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In vitro assays for bioactivity-guided isolation of endocrine active compounds in *Vitex agnus-castus*

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Abstract

Introduction: Extracts of *Vitex agnus-castus* (VAC) are used for the treatment of premenstrual symptoms. The mechanism of action was proposed to be dopaminergic and estrogenic in nature. To isolate these endocrinologically active substances, receptor ligand binding assays and cell cultures were used as *in vitro* systems to monitor the bioactivity-guided chromatographic separation.
Methods: Estrogen receptor (ER) and Dopamine D_2-receptor ligand binding assays were performed with human recombinant receptor protein. Both known ERs (ERα and ERβ) were used. Cultures of anterior pituitary cells were prepared from 3 months old female rats. The concentrations of prolactin (PRL) and of cAMP in the supernatants were measured by radio immunoassay (RIA). Starting material for the isolation of single constituents was the aqueous ethanol 70% (v/v) VAC extract BNO 1095. The fractionations were performed with high speed counter current or Sephadex-LH-20 chromatography. Structure elucidation was conducted with GC–MS, LC–MS, ^{1}H NMR and ^{13}C NMR.
Results: The bio-guided fractionation of BNO 1095 resulted in the isolation of dopaminergic bicyclic diterpenes. The fraction with the highest dopaminergic activity was a mixture of diterpenes of the clerodane type, designated as "BNO-diterpenes". These newly isolated diterpenes inhibited cAMP formation and PRL-release in rat pituitary cell cultures. The estrogenic compounds in BNO 1095 were identified as the flavonoids penduletin and apigenin. Both substances are specific ligands for the ERβ.
Conclusion: Using the strategy of bioactivity-guided fractionation we were able to isolate new dopaminergic diterpenes with a high specific activity which contribute significantly to the PRL-lowering activity of VAC. The therapeutical potential of the ERβ specific ligands penduletin and apigenin needs further investigation.

Keywords: Cell culture; Binding assays; Prolactin; Dopamine; Estrogen

1. Introduction

The term "premenstrual syndrome (PMS)" subsumes various symptoms that occur during the 7–10 days before menstruation and disappear a few hours

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after the onset of menstruation. A prevalent symptom is mastodynia, which is most likely due to a latent hyperprolactinemia, i.e. PMS-patients respond to stressful situations and during deep sleep phases with a hypersecretion of prolactin (PRL) which causes an unphysiological stimulation of the mammary gland [1]. PRL secretion from the lactotrophic cells of the anterior pituitary is controlled by a yet unknown Prolactin-Releasing Factor (PRF) and by the catecholamine dopamine (DA) which is the physiological PRL-Inhibiting Factor (PIF). Both factors are released from the hypothalamus into the portal vessels of the pituitary stalk [2].

Because of the PRL inhibiting properties, dopaminergic drugs are an efficient treatment to normalize the latent hyperprolactinemia associated with PMS [3]. As proven in several clinical studies, premenstrual symptoms, in particular premenstrual mastodynia are also beneficially influenced by extracts of the fruits of chaste tree (*Vitex agnus-castus* = VAC) [4,5] From *in vitro* experiments with rat anterior pituitary cell cultures and dopamine receptor binding assays with membranes from rat striatum it is known, that VAC contains a dopaminergic principle, which is relevant for the amelioration of premenstrual symptoms [6].

In addition to this dopaminergic principle, there is recent evidence, that VAC appears to contain also estrogen-like compounds, i.e. phytoestrogens. This assumption is based on cell culture data demonstrating an estrogenic action of a VAC extract on steroid release in cultures of human granulosa cells [7] and results by Dixon-Shanies and Shaikh [8] who reported inhibitory effects of phytoestrogens and herbal extracts including VAC on the growth of human breast cancer cells.

To investigate dopaminergic or estrogenic activity of a test compound or an herbal extract well-established *in vivo* models, primarily performed with rodents, are available. For example, the suppression of stress induced PRL release in intact male rats is a suitable approach to assess dopaminergic activity [9]. The standard test for measurement of estrogenic activity is the rodent uterotrophic assay. Following ovariectomy, the uterus of an adult rat becomes atrophic. Application of an estrogenic compound reverses this massive loss of uterus weight [10].

Undoubtedly, *in vivo* experiments with laboratory animals are an excellent experimental tool to describe the over all pharmacological/endocrine activity of an extract, however, this approach has limitations if it is intended to be used as a monitoring system to isolate active compounds from herbal extracts. Animal studies are: (a) time consuming, (b) expensive, (c) they raise ethical issues, (d) the observed effects cannot be unequivocally attributed to a specific effect directly exerted on the target organ/-tissue/-cell. For example, the PRL-reducing effect of VAC described *in vivo* may be due to a direct action of compounds on the lactotropic cells of the pituitary or it may result from an alteration of hypothalamic DA- or PRF release. Both modes of action will result in lowered PRL serum levels.

To overcome these obstacles we used following *in vitro* systems in order to isolate both types of endocrine active principles in VAC: the dopamine D_2-receptor ligand binding assay (DA-LBA) and the PRL release from primary cultures of rat pituitary cells were employed to isolate the dopaminergic principle in VAC. An estrogen-like action of a phytoestrogen requires binding to estrogen receptors (ER). A well-established method to study the binding of a test compound to the ER is the ER ligand binding assay (ER-LBA) with a cytosol preparation obtained from the uterus. Since the pioneering work of Kuiper et al. [11], discovering a second type of ER called ERβ, the knowledge about steroid receptor biology has considerably extended. The formerly known estrogen receptor was renamed ERα. Uterus cytosol contains at least both types of ER plus the so called Estrogen receptor related receptors [12]. To investigate whether an extract of VAC and compounds contained therein bind to ER and to decipher whether the putative estrogenic compounds bind to both or only to one subtype of subtype of ER receptor, ligand binding assays were performed with recombinant human ERα or ERβ to monitor the bioactivity-guided fractionation.

2. Materials and methods

2.1. Chemicals

The tracers ^{125}I-estradiol and ^{125}I-sulpiride were supplied by NEN (Dreieich, Germany). Recombinant human ERα and ERβ were obtained from Panvera (Madison, USA), recombinant human D_2-receptors