-cAMP [21]. All these data confirm the great potential of 20E for glycemia regulation as it should also be noted that 20E does not modify the normal glycemia of healthy control animals [2].

Recently, a proteomic study was engaged to identify all the genes, of which the expression is modified in HepG2 cells by 20E treatment [22] and this approach should lead to further understanding of the mechanism of action of 20E. It would certainly be of great interest to have similar data on glucose metabolism in other cell types (myocytes, adipocytes, etc.), as we know that 20E also stimulates glucose use by peripheral tissues [23], and that regular uptake of 20E increases glycogen content not only in liver, but also in heart and skeletal muscles [24].

2-Effects on lipid metabolism

This area is much less well documented than the previous one. The first experiments [2]) analyzed rat serum parameters (triglycerides, free fatty acids and cholesterol); they showed that 20E-treatment did not change triglyceride content. Similarly, the low levels of free fatty acids were not changed in normal animals, but the elevated levels induced by 48-hour starvation or by alloxan-induced diabetes were rapidly lowered by 25–30% after 20E-treatment. On the other hand, daily 20E treatment over 7 days resulted in a significant decrease of cholesterol content of serum and liver, owing to both a reduced synthesis (assessed from *in vivo* ^{14}C-acetate incorporation) and enhanced degradation. Enhanced cholesterol degradation and bile acid excretion was further demonstrated by Syrov et al. [25, 26] in animals previously treated with triton WR1339 (tyloxapol, a non-ionic detergent known to stimulate cholesterol synthesis in rodent liver [27]).

At the whole animal level, it is well established that 20E regular uptake favours the increase of lean mass [28, 29]. These data were further confirmed and extended by recent experiments using a diet-induced obesity model: when given a high-fat diet, mice rapidly became obese, but when simultaneously given 20E (5 or 10 mg/kg per day), they showed a much lower fat mass increase [21, 30, 31]. This effect does not result from a reduction of food intake. Reduced fat mass corresponds to a reduction of adipocyte size and not of their number, as a consequence of their reduced capacity of fatty acid uptake; at the same time, markers of tissue inflammation and the levels of cytokines involved in adipocyte differentiation/growth are much reduced [31]. A similar reduction of adiposity was observed in ovariectomized female rats receiving 20E [32, 33]. Although it is clear that fat mass develop-
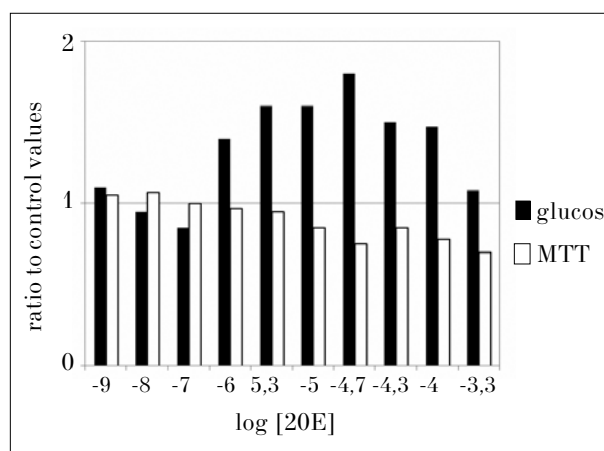

Fig. 1. Effect of various 20E concentrations on glucose consumption by HepG2 liver cells *in vitro* [16] (drawn from the data of Chen et al., 2006a). *Solid bars*: rate of glucose uptake by hepatocytes; *open bars*: proportion of viable cells as measured by the MTT test

Table 1

Effect of a 7-day treatment of mice with 20E (5 mg/kg, daily) on muscle development (mean ± s.e.m.) [4].

Muscle type	Treatment	Muscular mass, mg	Protein content, mg
M. *soleus*	Control	51,5 ± 1,4	9,3 ± 1,1
	20E	55,6 ± 1,8*	11,9 ± 0,9
M. *extensor digitorum longus*	Control	60,7 ± 2,6	10,5 ± 1,5
	20E	67,2 ± 2,4*	15,7 ± 1,1*

Significantly different from control (p < 0,05).

Table 2

Effect of a 7-day treatment of mice with 20E (5 mg/kg, daily) on swimming duration upon exhaustion (mean ± s.e.m., n = 10-12) [4].

Treatment	Body weight, g	Swimming time, % of control
Control	20,3 ± 0,2	100 ± 15
20E	20,5 ± 0,2	180 ± 22*
Training	20,3 ± 0,3	131 ± 20
20E + training	21,8 ± 0,4*	190 ± 31*

Significantly different from control (p < 0,05).

ment is modified upon 20E treatment, the lack of in vitro studies does not allow one to conclude whether this corresponds to a direct effect of ecdysteroids on adipocytes or to a consequence of their effets on other tissues (liver, muscles etc.).

3-Effects on protein synthesis

The stimulatory effect of phytoecdysteroids on protein synthesis is well documented: it corresponds to a general stimulation at the translational level. This effect is rapid and reaches its maximum value 4 hours after in vivo 20E administration in mice, and it is observed with many ecdysteroids [1]. Interestingly, while all tested ecdysteroids (0,5 mg/kg body weight) bearing a 20,22-diol were effective, as well as rubrosterone (an ecdysteroid lacking the side-chain), ecdysone showed no activity. Japanese research mainly focused on protein synthesis in liver, but it was later shown that the stimulation of protein synthesis concerned also muscles. Thus, Chermnykh et al. [4] treated mice by daily intraperitoneal injections of 20E (5 mg/kg) and analyzed the effects on two muscle types, the *soleus* (aerobic) and the *extensor digitorum longus* (anaerobic). After 7 days of treatment, they noticed a significant increase of the weight and protein content of both muscles (Table 1). Moreover, after separation of myofibrils and sarcoplasm, they showed that the increase of protein content concerned only myofibrils. The increase of muscle mass was accompanied by significant improvement of physical performance, which took place even in the absence of training (Table 2). In addition, 20E treatment results in an increase of dietary nitrogen retention, possibly owing to a reduction in protein catabolism [29]; whether this results from a reduction of stress (hence of glucocorticoid plasma levels) remains to be established.

Recent studies with mouse C2C12 myocyte cell lines [34] reported a similar stimulation of protein synthesis *in vitro*: the effect on [3H] leucine incorporation was dose-dependent and rapid (maximum effect was observed after 2 hours exposure) and it required only low (≤ 1 μM) concentrations of 20E (Fig. 2). Several ecdysteroids were tested, among which 20E and turkesterone (11α-hydroxy-20-hydroxyecdysone) were the most active, and semi-purified extracts from ecdysteroid-containing plants (spinach or *Ajuga turkestanica*) were also effective. Similar effect was observed with human skeletal muscle cells treated with 20E. Several pharmacological exper-
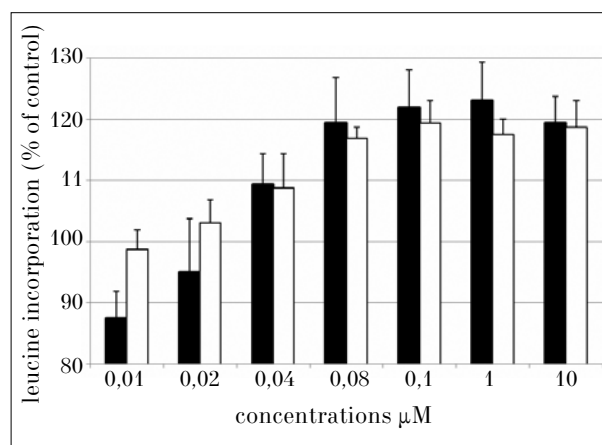

Fig. 2. Effect of two ecdysteroids on [3H]leucine incorporation into proteins by C2C12 myotubes *in vitro* [34]. *Solid bars*: 20-hydroxyecdysone; *open bars*: turkesterone

iments allowed the authors to demonstrate that the effect of 20E involved the PI3K/Akt system and calcium ions, and to propose a model for the mechanism of action involved [35] (Fig. 3). In this model, the membrane receptor(s) of 20E remains to be identified. These recent experiments included also *in vivo* studies. Gorelick-Feldman et al. [34] found an increase of rat grip strength by 18-24 % (p < 0,05) after 28-day treatment with 20E or an equivalent spinach extract.

Tóth et al. [36] used another approach to the problem by using male Wistar rats which received subcutaneous daily injections of 20E (5 mg/kg bwt) in the left thigh over 8 days. At the end of the experiment, the treated animals showed significant (p < 0,001) weight increase as compared to controls, the *soleus* and *extensor digitorum longus* muscles were significantly enlarged on both sides. The *soleus* muscle contains two fibre types, I and IIa, and the cross-sectional area of both types was significantly enlarged (Fig. 4). The situation was less clear-cut in the case of *extensor digitorum longus* muscle, which contains four fibre types, where types IIB and IIx predominate. Moreover, 20E increased the number of fibre nuclei, which suggests an acti-

vation of satellite cells. The same authors used also a model of regenerating muscle [37] where snake toxin (notexin, neurotoxic and myotoxic phospholipase A2 isolated from the venom of the Australian tiger snake, *Notechis scutatus*) is injected in the muscle, and this allowed them to show that 20E-treatment increases the growth rate of regenerating *soleus*, but also that the presence of a regenerating muscle modifies the response of the other muscles to 20E treatment.

From the above data, it is clear that 20E and related molecules efficiently promote muscle development and increase physical performance and endurance. In this respect they might be considered as anabolic substances. However they differ from the classical muscle promoting steroids, as they do not interfere with androgen receptors (see Section 5). Several ecdysteroid-containing medicinal plants are indeed used for improving general stamina, such as *Rhaponticum carthamoides* (Russia and Eastern Europe) and *Pfaffia paniculata* (Brazil), and many ecdyster-oid-containing preparations are available for increasing muscle mass of bodybuilders. The protein synthesis promoting ability of ecdys-teroids also makes them attractive for medical
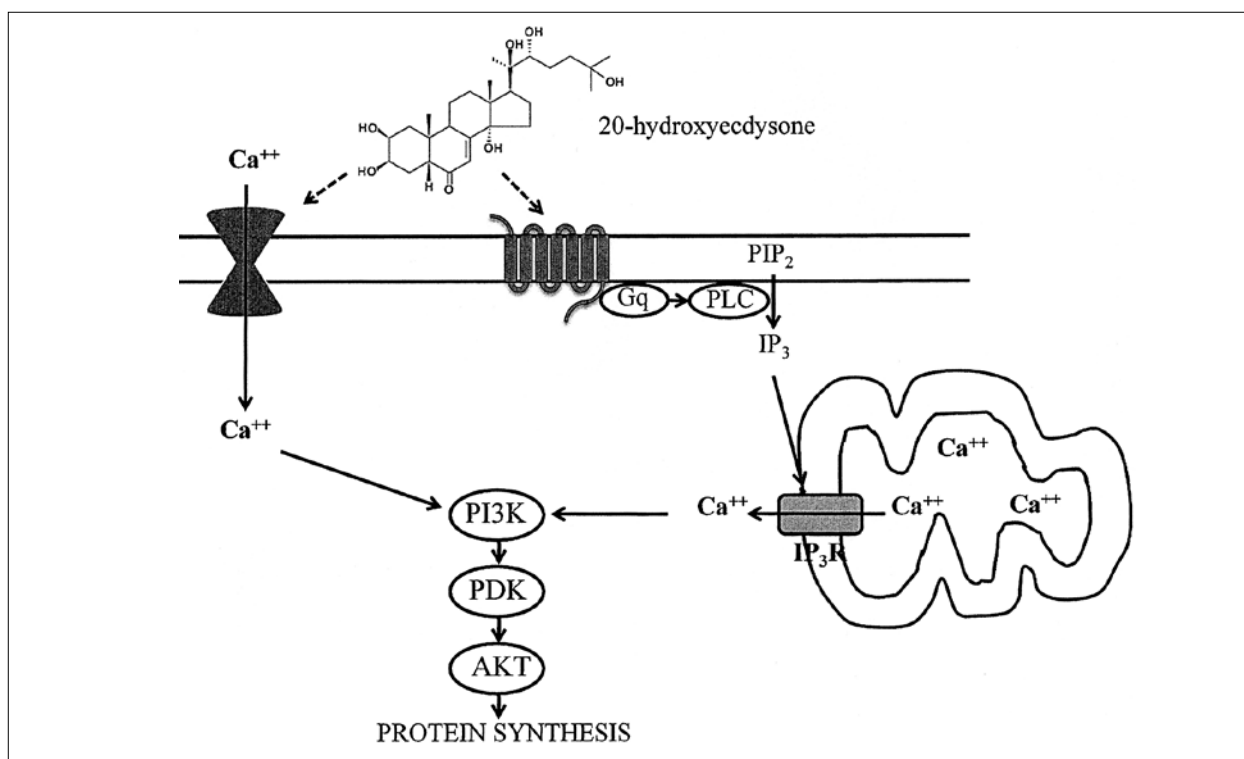

Fig. 3. Proposed mechanisms for the stimulation of protein synthesis by 20E (redrawn from [35], modified). 20E produces an increase of free Ca^{2+} originating both from internal stores (through the action of a membrane receptor coupled to a G protein that activates PLC) and from the extracellular space (through a calcium channel), and this activates the PI3K/AKT system, which finally results in a stimulation of protein synthesis.
AKT = protein kinase B (PKB); IP3 = inositol triphosphate; IP3R: receptor of IP3; PI3K = Phosphatidylinositol-3 Kinase; PDK = protein serine/threonine kinase 3'-Phosphoinositide-Dependent Kinase; PLC = Phospholipase C

applications, e.g. for preventing the age-related decline of muscle mass (sarcopenia).

4-Effects on osteoporosis

Syrov et al. [38] observed that the treatment of rats (5 mg/kg, orally) after an experimental bone fracture was able to accelerate healing processes. This observation received little attention until recently, where several laboratories engaged in detailed studies of ecdysteroid effects on bone metabolism and particularly in the context of osteoporosis connected with ageing [32, 33, 39 – 42].

The group of Prof. Wuttke in Göttingen developed a model of ovariectomized female rat which mimics women's menopause, which is classically accompanied by osteoporosis and is often treated by hormonal substitution, which is not devoid of unwanted side-effects. They first analyzed the effects of a plant (*Tinospora cordifolia*) extract over 4 weeks and observed significant osteoprotective effect, resembling that of estrogens on bones, but without any effect on uterus and mammary glands. Analysis of the active ingredients in this extract allowed the isolation of 20E, and further experiments were made with this molecule. Daily treatment of ovariectomized rats with 20E (18-116 mg/animal) showed its strong anti-osteoporotic activity, which was independent of the estrogen receptor (it did not increase uterus weight) [33, 40]. Similar results were obtained by Dong et al. [41, 42] and He [43] using a 20E-containing extract from *Achyranthes bidentata*, another medicinal plant. Gao et al. [44] and Dong et al. [42] showed that 20E accelerates the proliferation of

bone marrow mesenchymal stem cells, and Gao et al. [45] showed that 20E induces osteogenic differentiation in the same cells, thus taken together these data provide a rational explanation for the efficient effect of 20E.

5-General mechanisms of signalling

From the above data, it is clear that ecdysteroids display a lot of pharmacological effects, and the list above is far from complete, as it could include many other areas [5]. Such pleiotropic effects are not unique, and other examples are known of molecules involved in plenty of physiological processes, as e.g. vitamin D_3. The metabolic fate of 20E is not fully known at the moment, but is is already established that this molecule undergoes a complex set of metabolic conversions, which might explain in part the diversity of its *in vivo* effects. On the other hand, the rapid effects of 20E observed in several *in vitro* systems are consistent with the direct activity of the unconverted molecule. Nevertheless, it would make sense to check the metabolism of the applied ecdysteroid either in vivo or in vitro in order to allow definitive conclusions to be drawn.

It is generally considered that 20E does not bind vertebrate hormone nuclear receptors [6, 32 – 34]. This applies mainly for the receptors of sex steroid hormones (estradiol, testosterone) and for the glucocorticoid receptor. In fact this situation is not so clearcut for the androgen receptor [6], and this does not necessarily apply to all 20E metabolites. When performing SAR studies, Báthori et al. [6] noticed significant binding of 20E and polypodine B (5ß-OH 20E) to
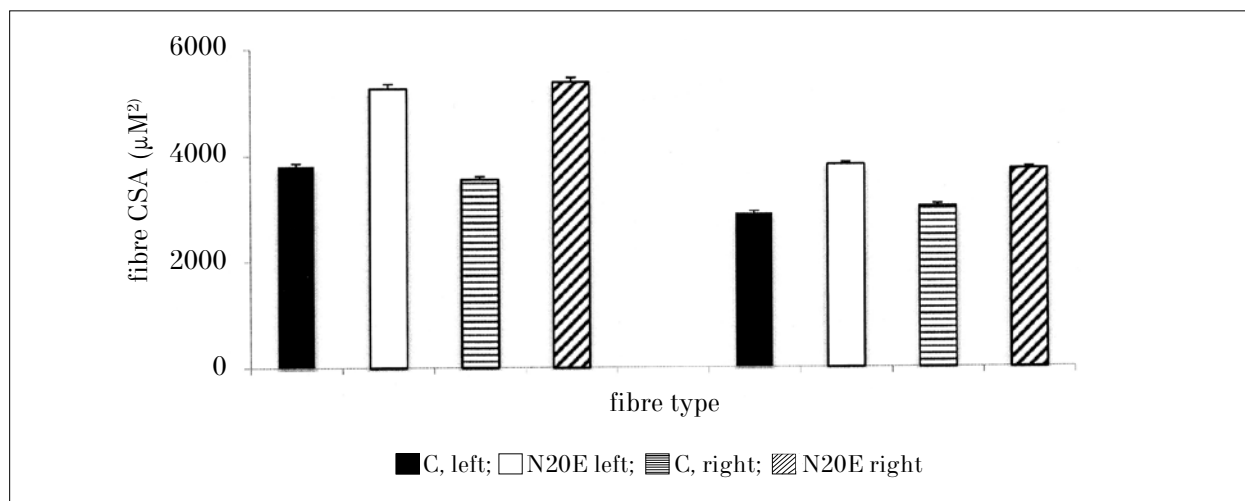

Fig. 4. Effect of 20E treatment on fibre size n the muscle soleus.
C: control animals; N20E: animals treated by 20E injections in the left thigh. Left and right muscles were analyzed at the end of the treatment and fibre cross-sectional area (CSA) was measured for each fibre type (I and IIa) [36]
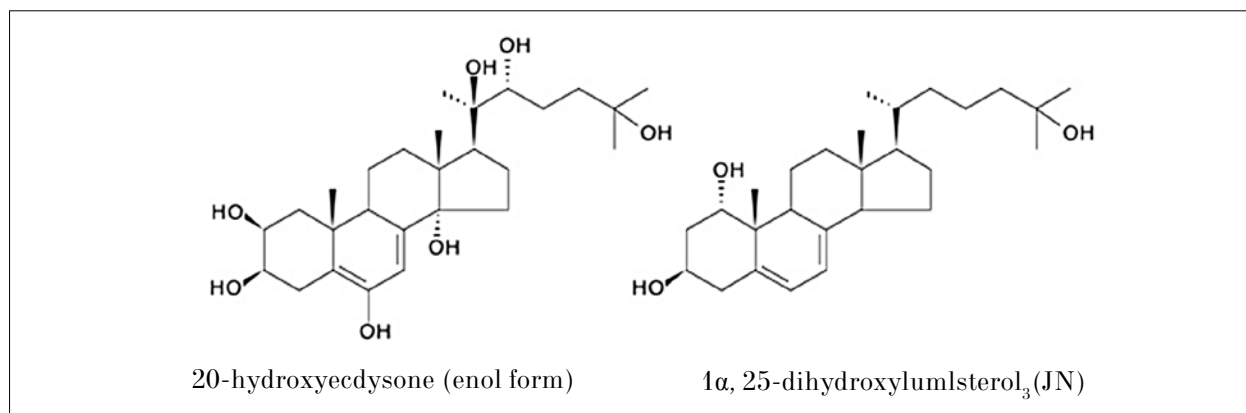
20-hydroxyecdysone (enol form)   1α, 25-dihydroxylumlsterol$_3$(JN)

Fig. 5. Structure comparison of lumisterol, a specific ligand of the membrane vitamin D receptor and of 20E in its enol form

the androgen receptor, and a side-chain cleavage product (9,11-didehydropoststerone) showed an even higher binding affinity ($K_i = 5,15\ 10^{-7}$ M); this finding may be relevant when considering that one of the metabolic conversions of 20E in mice is the side-chain cleavage between C20-C22, leading to poststerone. Of course there are many more nuclear receptors involved in general metabolic regulations, some of which bind bile acids or a wide array of steroids (e.g. FXR, PXR), and others for which the ligands are still unknown (e.g. RORα), and it would be worthwhile to test their ability to bind 20E (or any of its metabolites).

It is now clearly established that steroids may have rapid actions on cell membranes, and this has been demonstrated for many of them, e.g. vitamin D$_3$ [46]. The ligand specificity of these receptors differs from that of the corresponding nuclear receptors. A striking parallel has been regularly made between the effects of 20E and those of calcitriol [1,25-(OH)D$_3$] [11, 47, 48], and it was proposed recently that 20E could bind to the non-genomic (membrane) receptor of vitamin D [11, 49]. This assumption is based on in silico docking studies [49] showing possible binding of 20E in its enol form (Fig. 5) to this receptor.

After binding to its putative membrane receptor(s), 20E activates/modulates a complex set of secondary messengers; as those are strongly interconnected, recorded changes concern both cyclic nucleotides, phosphoinositides, calcium ions and it is therefore difficult at the moment to decide which system is the primary target of 20E. At a later step, it is now clear that the PI3K/Akt system is involved, as previously proposed [5, 50] and confirmed by all the data presented above. The PI3K/Akt system represents a key regulator of cellular activity [50].

Conclusions

There is a growing interest in the pharmacological effects of ecdysteroids on mammals. Their interest is not restricted to the areas described above, and it includes also anti-ageing properties, cosmetic uses, radioprotection etc., in fact the whole long list is impressive. This is illustrated by the list of recent patents included after the bibliography of this article.

Recent experiments have confirmed «old» data from Japan, Uzbekistan, Russia and Ukraine, including the similarity of the effects with those of vitamin D. These new experiments take advantage of recently available molecular tools and should allow a better understanding of the mechanisms of action of 20E and its analogues or metabolites. This could provide the basis for a forthcoming use of ecdysteroids in mammalian or human medicine.

The earlier studies suffered from a strong language barrier and the difficulty of accessing literature published in the Russian language, even though they represent more than 30 years of active research. More recently, this situation is being replicated with the proliferation of scientific articles published in Chinese, so we may hope that a systematic translation system will make those data more readily available to the scientific community.

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