Ecdysteroids: A novel class of anabolic agents?

AUTHORS: Parr MK[1], Botrè F[2], Naß A[1], Hengevoss J[3], Diel P[3], Wolber G[1]

[1] Institute of Pharmaceutical and Medicinal Chemistry, Department of Biology, Chemistry, Pharmacy, Freie Universität Berlin, Germany
[2] Laboratorio Antidoping, Federazione Medico Sportiva Italiana, Rome, Italy
[3] Department of Cellular and Molecular Sports Medicine, Institute of Cardiovascular Research and Sports Medicine, German Sport University Cologne, Germany

ABSTRACT: Increasing numbers of dietary supplements with ecdysteroids are marketed as "natural anabolic agents". Results of recent studies suggested that their anabolic effect is mediated by estrogen receptor (ER) binding. Within this study the anabolic potency of ecdysterone was compared to well characterized anabolic substances. Effects on the fiber sizes of the soleus muscle in rats as well the diameter of C2C12 derived myotubes were used as biological readouts. Ecdysterone exhibited a strong hypertrophic effect on the fiber size of rat soleus muscle that was found even stronger compared to the test compounds metandienone (dianabol), estradienedione (trenbolox), and SARM S 1, all administered in the same dose (5 mg/kg body weight, for 21 days). In C2C12 myotubes ecdysterone (1 μM) induced a significant increase of the diameter comparable to dihydrotestosterone (1 μM) and IGF 1 (1.3 nM). Molecular docking experiments supported the ERβ mediated action of ecdysterone. To clarify its status in sports, ecdysterone should be considered to be included in the class "S1.2 Other Anabolic Agents" of the list of prohibited substances of the World Anti-Doping Agency.

CITATION: Parr MK, Botrè F, Naß A, Hengevoss J, Diel P, Wolber G. Ecdysteroids: A novel class of anabolic agents? Biol Sport. 2015;32(2):169–173.

Received: 2015-02-09; Reviewed: 2015-02-23; Re-submitted: 2015-02-24; Accepted: 2015-02-27; Published: 2015-03-15.

Corresponding author:
Maria Kristina Parr
Freie Universität Berlin
Institute of Pharmacy
Königin-Luise-Str. 2+4
14195 Berlin
Germany
fon +49(0)30 838 57 686
fax +49(0)30 838 457 686
E-mail: maria.parr@fu-berlin.de

Key words:
ecdysteroids
doping in sport
anabolic effect
estrogen receptor beta
beta-ecdysone
muscle hypertrophy

INTRODUCTION

Ecdysteroids are widely marketed to athletes as dietary supplement, advertising to increase strength and muscle mass during resistance training, to reduce fatigue and to ease recovery. Several studies have reported a wide range of pharmacological effects of ecdysteroids in mammals, most of them beneficial to the organism. In the 1980s the most active phytoecdysteroid, ecdysterone (beta-ecdysone, a "Russian secret"), was suspected to be used by Russian Olympic athletes. The levels of ecdysteroids in western diet are generally low (usually in the range of less than 1 mg·day^{-1}), while the doses used by bodybuilders are stated in a range of up to 1000 mg·day^{-1}.

Extensive investigations on the possible growth-promoting effects of ecdysterone in various animal species (rats, mice, Japanese quail and cattle) and in humans [1-13] were reported and lots of rumors on its misuse by athletes are circulating since then. Ecdysterone has been demonstrated to increase protein synthesis in skeletal muscle [14]. Gorelick et al. proposed direct or indirect stimulation of the PI3K/Akt signaling pathway as mechanism for this increased protein synthesis [9, 15].

Conversely to anabolic-androgenic steroids (AAS) that increase muscle mass mainly through their binding to the androgen receptor (AR), no nuclear receptor that is homologous to the ecdysone nuclear receptor found in insects has yet been described in mammals so far [9]. Only recently, binding of ecdysterone to the human ERβ (ED50 = 13 nM) could be shown in cell culture experiments and induction of hypertrophy in C2C12 cells was shown to be mediated by the ERβ activation [2]. The aim of this study was to elucidate the anabolic potency of ecdysterone in comparison to other known anabolic agents and to support the hypothesis of ERβ mediated action by *in-silico* modelling.

MATERIALS AND METHODS

In-Vitro Hypertrophy Model. The anabolic properties of ecdysterone were tested by incubation of C2C12 derived myotubes with the test compounds and determination of diameters of 47 myotubes per group (mean of measurements every 10–20 μm along the myotube) as described before [2]. Concentrations applied in this study were 1 μM for ecdysterone and dihydrotestosterone (chemical structures in Figure 1) and 1.3 nM for the growth factor IGF-1.

Animal Study

Male Wistar rats (n=42, Janvier, Le-Genest St-Isle, France) were randomly allocated to verum and control groups. The animals were

kept under controlled conditions (T=20 ± 1°C, relative humidity Φ=50–80%, 12-h light/12-h dark) with free access to standard diet (SSniff GmbH, Soest, Germany) and water. To mimic the situation in athletes, intact (i.e. non-castrated) animals were treated with 5 mg·kg^{-1} body weight of the respective substance once daily for 21 days. Verum groups received injections of ecdysterone, metandienone, estradienedione, or S-1, each diluted in a solution of 20% DMSO and 80% peanut oil. The control groups were injected with vehicle only. The animals were handled in compliance with accepted veterinary medical practice and with the approval of the Animal Welfare Committee. Further experimental details were performed as described earlier [2].

The anabolic potency of ecdysterone was determined using the muscle fiber size of the soleus muscle of male Wistar rats as measure. The effect was compared to the anabolic androgenic steroids metandienone (dianabol) and estradienedione (trenbolox) as well as the selective androgen receptor modulator S-1 (chemical structures in Figure 1).

In-silico Modeling of Steroid Receptor Binding

Molecular modeling experiments were conducted to support the data that the anabolic effect of ecdysterone is mediated by ERβ binding, rather than AR, as known for classical anabolic androgenic steroids. Thus, ecdysterone was docked into crystal structures of the two subtypes ERα and ERβ as well as the AR (PDB entries 3UUD [16] for ERα, 3OLL [17] for ERβ, and 2AM9 for AR [18], respectively). These crystal structures represent complexes with estradiol for the ER and testosterone for the AR to make sure they represent the protein conformation relevant for agonism. The docking experiment was performed using the software GOLD [19] allowing side chain flexibility in the binding regions to allow adaptions to the significantly larger ligand ecdysterone. Binding poses were analyzed for key interaction features using the 3D pharmacophore modeling platform LigandScout [20, 21].

Statistics

Statistical data evaluation was performed by Kruskal–Wallis test followed by pair-wise comparison with the Mann–Whitney U-test. Box and whisker plots in Figure 2 and Figure 3 represent minimum, 25th, 50th (median), 75th percentile, and maximum of the distribution. Significance level was established at $p \leq 0.05$.

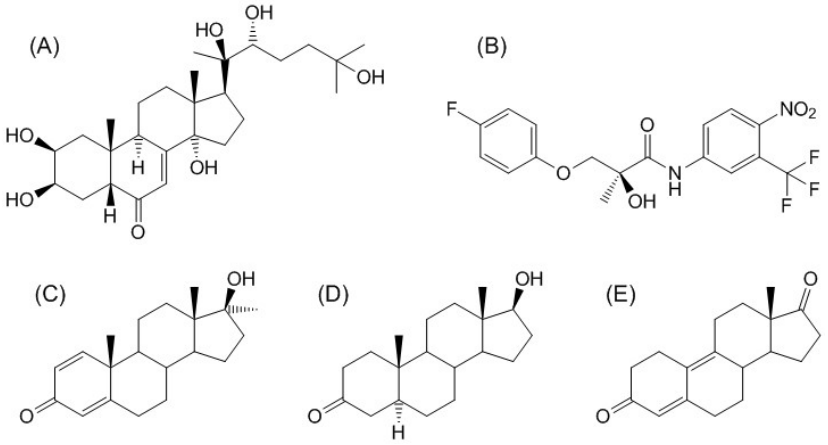

FIG. 1. Chemical structure of ecdysterone (A) as well as of the other anabolic agents used for comparison (B: selective androgen receptor modulator (SARM) S-1, C: anabolic androgenic steroid (AAS) metandienone, D: AAS dihydrotestosterone, E: AAS estradienedione)

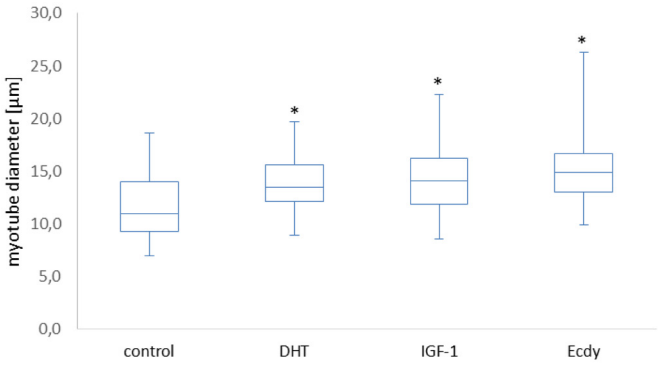

FIG. 2. Effects of DHT (10^{-6} M), IGF-1 (10 ng·mL^{-1}), and ecdysterone (Ecdy, 10^{-6} M) on the diameter of C2C12 myotubes. Determination of diameters of 47 myotubes per group.
No significant differences within treatment groups, *significant versus control, $p \leq 0.05$ by Kruskal–Wallis H-test and Mann–Whitney U-test

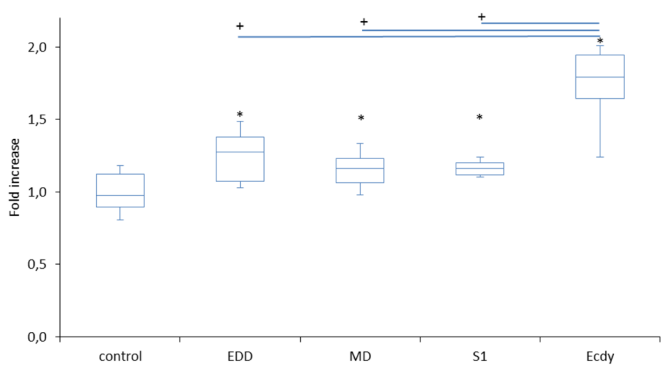

FIG. 3. Anabolic effect of ecdysterone (Ecdy) expressed as fiber size of soleus muscle in intact rats.
Significantly higher increase of fiber size of soleus muscle after Ecdy compared to estradienedione (EDD), metandienone (MD) and SARM S 1 (S1), *significant versus control, + significant versus Ecdy, $p \leq 0.05$ by Kruskal–Wallis H-test and Mann–Whitney U-test

RESULTS

Determination of Anabolic Properties. C2C12 derived myotubes were used as *in-vitro* model for testing anabolic activities. Incubation with ecdysterone showed significantly increased myotube diameters compared to vehicle treated control cells (Figure 2). Comparing the effect with the endogenous anabolic androgenic steroid dihydrotestosterone at the same concentration and the anabolic growth factor IGF-1 (concentration for comparison was 1.3 nM) a slightly higher (not statistically significant) effect was observed.

To mimic the situation in athletes, intact male Wistar rats were treated with the test components for 21 days. Significantly increased fiber sizes of the soleus muscle were determined following treatment with ecdysterone. Comparison with the animals treated with the anabolic androgenic steroids metandienone or estradienedione and the SARM S-1 yielded a significantly higher effect in the ecdysterone treated animals when the same doses were applied (Figure 3).

In-silico Investigation of Steroid Receptor Binding

To underline the plausibility of the proposed ERβ mediated anabolic mechanism, ecdysterone molecular docking experiments into the human sex steroid receptors were conducted to derive mechanistic insights into ecdysterone binding.

Compared to the x-ray structure of the AR/testosterone complex, *in-silico* molecular docking of ecdysterone in the AR revealed an

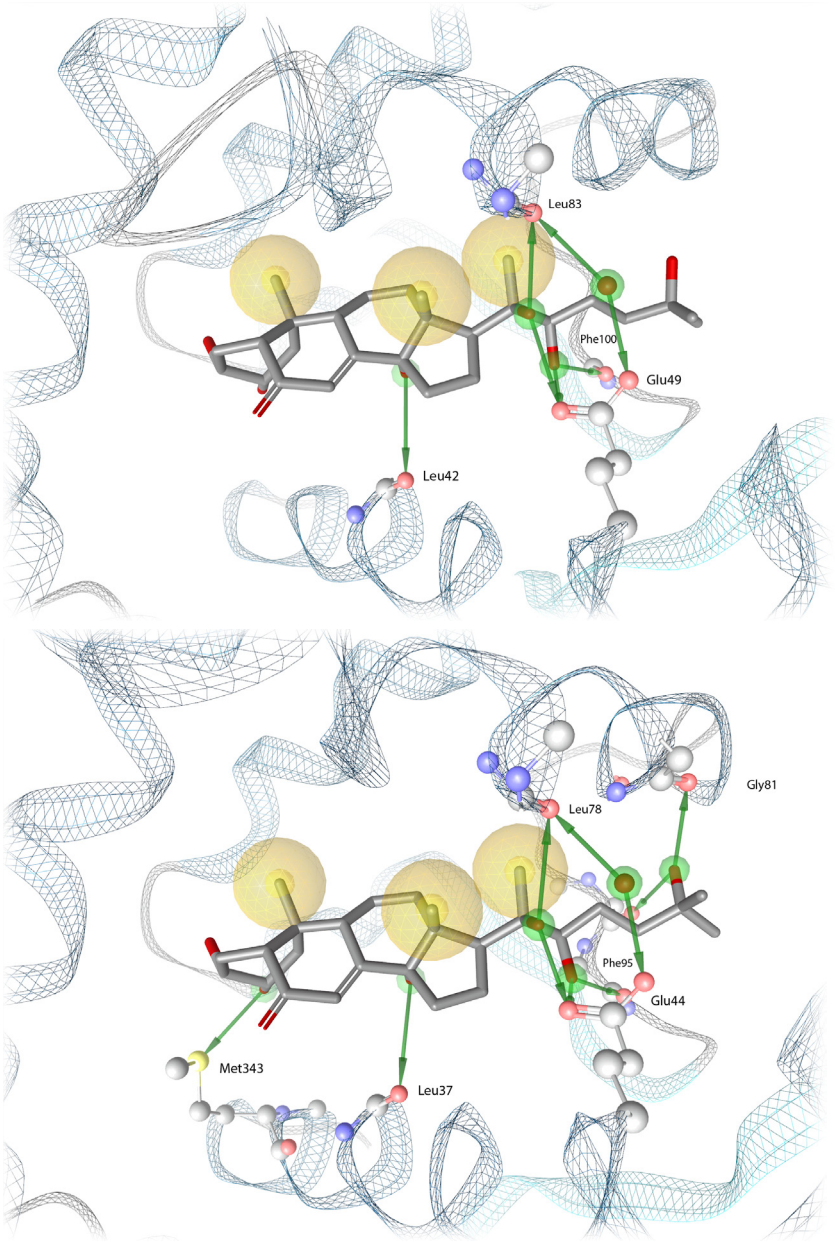

FIG. 4. ERα (PDB 3UUD [16], top) and ERβ (PDB 3OLL[17], bottom) with in-silico docked ecdysterone.
ERβ forms additional hydrogen bonds with Met343 to a hydroxyl group of ecdysterone and with the protein backbone to the terminal alkyhydroxyl group. Arrows indicate hydrogen bonds (green: donor, red: acceptor), while yellow spheres show hydrophobic interactions. Residue numbering was chosen according to the used PDB entries.

unfavorable binding pose and a completely different three dimensional orientation in the AR/ecdysterone complex: At those positions where methyl groups are found for the endogenous ligand testosterone, hydroxyl groups sterically interfere with the receptor in case of ecdysterone. In addition, a water molecule mediating important interaction in the testosterone complex is replaced by a methyl group for the ecdysterone docking pose resulting in an unfavorable interaction pattern.

In contrast, both ER isoforms offer a binding side shape more suitable to accommodate ecdysterone. Docking poses in both subtypes share several interactions shown in Figure 4. Three additional hydrogen bonds are exclusively formed in the docking pose of ecdysterone in ERβ, which may explain the experimentally observed isoform preference [2, 22]: In ERβ the ligand can adapt a conformation close enough to Met343 and form a hydrogen bond with the hydroxyl group in position 2 of ecdysterone. Additionally, two more hydrogen bonds with the terminal hydroxyl group to the backbone of the β-subtype (Leu346, Phe404 and Leu387, respectively) could be observed.

DISCUSSION

The data reported in here demonstrate that ecdysterone induces hypertrophy of muscles with a comparable or even higher potency as shown for anabolic androgenic steroids, SARMs or IGF-1. Analogous findings were also reported by Syrov *et al.* [14]. They reported increased body weight gain and weight as well as increased protein content of the tibialis muscle in rats after ecdysterone administration (5 mg·kg^{-1} BW, orally, for 10 days).

The generated docking poses support the hypothesis that ecdysterone shows no significant binding at the AR, but to ER with preference to the ERβ subtype.

CONCLUSIONS

An anabolic activity of ecdysterone was clearly confirmed by our investigation. The anabolic potency of the ecdysterone was comparable or even higher as found for the anabolic androgenic steroids, SARMs or IGF-1. Moreover *in-silico* docking experiments support the postulated non-androgenic mechanism of ecdysterone. More likely and in agreement to the experimental data anabolic activity of ecdysterone is mediated via binding to the ER particularly ER beta. With respect to doping prevention the high anabolic potency of ecdysterone justifies its classification as an anabolic agent and therefore needs to be listed in the category "S1 Anabolic Agents" of the list of prohibited substances of the World Anti-Doping Agency

Conflict of interests: the authors declared no conflict of interests regarding the publication of this manuscript.

REFERENCES

1. Haupt O, Tchoukouegno Ngueu S, Diel P, Parr M. Anabolic effect of ecdysterone results in hypertrophy of C2C12 myotubes by an estrogen receptor mediated pathway. In: Schänzer W, Geyer H, Gotzmann A, Mareck U (eds). Recent Advances in Dope Analysis (20). Sport und Buch Strauß: Cologne, 2012.
2. Parr MK, Zhao P, Haupt O, Ngueu ST, Hengevoss J, Fritzemeier KH, Piechotta M, Schlörer N, Muhn P, Zheng WY, Xie MY, Diel P.. Estrogen receptor beta is involved in skeletal muscle hypertrophy induced by the phytoecdysteroid ecdysterone. Mol Nutr Food Res. 2014;58:1861-1872.
3. Wilborn CD, Taylor LW, Campbell BI, Kerksick C, Rasmussen CJ, Greenwood M, Kreider RB.Effects of methoxyisoflavone, ecdysterone, and sulfo-polysaccharide supplementation on training adaptations in resistance-trained males. J Int Soc Sports Nutr. 2006;3:19-27.
4. Zwetsloot KA, Shanely AR, Merritt EK, McBride JM. Phytoecdysteroids: a novel, non-androgenic alternative for muscle health and performance. J Steroids Horm Sci. 2013; s12: 10-12.
5. Dinan L. The Karlson Lecture. Phytoecdysteroids: what use are they? Arch Insect Biochem Physiol. 2009;72:126-141.
6. Dinan L, Lafont R. Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals. J Endocrinol. 2006;191:1-8.
7. Lafont R, Dinan L. Practical uses for ecdysteroids in mammals including humans: an update. J Insect Sci. 2003;3:7.
8. Courtheyn D, Le Bizec B, Brambilla G, De Brabander HF, Cobbaert E, Van de Wiele M, Vercammena J, De Waschd K. Recent developments in the use and abuse of growth promoters. Anal Chim Acta. 2002;473:71-82.
9. Gorelick-Feldman J, Maclean D, Ilic N, Poulev A, Lila MA, Cheng D, Raskin I. Phytoecdysteroids increase protein synthesis in skeletal muscle cells. J Agric Food Chem. 2008;56: 3532-3537.
10. Toth N, Szabo A, Kacsala P, Heger J, Zador E. 20-Hydroxyecdysone increases fiber size in a muscle-specific fashion in rat. Phytomedicine. 2008;15:691-698.
11. Bathori M, Toth N, Hunyadi A, Marki A, Zador E. Phytoecdysteroids and anabolic-androgenic steroids-structure and effects on humans. Curr Med Chem. 2008;15:75-91.
12. Slama K, Koudela K, Tenora J, Mathova A. Insect hormones in vertebrates: anabolic effects of 20-hydroxyecdysone in Japanese quail. Experientia. 1996;52:702-706.
13. Slama K, Kodkoua M. Insect hormones and bioanalogues: their effect on respiratory metabolism in Dermestes vulpinus L. (Coleoptera). Biol Bull. 1975;148:320-332.
14. Syrov VN. Comparative experimental investigation of the anabolic activity of phytoecdysteroids and steranabols. Pharm Chem J. 2000;34:193-197.
15. Gorelick-Feldman J, Cohick W, Raskin I. Ecdysteroids elicit a rapid Ca2+ flux leading to Akt activation and increased protein synthesis in skeletal muscle cells. Steroids. 2010;75:632-637.
16. Delfosse V, Grimaldi M, Pons JL, Boulahtouf A, le Maire A, Cavailles V, Labesse G, Bourguet W, Balaguer P. Structural and mechanistic insights into bisphenols action provide guidelines for risk assessment and discovery of bisphenol A substitutes. Proc Natl Acad Sci USA. 2012;109:14930-14935.
17. Möcklinghoff S, Rose R, Carraz M, Visser A, Ottmann C, Brunsveld L. Synthesis and crystal structure of a phosphorylated estrogen receptor ligand binding domain. Chembiochem. 2010;11:2251-4.
18. Pereira de Jésus-Tran K, Côté P-L, Cantin L, Blanchet J, Labrie F, Breton R. Comparison of crystal structures of human androgen receptor ligand-binding domain complexed with various agonists

reveals molecular determinants responsible for binding affinity. Protein Sci. 2006;15:987-999.
19. Verdonk ML, Cole JC, Hartshorn MJ, Murray CW, Taylor RD. Improved protein–ligand docking using GOLD. Proteins. 2003;52:609-623.
20. Wolber G, Langer T. LigandScout: 3-D pharmacophores derived from protein-bound ligands and their use as virtual screening filters. J Chem Inf Model. 2004;45:160-169.
21. Seidel T, Ibis G, Bendix F, Wolber G. Strategies for 3D pharmacophore-based virtual screening. Drug Discov Today Technol. 2010;7:e221-e228.
22. Parr MK, Haupt O, Ngueu ST, Fritzemeier K-H, Muhn P, Diel PR. Estrogen Receptor Beta Mediated Anabolic Effects - Insights from Mechanistic Studies on the Phytoecdysteroid Ecdysterone and Selective Ligands. Endocr Rev. 2013:SAT-340-SAT-340.