DISSERTATION

Titel der Dissertation

„Optimization of a Fluorimetric Assay for the Search for Histone Deacetylase Modulators from Plant Origin and its Application in Bioactivity-Guided Isolation“

Verfasserin

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A Introduction

A.1 Structure and Function of Eukaryotic Chromatin

The major function of DNA is to carry genes. DNA interacts with proteins that fold and pack the molecule into a compact structure in the nucleus. The genome of eukaryotes is structured in chromosomes, a dynamic complex of DNA and proteins. In addition to the proteins involved in packaging DNA, chromosomes are also associated with many proteins required for the processes of gene expression, DNA replication and DNA repair. The proteins binding to DNA to associate eukaryotic chromosomes are divided into two classes, the histone and the nonhistone chromosomal proteins. The compression administrated by both classes of proteins with DNA is called chromatin. Histones are present in high quantities so that their total mass is almost equal to the mass of DNA.

Histones are responsible for the basic level of chromosome organization, the nucleosome. A nucleosome is built by a histone octamer, known as “nucleosome core”. Each individual nucleosome core consists of a complex of two molecules each of the histones H2A, H2B, H3 and H4 and the DNA molecule. 146 base pairs of DNA are wrapped around the histone octamer in 1.65 turns and in a left-handed coil. The total chromatin is compactly packed in a 30 nm fiber, Figure 1 (Albert et al., 2006).
A.2 Covalent Modifications of the Histone Tails

The four basic histones are composed of an N- and C-terminal tail and a globular domain, responsible for the histone folding. The histone N-tails extend from the DNA-histone core and are easily accessible for a variety of covalent post-translational modifications (PTMs), which control many aspects of the chromatin structure (Scharf and Imhof, 2007). Histones are synthesized in the cytosol and then assembled in the nucleus. Some modifications of the tails occur after synthesis and before nucleosome assembly, others after the nucleosome has been assembled (Albert et al., 2006).
PTMs have several important consequences such as the regulation of the functional state of chromatin, the stability of the 30 nm chromatin fiber and the gene expression. The modified histone tails have the ability to affect specific proteins to a stretch of chromatin and in this way can either cause higher compaction of the chromatin or can facilitate access to the DNA (Scharf, 2009). The types of PTMs studied currently are acetylation of lysines, phosphorylation of serines and threonines, methylation of arginines and lysines, ubiquitylation and sumoylation of lysines and ADP-ribosylation of glutamic acid (Imhof, 2006).

A.3 PTMs and Epigenetics

Epigenetics refer to heritable variations in gene expression without sequence changes of the DNA (Imhof, 2006). They are stable and essential for the cell fate. Epigenetics are dependent on cell genotype, cell development and environment and allow cells to respond and adapt to environmental stimuli (Heintzman et al., 2009). Epigenetic mechanisms regulate all biological processes, including genome reprogramming during early embryogenesis, cell differentiation and cell fate (Barros and Offenbacher, 2009, de Sario, 2009). Key epigenetic players are DNA methylation and histone post-translational modifications, which interplay with each other, with regulatory proteins and with non-coding RNAs, to remodel chromatin and to achieve nuclear compartimentalization (Delcuve et al., 2009). PTMs could act in a combinatorial manner and heritably alter gene expression without a change in the DNA sequence (Cedar and Bergman, 2009). Thus, this mechanism allows facilitation of epigenetic therapy for different diseases and cancer (Egger et al., 2004, Pogribny et al., 2007) and interventions to treat a variety of neurological and psychiatric conditions (Jiang et al, 2008). The importance of epigenetics in regulating immune and inflammatory responses has been studied as well (Wilson, 2008).


A.4 Histone Acetylation and Histone Deacetylation

Acetylation of lysines within the histone tails has an important role in the transcription. The acetylated state of chromatin is regulated by two counterpart group of enzymes, the histone acetyl transferases (HATs) and the histone deacetylases (HDACs) (Cheung et al., 2000), (Figure 2).

Figure 2. Histone acetylation catalyzed by HATs and deacetylation process regulated by HDACs (Scharf, 2007)

Currently, studies show that 11 positions of lysines are acetylated: at H4 the positions K8, 12 and 16, at H3 the positions K9, 14, 18, 23 and 27, at H2A K7 and at H2B K11 and 16 (Scharf, 2009).

Histone acetylation is a reversible modification of lysine residues within the amino terminal domains of core histone. The acetylation process is triggered by the transfer of an acetyl-group from acetyl-coenzyme A to the ε-amino group of the lysine residue catalyzed by HATs. The opposite process, by which the acetyl
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Histone acetylation tends to destabilize histone-DNA interactions (Ropero et al., 2007). Histone acetylation of promoter regions correlates with the transcriptional activity of corresponding genes; in general, high levels of histone acetylation, i.e. hyperacetylation, are observed at the promoters of transcribed genes, whereas hypoacetylation correlates with repression of gene expression (Egger et al., 2004). Indeed, many of the known transcriptional co-regulators possess either HAT or HDAC activities (Frew et al., 2009). The processes of acetylation and deacetylation are highly dynamic (Katan-Khaykovich and Struhl, 2008).

The mechanism how other modifications, for example methylation or phosphorylation, interact with acetylation and other transcriptional factors is largely unknown. This research area is of a great importance for a better understanding how genes are differently regulated and how more specific gene manipulation might be achieved.

A.5 Histone Deacetylases

Knowledge about structure, function and tissue distribution of the classical HDAC family members is needed to understand the mechanism of gene regulation via HDAC activity. In humans, 18 HDAC enzymes have been identified and categorized. Generally, HDACs comprise a family of enzymes that can deacetylate histones but also transcription factors including p53, FOXO, NF-kB, Ku70, Hps90. Thereby, HDACs regulate gene expression and cell cycle progression (Kim et al., 2009, Lin et al., 2006, Meraner et al., 2008).

In humans eleven histone deacetylases (HDACs) and seven sirtuins (SIRTs) have been identified and partly characterized until now. The HDACs are divided into four classes. Molecular masses of class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8) range from 42-55 kDa. Class II HDACs are subdivided in class Ila with the representatives HDAC4, HDAC5, HDAC7 and HDAC9 and class IIb with HDAC6 and HDAC10. All class II enzymes have molecular masses between 120-130 kDa. Furthermore, class I and II HDACs are zinc-dependent enzymes and
their tissue distribution changes during the development of an organism. Class I HDACs are mainly present in the nucleus, whereas class II HDACs shuttle between cytoplasm and nucleus. HDACs of class III, the sirtuins (SIRT 1-7) are NAD-dependent and do not have histone as their primary substrate. Very little information is available for HDACs of class IV, HDAC11 is the only representative enzyme of this group (De Ruijter et al., 2003, Gregoretti et al., 2004, Witt et al., 2009).

HDACs do not bind directly to DNA, they are recruited to multi-protein complexes associated with DNA which differ in their subunit composition. The activities of HDACs appear to be regulated in part by protein-protein interactions. In addition, HDACs are regulated by gene expression, subcellular localization and other PTMs (phosphorylation, sumoylation), as well as by processes as proteolysis and the availability of metabolic cofactors (Marks et al., 2009).

The role of HDACs on the physiological function in normal tissues and in organism development has been studied by generating knock out mice. Phenotypes of class I (HDAC1, 2 or 3) knock out mice resulted in defects of embryonic stem cell proliferation and multiple cardiac defects and led to an early embryonic lethality (Bhaskara et al., 2008, Haberland et al., 2009, Witt et al., 2009). Class II knock out mice developed defects in different tissues and organs, and the loss of HDAC7 leads to embryonic death as well. Thus, HDAC enzymes of class II have been shown to be essential for muscle differentiation and T-cell differentiation and apoptosis (Fischle et al., 2001, Wang et al., 2009). The studies confirmed that HDACs regulate the appropriate maturation of heart, smooth muscles, brain, kidney, liver, spleen and pancreas (Minucci and Pelicci, 2006, Haberland et al., 2009, Witt et al., 2009).

Imbalances in histone acetylation and/or deacetylation are assumed to play a crucial role in many “Western diseases” such as cancer, inflammation (Barnes 2006, Heo et al., 2009, Ito et al., 2002, Zhang et al., 2009) and viral infections (Senese et al., 2007, Zhang et al., 2009). A contribution of HDACs in cardiovascular diseases is under investigation (Granger et al. 2008, Haberland et al., 2009, Oka et al., 2009, Olson and Schneider, 2003, Urbich et al., 2009).
Lately, the influence of HDACs on different neural processes, namely memory enhancement, synaptic plasticity (Guan et al., 2009, Li and Richardson, 2009, Stefanko et al., 2009, Taniura et al., 2007) and Parkinson’s diseases (Jiang et al., 2008) has been discussed.

A.6 Histone Deacetylase Inhibitors

Recently, histone deacetylase inhibitors (HDACi) are the focus of tremendous interest as anti-cancer drugs (Frew et al., 2009, Ishihara et al., 2004, Lin et al., 2006, Marks and Xu, 2009, Ocker and Schneider-Stock, 2007, Rosato et al. 2003, Place et al., 2005), neuropsychiatric agents (Kumral et al, 2009, MacDonald et al., 2009) and immunomodulators (Bhat et al., 2008, Wang et al., 2009).

Generally, HDACi are known to be able to induce growth arrest, differentiation or apoptosis and autophagocytotic cell death of cancer cells in vitro and in vivo (Bolden et al., 2006, Condorelli et al., 2008, Dokmanovic and Marks, 2005, Frew et al., 2009, Ishdorj et al., 2008, Marks and Xu, 2009, Oh et al. 2008, Oka et al., 2009, Ropero and Esteller, 2007, Smith and Workman, 2009).

They are structurally diverse molecules with the ability to inhibit HDACs with varying efficiencies from nanomolar to micromolar range (Liu et al., 2006). By epigenetic chromatin remodeling they influence tumor growth by repression of angiogenesis or by increasing tumor cell immunogenicity (Liu et al., 2006, Ogbomo et al., 2007, Savickiene et al., 2006). In many clinical trials effects of several HDAC inhibitors in different cancers are studied (Akare et al., 2006, Bolden et al., 2006, Carew et al. 2008, Dashwood et al., 2006, Frew et al., 2009, Garber, 2007, Habold et al., 2008, Marks and Xu, 2009, Oka et al., 2009, Ogbomo et al., 2007).

However, most of the known HDAC modulators exert a rather unselective impact on HDAC activity (Bhaskara et al., 2008). Some pan-inhibitors, either natural or synthetic compounds already applied in clinical trials against cancer, induced
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different cardiac complications, which are major disadvantages for a frequent use in therapy.

The majority of these HDACi are microbial metabolites: e.g. romidepsin or trichostatin A, antibiotics with anti-fungal effects, are of bacterial origin, whereas depudecin, trapoxin A and B have been isolated from fungi. Novel marine natural products such as largozole, apicidine, azumamides and psammaplin A have shown HDAC-inhibitory activity at nanomolar concentrations (Newkirk et al., 2009).

Until now, only few plant metabolites, which possess HDAC inhibitory effects, are known. Flavone and flavone derivatives from Feijoa sellowiana Bert. exerted ability to inhibit HDAC 1 activity (Bontempo et al., 2007). A sulfur compound from the roots of Pleuropterus ciliinervis Nakai is a potent HDACi, with an IC\textsubscript{50} value of 1.43 µM (Son et al., 2007). Investigations of the rhizomes of Zingiber zerumbet L. have brought a new insight into the anti-cancer efficacy of this plant. Two major sesquiterpenoids, 6-methoxy-2E,9E-humuladien-8-one and zerumbone, were tested \textit{in vitro} for HDAC inhibition and IC\textsubscript{50} values of 1.25 and 8.35 µM, respectively, were determined (Chung et al., 2008). A recent study of a methanolic extract of the stem of Microtropis japonica (Franchet & Savatier) has revealed the potential of triterpenoids and ursolic acid to inhibit HDAC activity at concentrations of 20 µg/ml as shown by the increase in the acetylated state of histone 3 (Chen et al., 2009).

Further investigations on the specific functions of individual HDACs as well as the mechanism of action and the role of HDACi in normal cells are needed in order to ensure less adverse effects of HDACi in therapy.

The challenge in the field is to develop new compounds, which are effective and have particular function on “disease” related HDACs. First examples of active plant compounds underline that in this search for selective HDAC modulators the almost infinite structural biodiversity of plant kingdom could provide new promising substances for further development. Natural products have been a
highly important source of leads for the development of drugs. The expectations are that a more efficient and effective search for natural products will lead to discovery of a successful drug (Harvey, 2008). To speed-up the isolation of respective candidates in a bioactivity-guided approach, a suitable test method is indispensable.

A.7 HDAC Assay

A fluorimetric in vitro enzyme assay has been developed for the investigation of the influence of pure compounds on the activity of class I and class II HDACs. The principle of the assay is based on the following reactions: an ε-acetylated lysine-based substrate C-terminally coupled with 4-methyl-coumarine-7-amide (MCA) is deacetylated by HDAC. The deacetylated substance is recognized as a substrate by endopeptidase which releases 7-amino 4-methylcoumarin (AMC). Free AMC, in contrast to acetylated MCA, is highly fluorescent and can be monitored (Ciossek et al., 2008, Hildmann et al., 2006, Mazitchek et al. 2008, Riester et al., 2007, Wegener et al., 2003). The HDAC assay reactions are presented in Figure 3, page 10.

To the best of our knowledge, this method had not yet been tested nor been optimized and validated for the investigation of complex plant extracts and their ability to modulate HDACs.
Figure 3. Schematic overview of the HDAC assay reactions (Wegener et al., 2003)

**Introduction**

ε-acetylated lysine-based substrate C-terminally coupled with MCA

\[
\text{Tos-Gly-Pro} \quad \text{(CH}_2)_4 \text{NH} \quad \text{NH} \quad \text{O} \quad \text{COC} \quad \text{NH} \quad \text{(CH}_2)_4 \text{NH} \quad \text{H}_3\text{CO} \quad \text{NH} \quad \text{CH}_3
\]

Step I
HDAC

\[
\text{Tos-Gly-Pro} \quad \text{(CH}_2)_4 \text{NH} \quad \text{NH} \quad \text{O} \quad \text{COC} \quad \text{NH} \quad \text{(CH}_2)_4 \text{NH} \quad \text{H}_3\text{COO} \quad +
\]

step II
Endopeptidase

\[
\text{Tos-Gly-Pro} \quad \text{(CH}_2)_4 \text{NH} \quad \text{NH} \quad \text{O} \quad \text{COC} \quad \text{NH} \quad \text{(CH}_2)_4 \text{NH} \quad \text{H}_3\text{COO} \quad + \quad \text{H}_2\text{N} \quad \text{O} \quad \text{CH}_3
\]

7-amino 4-methylcoumarin (AMC)
(highly fluorescent product)
A.8 HDACs and Cardiovascular Diseases

Patients with chronic heart failure develop cardiac hypertrophy under enlargement of the heart. Cardiac hypertrophy is the heart’s response to a variety of extrinsic and intrinsic stimuli that impose increased biomedical stress (Barry et al., 2008, Force, 2008).

The causes and effects of cardiac hypertrophy have been precisely documented, although the molecular mechanisms, the responsible signals to cell changes, or cardiac signaling pathways are still poorly understood (Bradner, 2009). The modulation of myocardial growth was studied as a potential approach in the prevention and treatment of heart failure. Cardiac hypertrophy and heart failure are associated with activation of multiple pathways, confirmed by in vivo experiments (Force, 2008).

Studies have shown that HATs and HDACs participate in the regulation of hypertrophic heart response (Frey and Olson, 2003, Kook et al., 2003). The results suggest a potential role of HDACi as a therapy to control cardiac hypertrophy (Kong et al., 2006, Olson et al., 2003, Wei et al., 2008). Nevertheless, further investigations in this area are needed.

A.9 Leonuri Herba as Traditional Remedy against Heart Complaints

Leonuri herba consists of whole or cut, dried flowering aerial parts of Leonurus cardiaca L. (Lamiaceae). Other common names for the plant are motherwort, lion’s ear, lion’s tail or throw-wort.

The geographical areas of growth are western, eastern and central Asia, the plant is broadly distributed in central and eastern European regions, as well as in North Africa and North America. The plant prefers light (sandy), medium (loamy) and heavy (clay) soils and can grow under nutritionally poor condition, it requires moist and light (WHO, 2006).
Introduction

The plant can grow to a height of up to 1.2 m. The stem is erect, quadrangular, grooved, hollow, pale green or purplish brown with usually hairy, opposite and decussate leaves. The leaves have serrated margins and are palmately lobed with long petioles, basal leaves are wedge shaped with three points while the upper leaves are more latticed. Flowers appear in leaf axils on the upper part. The 6 to 12 flowers are small, with green, bell shaped calyx with 5 equal pointed lobes, the corolla is 2-lipped pubescent, white with purplish spots on the furry lower lip. The fruit is a brown, triangular nutlet, 2.5 to 3 mm long with a tuft of hair at the tip, (WHO, 2006), Figure 4.

Figure 4. *Leonurus cardiaca* L.

Main constituents of *Leonuri herba* are flavonoids, at minimum 0.2%, mainly O-glycosides of apigenin, kaempferol and quercetin. An overview of the
characterized constituents of Leonuri herba is presented in Table 1 (Melichar, 2007).

### Table 1: Constituents of Leonuri herba

<table>
<thead>
<tr>
<th>Class</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diterpenes</td>
<td>leocardin, leosibricin</td>
</tr>
<tr>
<td>Iridoides</td>
<td>ajugoside, ajugol, galiridoside, reptoside</td>
</tr>
<tr>
<td>Flavones</td>
<td>rutin, quercitrin, isoquercitrin, hyperoside, genkwanin</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>betonicine, stachydrine</td>
</tr>
<tr>
<td>phenolic acids derivatives</td>
<td>caffeic-acid-4-O-rutinoside, leonurin</td>
</tr>
<tr>
<td>essential oil</td>
<td>0.001 – 0.002%</td>
</tr>
<tr>
<td>Triterpenes</td>
<td>ursolic acid, oleanolic acid</td>
</tr>
</tbody>
</table>

Pharmacological studies on the cardiovascular effects of an ethanolic extract of Leonuri herba showed direct myocardial actions, inhibiting the effects of calcium chloride and stimulating alpha and beta adrenoceptors in the heart (Melichar, 2007).

A sedative effect of Leonuri herba was shown by a significantly prolonged sleeping time in mice (ESCOP, 2009).

For ursolic acid, a terpenoid constituent of Leonuri herba anticancer properties and cytotoxic properties were described (Ikeda et al., 2006, Ma et al., 2005, Tian et al., 2006, Yim et al., 2006).

In European traditional medicine Leonuri herba has been used for long against hearth complaints in order to strengthen the heart, as well as in nervous cardiac disorders (Melichar, 2007).

Due to these facts, this drug was selected as an example for a bioactivity-guided fractionation and detailed study in the search of novel HDACi.
A decoction of a preparation from traditional Chinese medicine (TCM) containing four different herbal drugs has been used in the treatment of cardiovascular diseases. This preparation has shown a significant increase in the survival rate of mice after experimental cardiac infarction (Ma, unpublished data).

Thus, the effect of this decoction and its ingredients on HDAC activity was of interest.
B  Aim of the Work

Recently, histone deacetylase inhibitors (HDACi) have received high interest as anti-cancer agents. In many clinical trials effects of several HDACi on different tumors are studied at the moment. Plant kingdom provides an almost infinite structural biodiversity. Thus, the purpose of this work was the establishment and optimization of an \textit{in vitro} HDAC assay for determination the enzyme activity of histone deacetylases.

The optimization process should assure high efficacy and improvement of the experimental design to make the assay a powerful tool for primary screening of complex extracts and fractions from plant origin for modulators of HDAC activity. The influence of diverse factors on the enzyme activity such as temperature, pH range, buffer composition, incubation times and enzyme sources had to be investigated. The comparison between normal and cancer cells, human versus rodent cells and epithelial, endothelial or muscle cell can provide significant information for the application of the assay searching for new leads in defined diseases.

The assay design should not only allow the use of nuclear extracts containing mixtures of HDACs but give insights on the influence of potent analytes on single HDACs as well.

The influence of primary and secondary plant’s metabolites, which might negatively influence the assay due to autofluorescence interfering with the wavelength of emission or due to precipitation of the enzyme, had to be studied. After successful validation of the test system the application for bioactivity-guided searches should be studied on an example. For this purpose Leonuri herba was selected from the VOLKSMED database, in which knowledge of Austrian traditional medicine is compiled. Due to the fact that HDACs seem to be involved in cardiac complaints this drug seemed to be a good candidate to prove the applicability of the assay on extracts, fractions and purified compounds.
C Materials and Methods

C.1 Plant Materials

C.1.1 Components of a Herbal Mixture from TCM

We received the single herbs (TCM 1 – TCM 4) from Dr. Y. Ma, Department of Pathophysiology, Medical University of Vienna.

- TCM 1 roots of *Glycyrrhiza uralensis* Fisch ex DC.
- TCM 2 rhizomes of *Zingiber officinale* Roscoe
- TCM 3 tubers of *Aconitum carmichaelii* Debx.
- TCM 4 cortex of *Cinnamomum cassia* Nees
- TCM 5 mixture of the single herbs

C.1.2 Ratanhiae radix

The root of *Krameria triandra* Domb. Burd et Simp. was obtained by Mag. pharm. R. Kottas-Heldenberg & Sohn Drogenhandel GmbH, Vienna, Austria (Ch. No.: KLA60374).

C.1.3 Viburni cortex

The bark of *Viburnum opulus* L. was obtained by Heinrich Klenk GmbH & Co. KG, Schwebheim, Germany (Ch. No.: 323213).
Materials and Methods

C.1.4 Leonuri herba

The herb of Leonurus cardiaca L. was obtained by Mag. pharm. R. Kottas-Heldenberg & Sohn Drogenhandel GmbH, Vienna, Austria (Ch. No.: KLA70811), the botanical identification was carried out by Ass. Prof. Dr. Christa Kletter, Department of Pharmacognosy, University of Vienna.

C.2 Extraction and Fractionation

C.2.1 Extraction of the TCM-mixture and its Components

The herbs were powdered and 1.0 g was soaked in 10 ml water for 1 hour. Then, extraction under reflux at 100°C for 30 minutes followed. The extracts were filtered. The clear aqueous solutions were lyophilized and yielded the amounts shown in Table 2. The lyophilized extracts of the single herbs (TCM 1-TCM 4) and of the mixture (TCM 5) were tested for their effect on HDAC activity. For that purpose each extract was dissolved in 20 µl DMSO and 980 µl assay buffer to final concentrations of 2 mg/ml.

Table 2: Yields of TCM extracts

<table>
<thead>
<tr>
<th>TCM</th>
<th>yield, mg per g drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM 1</td>
<td>93.65</td>
</tr>
<tr>
<td>TCM 2</td>
<td>31.14</td>
</tr>
<tr>
<td>TCM 3</td>
<td>123.23</td>
</tr>
<tr>
<td>TCM 4</td>
<td>57.67</td>
</tr>
<tr>
<td>TCM 5</td>
<td>112.54</td>
</tr>
</tbody>
</table>
C.2.2 Extraction of Ratanhiae radix and Detannification of the Extract

2 g powdered plant material were extracted with 100 ml water under reflux for 30 min. After filtration the extraction was repeated under the same conditions. The combined extracts were lyophilized, the yield was 299.3 mg. 10 mg of the dried extract were dissolved in 1 ml solvent (20 µl DMSO and 980 µl assay buffer). Concentrations between 0.20 to 1 mg/ml were tested in the HDAC assay. For detannification 50 mg lyophilized extract were dissolved in 500 µl MeOH. Partition between 2 ml water-methanol (1+9) and 2 ml hexane was performed. After separation the apolar phase was discarded, subsequently 2 ml chloroform were added to the aqueous phase and partitioned. The detannified chloroform phase and the aqueous phase were dried under reduced pressure at 40°C, yielding 7 mg and 28.5 mg, respectively. The detannified chloroform fraction was tested at concentrations from 2 to 10 mg/ml for their effect on HDAC activity. For this purpose 5 mg were dissolved in 125 µl solvent (5 µl DMSO and 120 µl assay buffer and further diluted). The aqueous fraction enriched in tannins was tested at concentrations from 0.2 to 1 mg/ml, here 4 mg were dissolved in 1 ml solvent (20 µl DMSO and 980 µl assay buffer and further diluted).

C.2.3 Extraction of Viburni cortex

The dried plant material was milled and extracted in a solvent-series of increasing polarity with dichloromethane (CH$_2$Cl$_2$), ethyl acetate (EtOAc), methanol (MeOH) and water (H$_2$O).

8 g powdered plant material was extracted with 100 ml solvent under reflux for 30 min. After filtration the extraction of the residue was repeated twice under the same conditions. This procedure was used for each of the solvents. The combined extracts were dried by evaporation under reduced pressure at 40°C and the yields were 146.2 mg (CH$_2$Cl$_2$), 216.3 mg (EtOAc), 731.2 mg (MeOH) and 766.2 mg (H$_2$O), respectively. The dried residues were tested for their effect
on the HDAC activity. 10 mg of the dried extracts were dissolved in 250 µl solvent (5 µl DMSO and 245 µl assay buffer) and diluted to final concentrations of 10 mg/ml in the assay.

C.2.4 Extraction of Leonuri herba – Analytical Scale

The dried plant material was milled and extracted in a solvent-series of increasing polarity with dichloromethane (CH\textsubscript{2}Cl\textsubscript{2}), ethyl acetate (EtOAc), methanol (MeOH) and water (H\textsubscript{2}O). 9 g powdered plant material was extracted with 100 ml solvent under reflux for 30 min. After filtration the extraction of the residue was repeated twice under the same conditions. This procedure was used for each of the solvents. The combined extracts were dried by evaporation under reduced pressure at 40°C and the yields were 190.1 mg (CH\textsubscript{2}Cl\textsubscript{2}), 98.3 mg (EtOAc), 759.4 mg (MeOH) and 767.9 mg (H\textsubscript{2}O), respectively. The dried residues were tested for their effect on the HDAC activity. 10 mg of the dried extracts were dissolved in 250 µl solvent (5 µl DMSO and 245 µl assay buffer) and diluted to final concentrations of 10 mg/ml in the assay.

C.2.5 Extraction of Leonuri herba – Preparative Scale

1.0 kg of the powdered dry plant material (in portions of 250.0 g) was extracted under reflux with 5.5 l methanol for 30 minutes. The supernatant was removed and filtered. The residue was extracted for another 30 minutes under reflux with 5.5 l methanol. After filtration the combined extract solutions were evaporated under reduced pressure at 40°C and the yield was 93.1 g.

C.2.6 Removal of Chlorophyll from the Extract of Leonuri herba

It is known that chlorophyll interferes unspecifically with numerous proteins. In order to exclude a false result, chlorophyll was removed from the extract. The
The applied method was based on a liquid-liquid-partition of the extract between water and chloroform and subsequently water and ethyl acetate. The dried methanolic extract of Leonuri herba was dissolved in 500 ml water in an ultrasonic bath for 30 min at room temperature. The clear solution was partitioned twice with chloroform. The chloroform phases were removed and yielded 23.4 g after evaporation. The partition was further performed twice with ethyl acetate resulting in a yield of 1.7 g. The chlorophyll free aqueous extract was lyophilized. The yield was 67.8 g. All fractions were characterized by TLC and HPLC. The lyophilized chlorophyll-free extract was tested at a concentration of 10 mg/ml in the HDAC assay and bioactivity guided fractionation followed.

In a preliminary test, 4 g of the purified extract were fractionated by Solid Phase Extraction (SPE) on C18-cartridges into five different fractions (see C.2.7). Due to high costs of this method, the major part of the extract (60.0 g) was separated by column chromatography on Sephadex LH 20 (see C.2.8, page 21).

C.2.7 Solid Phase Extraction of the Purified Methanolic Extract from Leonuri herba

A portion of 4 g of the methanolic extract obtained by exhaustive extraction (C.2.6, page 19) were dissolved in 4 ml 40% methanol in an ultrasonic bath for 15 min at room temperature. Four cartridges (Varian Mega BE-C18; 5 g, 20 ml) were conditioned with two reservoir volumes (RV) methanol and two RV water. 1 ml of the extract was applied on each of the four cartridges which were dried by air purge for 10 min. The extracts were eluted from each cartridge with 5 RV each of water (F1), 20% MeOH (F2), 40% MeOH (F3), 80% MeOH (F4) and 100% MeOH (F5) at flow rates of 5 ml/min. The fractions of identical polarity were pooled. After removal of methanol from the obtained fractions by evaporation, the aqueous solutions were lyophilized. The yields were 2.70 g F1, 240 mg F2, 310 mg F3, 54 mg F4 and 49 mg F5.
Materials and Methods

20 mg of the dried extracts were dissolved in 500 µl solvent (10 µl DMSO and 490 ml assay buffer) and diluted to final concentrations of 1 mg/ml in the assay. The fractions were further analyzed and characterized by chromatographic methods.

Fraction F3 was further separated by centrifugal partition chromatography (see C.3.1). 86.0 mg of F3 were solved in an ultrasonic bath in 2 ml ethyl acetate-water (1:1) for 30 min. The sample fractionation was started at a flow of 1.5 ml/min. 70 fractions with a volume of 10.5 ml were collected. Then methanol was pumped into the system at flow rate 9 ml/min. 23 methanol fractions at a volume of 18 ml were collected additionally. All fractions were evaporated and analyzed by TLC.

C.2.8 Gel Permeation Chromatography of the Methanolic Extract of Leonuri herba

The major part of the extract of Leonurus cardiaca L. (60.0 g) after chlorophyll removal (C.2.6, page 19) was separated by column chromatography on Sephadex LH 20 with water – methanol as a mobile phase (C.3.2, page 22). Each fifth fraction was analyzed by TLC.

C.3 Chromatographic Methods

C.3.1 Centrifugal Partition Chromatography (CPC)

Instrumentation:
High Speed Countercurrent Chromatograph CCC 100, Pharma Tech Research, Baltimore, USA
Mode of operation tail to head
Stationary phase water
Mobile phase ethyl acetate
Materials and Methods

Distribution coefficient of F3  0.75
Flow rate   1.5 ml/min
Fraction size  15 ml

C.3.2  **Column Chromatography on Sephadex LH-20**

Column length   80 cm
Column diameter  3 cm
Stationary phase Sephadex LH-20
Mobile phase water (fraction 1 to 100)
                   20% methanol (fraction 101 to 200)
                   40% methanol (fraction 201 to 300)
                   80% methanol (fraction 301 to 400)
                   100% methanol (fraction to 401 to 500)
Amount of loading sample  60 g in two portions (31 g and 29 g)
Flow rate   40 ml/h
Fraction size  20 ml

C.3.3  **Thin Layer Chromatography (TLC)**

Thin Layer Chromatography was used for the characterization of the fractions.

*System 1*
Stationary phase  TLC plates 20 x 20 cm silica gel 60 F254; Merck, Darmstadt, Germany
Mobile phase ether acetic acid – formic acid – acetic acid – water (100+11+11+26)

*System 2*
Stationary phase  Cellulose F plates 20 x 20 cm, 250µ F254; Merck, Darmstadt, Germany
Mobile phase  30% acetic acid
### Materials and Methods

**System 3**

Stationary phase Polygram 20 x 20 cm polyamide plates 6 UV\textsubscript{254}; MN, Düren, Germany

Mobile phase toluene - petrol ether - butanone – methanol
(18.5+4.5+9+6)

Detection:
The plates were sprayed with
- **NEU reagent**: 1% methanolic diphenylboryloxyethylamine solution, followed by 5% ethanolic solution of polyethylene glycol 400 (PEG-400). Detection was performed under UV 365 nm (Wagner and Bladt, 1996).
- **Anisaldehyde sulfuric acid reagent**: mixture of 10 ml acetic acid, 85 ml methanol, 5 ml concentrated sulfuric acid and 0.5 ml anisaldehyde. Detection at day light followed heating at 105°C for 5 to 10 minutes (Wagner and Bladt, 1996).

**Sample Preparation for TLC**

5.0 – 10.0 mg samples were dissolved in 1 ml methanol and 10 - 20 µl were applied for analysis.

### C.3.4 High Performance Liquid Chromatography (HPLC)

**Instrumentation:**
- **Degasser**: Shimadzu DGU 20A5 prominence Degasser
- **Controller**: Shimadzu CBM 20A prominence Communication Bus Module
- **Autosampler**: Shimadzu SIL 20AC HT prominence Auto Sampler
- **Pump**: Shimadzu LC 20AD prominence Liquid Chromatograph
- **Detector**: Shimadzu SPD M20A prominence Diode Array Detector
- **Column oven**: Shimadzu CTO 20AC prominence Column Oven
- **Software**: LC Solution
Materials and Methods

**System 1**

Column: Hypersil BDS C18, 250 mm x 4 mm; 5µm Agilent, St. Clara, US

Eluent A: water adjusted to pH 2.8 with acetic acid

Eluent B: acetonitrile (acidified with appr. 3 ml/L conc. acetic acid)

Composition at the beginning of analysis: 85% A + 15% B.

Flow rate: 1 ml/min

Temperature: 30 °C

Table 3: Gradient program 1

<table>
<thead>
<tr>
<th>time, min</th>
<th>% eluent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 – 1.0</td>
<td>15</td>
</tr>
<tr>
<td>1.0 – 15.0</td>
<td>50</td>
</tr>
<tr>
<td>15.0 – 30.0</td>
<td>60</td>
</tr>
<tr>
<td>30.0 – 40.0</td>
<td>80</td>
</tr>
<tr>
<td>40.0 – 50.0</td>
<td>100</td>
</tr>
</tbody>
</table>

**System 2**

Column: ACE C18-AR, 150 mm x 2.1 mm; 3µm, ACE Aberdeen, Scotland

Eluent A: water adjusted to pH 2.8 with acetic acid

Eluent B: acetonitrile (acidified with appr. 3 ml/L conc. acetic acid)

Composition at the beginning of analysis: 88% A + 12% B.

Flow rate: 0.35 ml/min

Temperature: 35 °C

Table 4: Gradient program 2

<table>
<thead>
<tr>
<th>time, min</th>
<th>% eluent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 – 17.0</td>
<td>20.5</td>
</tr>
<tr>
<td>17.0 – 30.0</td>
<td>43.9</td>
</tr>
<tr>
<td>31.0 – 36.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

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Materials and Methods

System 3
Column ACE C18-AR, 150 mm x 2.1 mm; 3µm, ACE, Aberdeen, Scotland
Eluent A water
Eluent B methanol
Composition at the beginning of analysis 75% A + 25% B.
Flow rate 0.35 ml/min
Temperature 35 °C

Table 5: Gradient program 3

<table>
<thead>
<tr>
<th>time, min</th>
<th>% eluent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 – 17.0</td>
<td>60</td>
</tr>
<tr>
<td>17.0 – 22.0</td>
<td>100</td>
</tr>
</tbody>
</table>

Sample Preparation for HPLC
Samples were dissolved in methanol at a concentration of 1 mg/ml and centrifuged at 13’000 rpm for 10 min before injection into the HPLC system. 3 µl (System 2 and 3) or 10 µl (System 1) of the supernatant were used for HPLC-analyses.

C.3.5 Preparative HPLC

Instrumentation:
Controller Shimadzu CBM 20A prominence Communication Bus Module
Auto sampler Shimadzu SIL 10AP Auto Sampler
Pump 2 x Shimadzu LC 8A preparative Liquid Chromatograph
Detector Shimadzu SPD M20A prominence Diode Array Detector
Fraction collector: Shimadzu FRC10 fraction collector
Software LC Solution
Materials and Methods

Column  MN Nucleosil 100-7 C18, 30 cm x 3 cm; 7µm, Macherey and Nagel, Düren, Germany
Eluent A  water
Eluent B  methanol
Composition at the beginning of analysis 60% A + 40% B.
Flow rate  30 ml/min
Temperature  RT

Table 6: Gradient program 4

<table>
<thead>
<tr>
<th>time, min</th>
<th>% eluent B</th>
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</thead>
<tbody>
<tr>
<td>0.0 – 10.0</td>
<td>40</td>
</tr>
<tr>
<td>10.0 – 20.0</td>
<td>95</td>
</tr>
</tbody>
</table>

Sample Preparation
Samples were dissolved in 40% methanol at a concentration of 100 mg/ml and 250 µl were applied in each run.

C.3.6 HPLC/LC-MS<sup>n</sup> analysis

The LC-MS analyses were performed on an UltiMate 3000 Rapid Separation LC System (Dionex, Germering, Germany), coupled to a 3D quadrupole ion trap instrument equipped with an orthogonal ESI source (HCT, Bruker Daltonics, Bremen, Germany). The gradient program used for the analysis is summarized in Table 7, page 27.

Instrumentation:
Controller  Ultimate 3000 Dionex
Autosampler  Ultimate 3000 RS Dionex Autosampler
Pump  Ultimate 3000 RS Dionex Pump
Detector  Ultimate 3000 RS Dionex Diode Array Detector
190 – 400 nm
Materials and Methods

Column oven: Ultimate 3000 RS Dionex Column Compartment
Column: ACE C18-AR, 150 mm x 2.1 mm; 3 µm, ACE, Aberdeen, Scotland
Software: Chromelion Xpress
Eluent A: water
Eluent B: methanol
Composition at the beginning of analysis: 70% A + 30% B.
Flow rate: 0.35 ml/min
Temperature: 35 °C

Table 7: Gradient program for LC-MS\textsuperscript{n} analysis

<table>
<thead>
<tr>
<th>time, min</th>
<th>% eluent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 – 10.0</td>
<td>30</td>
</tr>
<tr>
<td>10.0 – 17.0</td>
<td>60</td>
</tr>
<tr>
<td>17.0 – 20.0</td>
<td>80</td>
</tr>
<tr>
<td>21.0 – 26.0</td>
<td>100</td>
</tr>
</tbody>
</table>

The ESI ion source parameters were as follows: capillary voltage: 4.0 kV, nebulizer: 20 psi (N\textsubscript{2}), dry gas flow: 7 L/min (N\textsubscript{2}), and dry temperature: 330 °C.

The mass spectrometer was operated in an automated data-dependent acquisition (DDA) mode where each negative ion MS scan (m/z 100-1500, average of 5 spectra) was followed by MS\textsuperscript{2} scans (m/z 40-1000, average of 5 spectra, isolation window of 4 Th, fragmentation amplitude of 0.7 V) of the two most intense precursor ions and MS\textsuperscript{3} scans (m/z 40-1000, average of 5 spectra, isolation window of 4 Th, fragmentation amplitude of 0.7 V) of the most intense fragment ion in each MS\textsuperscript{2} scan. Helium was used as collision gas.

Sample Preparation for HPLC/LC-MS\textsuperscript{n}
Samples were dissolved in methanol at a concentration of 1 mg/ml and centrifuged at 13'000 rpm for 10 min before injection into the HPLC system. 1 µl of the supernatant was used for HPLC/LC-MS\textsuperscript{n} analysis.
C.4 Cell Culture

C.4.1 Cell Lines and Cultivation

Primary rat vascular smooth muscle cells (VSMCs, kindly provided by Dr. A. Schwaiberger, University of Vienna) as well as HeLa cells (kindly provided by a.o. Prof. Dr. G. Krupitza, Medical University of Vienna) were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, penicillin (1000 U/l) and streptomycin (1 mg/l) (all from LONZA, Belgium) and incubated at 37°C under an atmosphere of 5% CO₂ in air.

Human umbilical vein endothelial cells (HUVECs, kindly provided by Dr. Y. Schilder, University of Vienna) were cultivated in endothelial basal medium (EBM) supplemented with 10% fetal calf serum, penicillin (1000 U/l), streptomycin (1 mg/l) and additives provided by the manufacturer (LONZA, Belgium) and incubated at 37°C under an atmosphere of 5% CO₂ in air.

C.4.2 Cell Storage, Freezing and Thawing

Cells were harvested for storage at 100% confluence. They were trypsinized and centrifuged (1’100 rpm, 10 min, 4°C), the supernatant was removed. Fresh medium was used to suspend the cells and 10% DMSO was added for cryoprotection. Aliquots of 1 ml containing approximately 10⁶ cells were frozen at -80°C in cryovials. After storage in a double walled Styrofoam box for several days they were transferred to liquid nitrogen at -196°C for long time storage.

When the cells were thawed, they were suspended into 15 ml growth medium, the solution was centrifuged (1’100 rpm, 5 min, 4°C) to remove any DMSO and the cells were suspended in fresh medium for further use.
C.4.3 Cell Viability

Cell cultures were observed under microscope to study any morphological changes when incubated with and without tested compounds as well as to examine for any microbial contaminations. Additionally, a cell counter was used to quantify the cell viability. 500 µl cells in incubation medium were placed in a vial. A Vicell XR cell viability analyzer, Beckman Coulter, automatically added 500 µl trypan blue dye to the vial and the cell number and the cell viability were determined according to the supplier’s protocol by 50 measurements.

C.5 Cell Based Assays

C.5.1 In vitro Cytotoxicity Assay

Resazurin was used to measure the metabolic activity of living cells. Viable cells continuously convert non-fluorescent resazurin to fluorescent resofurin, increasing the overall fluorescence and color of the medium. By measurement of fluorescence the cell viability was quantified.

HeLa cells were grown overnight in transparent 96 well plates (Greiner, Frickenhausen, Germany) at concentrations of $2 \times 10^4$ cells per well in 200 µl DMEM medium. The medium was removed and the cells were washed with PBS buffer. Then, the cells were incubated for 24 hours with starvation medium containing 1% C-source. After 24 hours the medium was discarded, the cells were washed with PBS buffer and 180 µl of fresh DMEM medium containing 10% C-source were added. The tested compounds were solved in DMEM medium at concentrations between 100 to 1000 µM and 20 µl of the solutions were added to the wells. The end concentration of the tested compounds was 10 to 100 µM, respectively. The incubation period was 48 and 72 hours, respectively.
For the resazurin assay, 40 µl resazurin solution (0.1 mg/ml) was added to each well. The incubation time was 4 hours at 37°C. The fluorimetric measurement was performed at 535 nm excitation and 590 nm emission in a TECAN Genious microtiter plate reader. Appropriate blanks (dye without cells) were subtracted from the values. All measurements were performed in duplicate.

C.5.2 Proliferation Assay

Generally, intact DNA incorporates the crystal violet dye. The intensity of the absorbance is in correlation with the cell proliferation rate.

Crystal Violet Staining
For staining with crystal violet the HeLa cells were grown and incubated with the tested compounds as described (C.5.1, page 29). After complete removal of the medium, 100 µl of crystal violet solution (0.5% crystal violet in 20% MeOH) were added to the wells. The incubation time was 15 min at RT. Then the 96 well plate was washed with distilled water and completely dried overnight at RT. By addition of 200 µl solution of 0.1 M sodium citrate in ethanol the reaction was started. The plate was shaken for 10 min at RT, and the absorbance was measured at 550 nm in a TECAN Sunrise microtiter plate reader. All measurements were performed in triplicate and appropriate blanks were subtracted from the values.

C.6 Western Blot Analyses and Gel Electrophoresis

C.6.1 Western Blot Analyses

Western blot analyses were performed to determine the acetylated state of histones (AcH3 and AcH4) and acetylated tubulin after treatment with potential HDACm as well as to study their influence on cell cycle progression by the investigation of the expression of protein p21. For p21 and tubulin a gel gradient 10% and for AcH3 and AcH4 a gel gradient 12% was used.
C.6.2 Gel Electrophoresis

Stock solutions used for gel preparation:
1.5 M Tris- Base pH 8.8 (Tris- Base 18.17 g/l, pH adjusted to 8.8 with HCl)
1.25 M Tris- Base pH 6.8 (Tris- Base 15.14 g/l pH adjusted to 6.8 with HCl)
SDS 10%
APS 10%

A total volume of 7.5 ml solution for the separating gel according to the composition given in Table 8 was placed in gel casters for polymerization. Next, a thin layer of isopropanol was added to assure gel homogeneity and to prevent air bubbles during polymerization.

Table 8: Components used for the preparation of the separating gels

<table>
<thead>
<tr>
<th>solutions</th>
<th>10% gel gradient</th>
<th>12% gel gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% PAA</td>
<td>2.5 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>1.5M Tris HCL 8.8</td>
<td>1.8 ml</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>75.0 µl</td>
<td>75.0 µl</td>
</tr>
<tr>
<td>distilled water</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>7.5 µl</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>37.5 µl</td>
<td>37.5 µl</td>
</tr>
<tr>
<td>total</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
</tr>
</tbody>
</table>

The solution for the loading gel was prepared according to Table 9, page 32. After the solution was poured, a comb was placed to create wells for the samples.
C.6.3 Protein Determination

The amount of protein was quantified by the method of Bradford (Bradford, 1976). The assay is based on the shift of the absorbance maximum for an acidic solution of Coomassie Brilliant Blue from 465 nm to 595 nm due to the binding to protein. Both hydrophobic and ionic interactions stabilize the anionic form of the dye causing a visible color change.

The reaction was started by mixing 10 µl of appropriately diluted sample solutions with 190 µl of Bradford solution (1:5 dilution in water, Roti® - Quant, Roth, Germany) in a transparent 96 well plate (Greiner, Frickenhausen, Germany) at room temperature. The samples were shaken for 15 min on a laboratory shaker and then the extinction was measured at 595 nm in a TECAN Sunrise microtiter plate reader. The standard curve was prepared using bovine serum albumin (BSA) at concentrations between 0 and 20 µg. All measurements were performed in triplicate and appropriate blanks were subtracted from the values.
C.6.4 SDS Electrophoresis and Western Blot

Buffers

Epho-buffer (3.0 g Tris-Base, 14.4 g glycine and 1 g SDS in 1 l water)
Blotting-buffer (15.2 g Tris-Base and 72.9 g glycine in 1 l water)
TBS-T (3.0 g Tris-Base, 11.1 g sodium chloride, and 1 ml of Tween 20 in 1 l water at pH adjusted to 8.0 with HCl)
ECL buffer (1 M Tris-Base pH 8.5)
p-coumaric acid (0.15 g in 10 ml DMSO)
luminol solution (0.44 g in 10 ml DMSO)
Detection buffer: 1 ml ECL buffer, 22 µl p-coumaric acid solution, 50 µl luminol solution and 3 µl 30% hydrogen peroxide were mixed and replenished to 10 ml with water.

30 - 40 µl samples with 20 µg proteins were mixed with 10 - 15 µl sample buffer (255 µl 10% SDS and 45 µl mercaptoethanol) and boiled at 95°C for five minutes. The pretreated samples and 5 µl native protein marker (Biorad, 20 - 250 kDA) were loaded into the wells of the gel. The gel was placed into the module tank, which was filled with epho-buffer. The gel was run for 1 hour at 30 mA.

The membrane was preconditioned by soaking in methanol to transparency, then was transferred into a mixture of 100 ml blotting-buffer with 200 ml methanol and 700 ml with water. After that the membrane was placed into the “sandwich” chamber with 2 fiber pads and 2 filter papers (all soaked in a blotting buffer). The blot was run at room temperature with blotting buffer. Running time was 90 minutes at 100 V. After the trans-blot block, the membrane was incubated with 5% nonfat dry milk in TBS-T for 1 hour, on a laboratory shaker at room temperature. The membrane was washed three times with TBS-T buffer for 10 min at RT on a laboratory shaker. Incubation with a primary antibody was performed for 1 hour at RT (Table 10, page 34). The membrane was washed with TBS-T buffer three times for 10 min on a laboratory shaker. A secondary antibody was placed in 5% BSA solved in TBS-T or TBS-T only for 1 hour at RT.
(Table 11). Finally, the membrane was three times washed with TBS-T and incubated with the detection buffer for 1 minute. Protein detection was performed with a LAS-3000™ luminescent image analyzer and quantification of bands with AIDA™ software.

Table 10: Used primary antibodies

<table>
<thead>
<tr>
<th>Target</th>
<th>Source</th>
<th>Dilution</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-H3 (lys9/lys 14)</td>
<td>rabbit</td>
<td>1:1000 in 5% BSA, TBS-T</td>
<td>Cell Signaling Technology Inc. (Danvers, MA, USA)</td>
</tr>
<tr>
<td>Ac-H4 (lys12)</td>
<td>rabbit</td>
<td>1:1000 in 5% BSA, TBS-T</td>
<td>Cell Signaling Technology Inc. (Danvers, MA, USA)</td>
</tr>
<tr>
<td>p21</td>
<td>rabbit</td>
<td>1:1000 in TBS-T</td>
<td>New England Biolabs (Beverly, MA, USA)</td>
</tr>
<tr>
<td>Ac-tubulin</td>
<td>mouse</td>
<td>1:1000 in TBS-T</td>
<td>Santa Cruz (Santa Cruz, CA, USA)</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>mouse</td>
<td>1:1000 in TBS-T</td>
<td>Santa Cruz (Santa Cruz, CA, USA)</td>
</tr>
</tbody>
</table>

Table 11: Used secondary antibodies, HRP linked antibodies

<table>
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<th>Target</th>
<th>Source</th>
<th>Dilution</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>rabbit IgG</td>
<td>goat</td>
<td>1:2500 in 5% BSA, TBS-T</td>
<td>New England Biolabs (Beverly, MA, USA)</td>
</tr>
<tr>
<td>mouse IgG</td>
<td>goat</td>
<td>1:2500 in TBS-T</td>
<td>Upstate (Charlottesville, VA, USA)</td>
</tr>
</tbody>
</table>

C.7 Preparation of Nuclear Extracts

Buffers
Buffer A (10 mM HEPES, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF pH adjusted to 7.5 with HCl and replenished to 1 l with water)
Materials and Methods

Buffer B (20 mM HEPES, 1 mM EDTA, 420 mM NaCl, 1 mM DTT, 0.5 mM PMSF, pH adjusted to 7.0 with HCl and replenished to 1 l with water)

Cells were grown in 100 mm dishes and harvested on ice by washing them twice with phosphate buffered saline (PBS) and scraping the cells into 1 ml of PBS. The cells were transferred to pre-cooled Eppendorf tubes and centrifuged (13’000 rpm, 10 min, 4°C), the supernatant was discarded. The pellet was incubated with 100 µl of buffer A for 15 min at 4°C on a laboratory shaker, centrifuged (13’000 rpm, 2 min, 4°C) and the supernatant, which contained cytosolic proteins, was removed. The pellet was further incubated with buffer B for 15 min at 4°C on a laboratory shaker and centrifuged (13’000 rpm, 5 min, 4°C) to precipitate DNA. The supernatant comprising the nuclear extract was used as source of HDAC proteins. Aliquots of 100 µl were stored at -80°C. Protein content was determined by the Bradford method.

C.8 Immunoprecipitation (IP) with A-Sepharose Beads

The protocol used for isolation of HDAC 5 was based on the application of protein A coupled to an insoluble A-sepharose resin. An antibody-HDAC 5 was used to capture HDAC 5 from the nuclear extract. Then, in the solution the antigen-antibody complex was built and bound to protein A on the A-sepharose resin. By centrifugation the complex was precipitated and separated from the lysate proteins. The complex was further characterized.

Sample Preparation
2 ml of nuclear extract, with a protein content of 1 µg/ml, was mixed with 2 ml affinity pure antibody-HDAC 5 (Sigma, H9663, 1 µg/ml protein). The incubation was performed overnight at 4°C. Then, 100 µl A-sepharose beads were added and the mixture was further incubated on a rotating device for 2 hours at 4°C. The mixture was centrifuged at 7’000 rpm for 10 minutes at 4°C. The beads were twice washed with PBS buffer. The beads were incubated with 100 µl lyses buffer
on a rotating device for 30 minutes at 4°C, and then again centrifuged at 7'000 rpm for 10 minutes at 4°C. The supernatant was used for further characterization in the HDAC assay. The beads were mixed with SDS buffer for Western blot analysis.

C.9 HDAC Assay

The assay was carried out in black 386-well plates (Greiner, Frickenhausen, Germany).

As a blank 10 µl assay buffer (25 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂) were mixed with 50 µM assay substrate (10 µl), an ε-acetylated lysine-based substrate C-terminally coupled with 4-methyl-coumarine-7-amide.

As a control (i.e. 100 % HDAC activity) 5 µl assay buffer were incubated with 5 µl of the enzyme source (40 µg/ml nuclear proteins) and 10 µl assay substrate.

TSA as a characterized HDAC inhibitor served as a positive control. Here, the assay buffer (5 µl) contained 4 µM TSA, the enzyme source (5 µl) and the assay substrate (10 µl).

The samples were prepared by addition of 5 µl of the assay buffer, containing different concentrations of the tested compounds (200 µM to 1 mM), with 5 µl of the enzyme source and 10 µl of the assay substrate.

By subsequent addition of 10 µl activator solution (solution of endopeptidase in presence of 1 µM TSA) the deacetylation reaction was stopped and the fluorophore was selectively released from the deacetylated substrate. The fluorophore was excited at 360 nm and its emission was measured at 465 nm in a TECAN Genios microtiter plate reader. All measurements were performed in duplicate. The activity of the enzyme was expressed in relative fluorescence units (RFU).

\[
\text{RFU} = \frac{[\text{RFU}_{\text{enzyme}} - \text{RFU}_{\text{blank}}] \times \text{slope} \times \text{reaction time} \times (V_{\text{enzyme}} + V_{\text{substrate}})}{V_{\text{enzyme}}}
\]

V- volume, µl
Control HeLa nuclear extract was purchased from Upstate (12-309) and used for the comparison of the activity of self-isolated nuclear extracts from VSMC, HUVECs and HeLa cells prepared according to the C.7, page 34.

For the study of natural compounds, extracts and fractions generally, all samples were primarily dissolved in dimethyl sulfoxide and further diluted with the assay buffer in ratio 1:50 prior to the application in the assay to obtain an end concentration of maximal 1% DMSO. Concentrations of 200 µM to 1 mM for pure compounds and of 1 mg/ml to 10 mg/ml for extracts and fractions were studied. IC\textsubscript{50} values of some HDAC modulators were determined by fitting a sigmoidal dose response curve with variable slope (GraphPad Prism).

C.10 Cell Imaging

C.10.1 Preparation of Poly-L-Lysine (PLL) Coated Cover Slips

Glass cover slips (12 mm) were heated with 1M HCl at 50 - 60°C overnight. Then they were cooled to room temperature. Three washing steps were performed for 30 min each, on a laboratory shaker. The cover slips were incubated overnight with 15 ml PLL solution (1 mg/ml in distilled water) in a Petri dish on a laboratory shaker at 4°C. Due to toxicity of free polyaminoacids the cover slips had to be precisely washed ten times with distilled water. They were dried on a filter paper and stored in a sterile Petri dish. Before use, the cover slips were rinsed in 100% EtOH at sterile conditions and dried properly for cell cultivation.

C.10.2 Cell Preparation for Imaging

HeLa cells were cultured overnight on the pretreated PLL coverslips at concentrations of 2 x 10^4 cells/ml in 6 well plates (Greiner, Frickenhausen, Germany).
Materials and Methods

HeLa cells were incubated with the active compounds or extracts for 24 and 48 hours. Studies of the cytoskeletal phenotypes were performed after treatment of the HeLa cells with the extract or fractions at three different concentrations (20.0, 30.0, 40.0 µg per 1 ml DMSO) whereas for the pure compounds (aloin, rutin, sennoside A and B, verbascoside and stachydrine) concentrations of 25, 37.5 and 50 µM were used. The cells were fixed and double immunofluorescent labeled according to an optimized protocol of Mitchison group (http://mitchison.med.harvard.edu).

Buffers
CB stock buffer (40 mM MES pH 6.1, 452 mM KCl, 12 mM MgCl₂ and 8 mM EGTA in 100 ml water)
CBS buffer (500 µl 16% formaldehyde solution, 500 µl CB stock buffer and 500 µl of 1.28 mM sucrose in 2 ml water)
Abdil buffer (TBS-0.1% TX, 2% BSA, and 0.1% azide in 100 ml water)

Sample Preparation
After incubation of HeLa cells with tested compounds for 24 or 48 hours, the DMEM medium was removed and the cells were fixed with 4% formaldehyde solution in CBS buffer for 20 minutes. Then the cells were rinsed with TBS buffer, 3 changes in 5 minutes were adequate. The cells were permeabilized with TBS-0.5% TX buffer for 10 minutes and again rinsed three times with TBS-0.1% TX buffer, 3 changes in 5 minutes were adequate. By addition of Abdil buffer the cells were blocked for 10 minutes, then the incubation with 1 µg/ml fluorescent phalloidin in Abdil buffer followed for 20 minutes at RT.

The tubulin cytoskeleton in fixed cells was observed by use of TRITC-conjugated rhodamine. After incubation of the HeLa cells the medium was removed and the cells were fixed and treated as described above, then the incubation with fluorescent rhodamine (1:500 in Abdil buffer) followed for 60 minutes at RT.
Materials and Methods

5 µg/ml Hoechst in Abdil buffer was used to stain the nuclei by incubation of the cells for 10 minutes at RT.

Finally, the cover slips were washed with TBS-0.1%TX and three times rinsed with TBS. The rest of TBS buffer was drained, and the cover slips rapidly dried under air stream, mounted and sealed on preparative glass covers. Mounting medium was 0.5% p-phenylenediamine (Sigma 87890) in 20 mM Tris, pH 8.8, 90% glycerol. The preparative glasses were stored in dark and cold (-80°C) conditions. Observations were performed by using a fluorescence microscope Olympus BX51, (Olympus Europe GmbH, Hamburg, Germany). The analysis parameters were FITC 900ms (actin), TRITC 6s (tubulin), and Dapi 200ms (DNA) after incubation of the cells for 24 hours. In contrary the experiments with 48 hours of incubation were studied by adjusting the analysis parameters as follows: FITC 600ms, TRITC 2s, and Dapi 90ms.

C.11 Other Technical Equipment and Software

Cell viability analyzer and cell counter Vi-Cell™ XR (Beckman Coulter, Fullerton, CA, USA)
TECAN Sunrise™ microplate reader (TECAN, Mannedorf, Switzerland)
TECAN GENios Pro™ microplate reader (TECAN, Mannedorf, Switzerland)
Fluorescence microscope Olympus BX51 (Olympus Europe GmbH, Hamburg, Germany)
Analyzing software: cell^F software (Europe GmbH, Hamburg, Germany)
Light microscope Olympus CKX31 (Olympus Europe GmbH, Hamburg, Germany)
Camera: Olympus Live View Digital SLR Camera E-330 (Olympus Europe GmbH, Hamburg, Germany)

Analyzing software: Cellprofiler software (www.cellprofiler.org)
Luminescence image analyzer LAS-3000™ (Fujifilm Global, Tokyo, Japan)

Analyzing software: AIDA™ (Fujifilm Global, Tokyo, Japan)
C.12 Statistics

The statistical analyses were performed using GraphPad PRISM™, version 4.03 (GraphPad Software Inc, San Diego, CA, USA). Data are expressed as means of at least three independent experiments including standard deviation or standard error.
D Results

D.1 Assay Validation and Optimization

Various parameters had to be determined in order to apply the fluorimetric assay for the study of the effects of complex plant extracts and mixtures on HDAC activity.

The assay optimization included detailed investigation of several factors, e.g. temperature, incubation times, the role of pH and cations and anions, the intra- and interday reproducibility, the applicability of different enzyme sources and HDAC substrate, as well appropriate positive control and influence of fluorescent compounds. The results obtained in the optimization process added important knowledge for the application of the HDAC assay as suitable tool for the in vitro search of HDAC modulators from plant origin.

D.1.1 Linearity

A linear standard curve of AMC was required for the reliable determination of the activity of the HDAC source and its modulation by the tested compounds. Optimal sensitivity for detection of AMC fluorescence was obtained at 360 nm excitation and 465 nm emission. The intensity of released fluorophore was measured after incubation of the test mixture and the activator solution. A standard curve using different concentrations of unacetylated AMC (0 - 20 µM) displayed excellent linear correlation ($r^2 = 0.9975$) between AMC concentration and the relative fluorescence units after correction for the appropriate blanks Figure 5, page 42.
Results

Figure 5. Standard curve of AMC at concentrations between 0 and 20 µM

D.1.2 Source of HDAC

Three different well-established cell types were used for the preparation of the nuclear protein, namely HUVECs, HeLa and VSMCs. By selecting these cell types, normal human cells (HUVECs) were compared with cancer cells (HeLa) and rodent cells (rat VSMCs). The use of different HDAC sources was used to study different implications of the HDACs in different cell types, for example in epithelial (HeLa), endothelial (HUVECs) and smooth muscle cells (VSMCs).

It has been proven that the total amount of HDACs is almost equally expressed in normal and in tumor cells, the HDACs content is differently distributed in various cell types (De Ruijter et al., 2003). By comparison of the HDAC activity in nuclear extracts of HeLa, HUVEC and VSMC cells a similar level was observed (Figure 6, page 43).

The activity of the enzyme was expressed in units, with one unit (U) corresponding to 1 µM deacetylated product released per minute at RT.
Results

Based on the quantification of the protein content in correlation to the HDAC activity a specific HDAC activity of approximately 2 U/mg nuclear extract was determined for the three different nuclear extracts. Data presenting the enzyme activity of three different experiments with VSMC, HeLa cells and HUVEC, each, are compiled in Table 12.

Figure 6. Enzyme activity of VSMC, HeLa cells and HUVEC in U/mg, (statistics: one-way ANOVA, no significant difference, ns = p, n = 3)

Table 12: Specific enzymatic activity in U/mg of single experiments with VSMC, HeLa cells and HUVEC nuclear extracts

<table>
<thead>
<tr>
<th></th>
<th>protein, mg/ml</th>
<th>Sp. activity, U/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSMC</td>
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</tr>
<tr>
<td>VSMC</td>
<td>2.00</td>
<td>1.57</td>
</tr>
<tr>
<td>VSMC</td>
<td>1.96</td>
<td>1.82</td>
</tr>
<tr>
<td>HeLa</td>
<td>2.25</td>
<td>1.82</td>
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<tr>
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</tr>
<tr>
<td>HeLa</td>
<td>2.14</td>
<td>1.69</td>
</tr>
<tr>
<td>HUVEC</td>
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</tr>
<tr>
<td>HUVEC</td>
<td>1.12</td>
<td>1.57</td>
</tr>
<tr>
<td>HUVEC</td>
<td>1.14</td>
<td>1.22</td>
</tr>
</tbody>
</table>
In conclusion, these experiments showed that the total HDAC activity of the three different sources for nuclear extract was similar and that these cell types can be used to study modulatory effects on HDAC activity.

Due to the easy cultivation conditions of HeLa cells, the enzyme stability during storage and as well the high HDAC activity of the nuclear extract, HeLa nuclear extracts were used for further studies as a main source of HDAC. 1 ml HeLa cells suspension at concentration $10^6$ cells/ml were cultivated in a 60 mm Petri dish with 12 ml DMEM medium, the cells grew rapidly and every second day HeLa nuclear extract was produced.

D.1.3 Influence of Trichostatin A on the HDAC Activity

Trichostatin A (TSA) is a well-characterized, very potent inhibitor of class I and class II HDACs. The IC$_{50}$ values of TSA for HDAC 1, 3, 4, 6 and 10 were reported to be 4.99 nM, 5.21 nM, 27.60 nM, 16.40 nM and 24.30 nM, respectively, whereas for recombinant human HDAC 8 an IC$_{50}$ of 486 nM was determined (Stefanko et al., 2009, Tatamyia et al., 2009).

To confirm the results obtained with the HeLa nuclear extract prepared as described (C.7, page 34) we used trichostatin A as a positive control.

In the set up used an IC$_{50}$ value of 44.04 nM for TSA was determined (Figure 7, page 45). As the HeLa extract comprised a mixture of different HDACs and other proteins this IC$_{50}$ value confirmed reliability of the assay.
Figure 7. IC\text{50} value of TSA for the HeLa nuclear extract in the HDAC assay (statistics: one-way ANOVA, no significant difference, n = 3)

\[ r^2 = 0.9730 \]

To secure the optimal activity of the enzymes, the avoidance of many thawing and freezing cycles as well as storage of the nuclear extract at -80°C were self-evident. Aliquots of 100 µl HeLa nuclear extracts were stored at -80°C and they were used only for one set of analyses at the same day.

Overall, the nuclear extracts of HeLa cells proved to be a very good and easily accessible source of HDAC for the investigation of HDAC modulators.

**D.1.4 Incubation Time**

Incubation time of the enzyme with substrate is a crucial parameter for the enzymatic reaction.

Different time courses were compared to determine the optimal incubation time in the assay-system. Different combinations of incubation time of the enzyme with the substrate in the first step I and further incubation of the samples with the activator solution in the second step II were monitored. The first set of
Results

experiments was performed using 10 min incubation time for both steps. In the second experiment the incubation times were increased to 30 min for both steps, the third experiment was performed 45 min for step I and 30 min for step II. In a fourth set 60 min of incubation for step I was followed by 30 min for step II. The incubation times in experiment five were 60 min for both steps; experiment six was performed with 60 min incubation time in step I and 120 min in step II (Figure 8). All experiments were performed three times in duplicates and appropriate blanks were subtracted.

From the experiments was deduced that obviously after 60 min of incubation for each of the two steps a plateau in the reaction was reached. Thus, 60 min incubation times were used for all further experiments.

Figure 8. Effect of different incubation times for step I and II on HDAC activity

<table>
<thead>
<tr>
<th></th>
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<th>Step II</th>
</tr>
</thead>
<tbody>
<tr>
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<td>10’</td>
</tr>
<tr>
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<td>30’</td>
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</tr>
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<td>4</td>
<td>30’</td>
<td>60’</td>
</tr>
<tr>
<td>5</td>
<td>60’</td>
<td>60’</td>
</tr>
<tr>
<td>6</td>
<td>60’</td>
<td>60’</td>
</tr>
</tbody>
</table>

n= 3
Results

D.1.5 **Incubation Temperature**

Temperature during incubation and measurement was studied due to its strong influence on enzyme activity and stability. The experiments were performed at 22°C, 30°C and 37°C at standard assay conditions (Figure 9).

Figure 9. Enzyme activity in dependence of the incubation temperature (statistics two-tailed t-test vs 37 °C, ** = p < 0.005, n=3)

![Bar chart showing enzyme activity across different temperatures](chart.png)

At 22°C the enzyme retained its activity and displayed a satisfying velocity of the reaction. Thus, all further experiments were performed at 22°C.

D.1.6 **pH Optimum for HDAC Nuclear Extract**

The optimum pH and the influence of metal ions on HDAC activity had been studied by other groups (Mazitschek et al., 2008). Thus, pH of 7.8 was used in all experiments as recommended. The presence of potassium and sodium salts was essential for the enzyme activity and guaranteed by use of respective buffers.
D.1.7 Intra- and Inter-day Reproducibility

Repeated intra-day (Figure 10) and inter-day (Figure 11, page 49) measurements were performed in order to investigate the reproducibility of the assay. For assessment of intra-day reproducibility three experiments were performed at 10 and 12 a.m., and 4 p.m. (in duplicate, each).

Inter-day reproducibility included measurements on three subsequent days. The experiments were performed in duplicate at 10 a.m., with three different lots of the HDAC source.

Figure 10. Intra-day reproducibility

![Graph showing RFU levels for different times and conditions with error bars for n = 3](image)

Standard deviations of ±1.82% for the positive control TSA and ±5.10% for the enzyme incubated with substrate only were determined for intra-day measurements.
Results

Figure 11. Inter-day reproducibility

![Graph showing inter-day reproducibility](image)

Standard deviations of ±2.60% for TSA and ±1.08% for the enzyme incubated with the substrate, respectively, were obtained for inter-day measurements.

The values of intra- and inter-day analysis confirmed the excellent reproducibility of the HDAC assay.

D.1.8 Influence of Autofluorescent Compounds on the Assay

Due to the traditional use of the cortex from *Viburnum opulus* L. in cardiac complaints (VOLKSMED), extracts of this drug should serve as an example for the bioactivity-guided fractionation based on the HDAC assay. Four extracts of different polarity were prepared with CH$_2$Cl$_2$, EtOAc, MeOH and H$_2$O (C.2.3, page 18). In the analyses of the extracts in the HDAC assay massive interferences were observed. It was not possible to determine the influence on HDAC activity. Therefore, TLC analyses were performed with the extracts. By detection under UV 365 nm several dark blue fluorescent zones with R$_f$ values 0.25, 0.34, 0.53, 0.66 and 0.85 were observed due to a high content of coumarin...
Results

derivatives (Figure 12). Obviously these compounds were responsible for the interferences. Viburni cortex contains scopoletin, scopolin, esculetin and 2'-p-coumaroyl-dihyropenstemid as major constituents (Teuscher et al., 2009).

Figure 12. TLC of CH$_2$Cl$_2$-, EtOAc- and MeOH- extracts from Viburni cortex. System 1 (C.3.3, page 22) and detection under UV 365 nm

As autofluorescence of some compounds in complex plant extracts was a parameter which influenced the precision of the measurements, the assay had to be adapted. Thus, in analyses of extracts, the measurement of the autofluorescence of the tested mixtures in presence of substrate is an indispensable prerequisite for the application of the assay in bioactivity-guided approaches.
By analyses coumarin derivatives, such as esculetin or herniarin, massive interferences with the fluorophore AMC even at low concentrations (approximately 100 µM) were proven, which could lead to false positive results for HDAC modulation. Thus, all extracts under investigation in the HDAC assay have to be checked for dark blue-fluorescent substances in suitable thin layer chromatography (TLC) systems before testing in the assay, in order to avoid falsification of the results.

Additionally, the assay has to include measurement for autofluorescence of the analyzed solution and the respective values have to be corrected.

D.1.9 Amount of Organic Solvents Applicable in the Assay

In the bioactivity-guided search for HDACm from plants, extracts of different polarity have to be studied. Thus, especially the solubility of apolar fractions or extracts for applicability in the assay had to be checked.

DMSO is a universal solvent for various compounds with different polarity. The use of DMSO in cell culture is ubiquitous as well. Nevertheless, numerous studies have shown that the cells drastically react under its influence. Depending on the cell lines and cell types, as well as the applied DMSO concentration and the time of incubation, the process of differentiation and apoptosis are induced or blocked. The effectiveness of DMSO on cell differentiation process has been demonstrated by a large numbers of studies. The investigation of the cellular mechanism of DMSO on cell cycle is still unclear (Bolduc et al., 2001, Nickels, 2004).

The influence of DMSO on the HDAC assay was tested at concentrations of 1, 1.25, 2, 2.5 and 5% (Table 13, page 52). The assay was robust against 5% DMSO. At all tested concentrations the modulation of the enzyme remained in the deviation of the method.
Results

Table 13: Influence of DMSO concentrations on the HDAC activity

<table>
<thead>
<tr>
<th>sample</th>
<th>concentration, %</th>
<th>activity, %</th>
</tr>
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<tbody>
<tr>
<td>DMSO 1%</td>
<td>1.00</td>
<td>108.0</td>
</tr>
<tr>
<td>DMSO 1.25%</td>
<td>1.25</td>
<td>96.6</td>
</tr>
<tr>
<td>DMSO 2%</td>
<td>2.00</td>
<td>111.4</td>
</tr>
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<td>DMSO 2.5%</td>
<td>2.50</td>
<td>98.9</td>
</tr>
<tr>
<td>DMSO 5%</td>
<td>5.00</td>
<td>112.8</td>
</tr>
</tbody>
</table>

DMSO proved to be the superior solvent for the use in the HDAC assay due to its high potency to dissolve various plant extracts with different polarity.

The assay substrate, AMC, TSA and all tested compounds were therefore dissolved in respective concentrations in DMSO and then diluted in a ratio 1:50 with the assay buffer to final DMSO concentrations of 2%.

Methanol was also tested as a solvent at concentrations of 1%, 2% and 5% to study its influence on HDAC assay. At concentrations of 1 and 2% it did not show any interactions with the enzymatic activity. The disadvantage of pure methanol was the poor solubility of several fractions.

Thus, DMSO was chosen as the superior solvent and was further used for all tests.

Although 2 to 5% DMSO can be added for the dissolution of extracts, fractions or pure compounds, the determination of the activity of very apolar primary and secondary plant metabolites such as fatty acids, sterols, carotenoids and fat-soluble vitamins was not possible. Due to the fact that the maximum amount of DMSO in the assay is 5%, the incomplete solubility of these compounds in mixtures of DMSO and assay buffer prevented the measurements.
Results

D.1.10 Influence of Different Classes of Natural Compounds on the HDAC Assay

To exclude interferences of other substances with the HADC assay, the influence on HDAC activity of primary and widespread secondary plant metabolites, such as sugars, vitamins, amino acids, phenolic and organic acids, tannins, chlorophyll, pigments etc. which are common and abundant constituents in extracts, were studied.

Monosaccharides such as glucose and fructose or the disaccharide saccharose at concentrations of 1 mM showed no obvious interference of sugars with the assay.

Chlorophyll (chlorophyll$_a$ 420 µM, chlorophyll$_b$ 350 µM) as well as other plant pigments e.g. purpurin (630 µM) did not affect the measurement as well.

Solutions of various organic acids like citric, ascorbic, gallic, salicylic, cinnamic, caffeic, ferulic, vanillic or isovanillic acids (1 mM) did not interfere with the assay.

The modulatory effect of some amino acids was studied at a concentration of 1 mM. The experiments were performed with nonpolar amino acids like alanine, methionine and glycine, polar amino acid e.g. arginine, and slightly polar amino acids such as cysteine and tryptophan. The results showed that at the tested concentration (1 mM) the influence on HDAC activity was marginal, appr. 5 % inhibition was determined.

The inhibitory potential of water-soluble vitamins was studied as well. Several representatives of the group of B vitamins (thiamine, riboflavin, niacin and nicotinamide, pyridoxine and pyridoxal, folic acid, and cyanocobalamin) at concentrations of 1 mM were tested and did not show an inhibitory effect.

Ubiquitous flavonoids such as hyperoside, rutin and quercetin influenced the activity of the histone deacetylases only at higher micromolar concentrations. IC$_{50}$ values of 603 µM, 420 µM and 560 µM, respectively, were determined. Less widespread flavonoids and phenols such as apigenin, chrysin, saponarin,
myricetin, myricitrin, taxifoline, catechin, epicatechin, biochanin A, daidzein, daidzin, formononetin, genistein, glycitein or glycitin showed no or only very marginal inhibitory effects at concentrations between 350 µM to 1 mM. Other phenolic compounds such as chlorogenic acid (1 mM) or verbascoside (400 µM) did not influence the HDAC activity.

For tannic acid and catechin gallate an inhibitory influence on HDAC activity at a concentration of approximately 300 µM was observed. This effect was due to an obvious precipitation of proteins and not a direct inhibition of the enzymatic activity.

Due to the observed effects of tannic acid and catechin gallate the general applicability of the assay to plant extracts which contain tannins had to be checked. For this purpose an aqueous extract of Ratanhiae radix (C.2.2, page 18) was prepared and tested, as this drug contains up to 15% tannins (Blaschek et al., 2009).

The aqueous extract displayed an inhibitory effect on HDAC activity, with an IC\textsubscript{50} of approximately 545 µg/ml. This inhibition, however, was again due to precipitation of the HDAC proteins. This was confirmed by the preparation of a detannified extract (C.2.2, page 18), which showed an inhibition of approximately 25% at a concentration of 10 mg/ml (Figure 13, page 55).
These findings confirmed the expected fact that tannins strongly inhibit HDAC activity due to precipitation of the enzymes. In order to avoid false positive results detannification of extracts rich in tannins before testing is indispensable.

D.2 Screening of Secondary Plant Metabolites for their Potential for HDAC Modulation

After the comprehensive validation of the fluorimetric HDAC assay several secondary plant compounds were selected due to their structural diversity and their relationship to known HDAC inhibitors for a screening.

D.2.1 Anthraderivatives

In some cancer treatments (leukemia, lymphomas, breast and esophageal carcinomas, osteosarcomas, Kaposi’s sarcoma, soft tissue, testicular, gastric, ovarian, liver, bile-dict, pancreatic and endometrial carcinomas) doxorubicin, an anthraderivate, is a powerful chemotherapeutic (Christiansen and Autschbach,
2006). Its mechanism of action is a direct interaction with DNA by intercalation. Recently, it was shown that some HDACi, e.g. valproic acid, increased the sensitivity of osteosarcoma to doxorubicin. The integration of HDACi into cytotoxic therapy against cancer might lead to more specific cancer treatment (Wittenburg et al., 2010).

Due to these facts the influence of three types of anthraderivatives on HDAC activity was studied and IC\textsubscript{50} values were determined. The aloines aloin A (IC\textsubscript{50} 270 µM) and B (IC\textsubscript{50} 300 µM), the anthraquinones aloemodin (IC\textsubscript{50} 420 µM) and emodin (IC\textsubscript{50} 370 µM) showed activity in the HDAC assay. The best effect was achieved with the dianthrones sennoside A (IC\textsubscript{50} 180 µM) and B (IC\textsubscript{50} 220 µM), although as well at micromolar concentrations only. These results pointed to a better activity of those anthraderivatives in which C\textsubscript{10} is blocked by a C-C bond.

D.2.2 Alkaloids, Betaines and Cardiac Glycosides

In contrary to these anthraderivatives, alkaloids like atropine, berberine, boldine, caffeine, cephaeline, chelidonine, quinine, emetine, eserine, papaverine, cardiac glycosides like digitoxin or gitoxin, and betaines like stachydrine or betonicine remained without effects up to concentrations of 400 µM.

D.2.3 Ginkgolic Acids

Due to the reports of inhibition of tumor cell proliferation by ginkgolic acids (Yang at al., 2004) a set of ginkgolic acids (Table 14, page 57) kindly provided by Dr. E. Koch (Dr. Willmar Schwabe GmbH & Co. KB, Karlsruhe, Germany) was tested for a modulatory effect on the HDAC activity.
Table 14: Ginkgolic acids tested in the HDAC assay

<table>
<thead>
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<th>Code</th>
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<th>MW</th>
<th>CAS No.</th>
</tr>
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<td>GS-007</td>
<td><img src="image6" alt="Chemical structure" /></td>
<td>374.57</td>
<td>C_{24}H_{38}O_{3} 69506-63-4</td>
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<tr>
<td>GS-008</td>
<td><img src="image7" alt="Chemical structure" /></td>
<td>376.58</td>
<td>C_{24}H_{40}O_{3} 20261-39-6</td>
</tr>
</tbody>
</table>
Results

The samples were analyzed in duplicates at concentrations of 1 mg/ml. The results showed that the ginkgolic acids did not possess modulatory activity on the enzymatic activity at this concentration, Table 15.

Table 15: Influence of ginkgolic acids on HDAC activity (n = 2)

<table>
<thead>
<tr>
<th>Sample</th>
<th>concentration, mM</th>
<th>activation, %</th>
</tr>
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<tbody>
<tr>
<td>GS-001</td>
<td>0.68</td>
<td>17.9</td>
</tr>
<tr>
<td>GS-002</td>
<td>0.73</td>
<td>7.6</td>
</tr>
<tr>
<td>GS-003</td>
<td>0.73</td>
<td>9.7</td>
</tr>
<tr>
<td>GS-004</td>
<td>0.73</td>
<td>4.9</td>
</tr>
<tr>
<td>GS-005</td>
<td>0.78</td>
<td>1.3</td>
</tr>
<tr>
<td>GS-007</td>
<td>0.68</td>
<td>1.3</td>
</tr>
<tr>
<td>GS-008</td>
<td>0.65</td>
<td>2.4</td>
</tr>
</tbody>
</table>

At the standard assay conditions the ginkgolic acids were not potential HDAC modulators.

D.2.4 Comparison of in silico HDAC Inhibition with the in vitro HDAC Assay

An in silico model has been developed by the research group of the Center for Molecular Biosciences Innsbruck, University of Innsbruck, for the search for HDAC inhibitors. According to the developed in silico model 27 pure compounds (indicated from 1 – 27) and 6 plant extracts (named A – F) were selected as potent inhibitors. To check the validity of the theoretical approach these compounds and extracts were tested in the HDAC assay (Table 16, page 59).
Results

Table 16: Inhibitory effect of \textit{in silico} hits in the in vitro HDAC assay in %

<table>
<thead>
<tr>
<th>N</th>
<th>Abbreviation</th>
<th>10µM</th>
<th>25µM</th>
<th>50µM</th>
<th>100µM</th>
<th>250µM</th>
<th>10mg/ml</th>
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<td>1</td>
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<td>2</td>
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<td>3</td>
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<td>4</td>
<td>NSC96573</td>
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<tr>
<td>5</td>
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<td>7</td>
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<tr>
<td>A</td>
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<tr>
<td>B</td>
<td>Lin-str-rd 2</td>
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<td></td>
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</tr>
<tr>
<td>C</td>
<td>Leo-het-hb 1</td>
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</tr>
<tr>
<td>D</td>
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<td>3.4</td>
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</tr>
<tr>
<td>E</td>
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</tr>
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<td>F</td>
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<td>45.6</td>
<td></td>
<td>0.0</td>
<td></td>
</tr>
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</table>

All compounds were tested at 250 µM, whereas the concentration of the extracts in the assay was 10 mg/ml in the first screening. Compounds with an inhibitory effect higher than 20% were tested further at concentrations of 100 µM, 50 µM, 25 µM and 10 µM.
Results

The compounds 2, 6, 7, 8, and 19 at a concentration of 250 µM showed the strongest effect with 45.1%, 79.9%, 42.5%, 43.1%, and 49.4% inhibition, respectively. Compound 6 was the most potent. Thus, this substance was further investigated and an IC$_{50}$ value of 115 µM was determined.

Extract F (Spa-sub-hb 2) at a concentration of 10 mg/ml exhibited an HDAC inhibition of 45.6%.

The chemical structures of the tested compounds are shown in Supplements, page 138.

The low HDAC inhibitory potential of the in silico hits can be explained by the fact that the in silico system was established for a study of the isoform HDAC 8, whereas in the in vitro assay HeLa nuclear extract was used as source of HDACs. Due to lack of detailed information on the content of HDAC 8 in the HeLa nuclear extract a close correlation of the in silico results to the in vitro results was not achieved. Nevertheless, some of the tested compounds seem to be potential inhibitors to other HDAC isoforms as well.

D.2.5 Effect of TCM Decoctions on the HDAC Activity

A preparation of traditional Chinese medicine is currently under investigation in a model of experimental cardiac infarction in mice. The verum group received a decoction prepared from the roots of Glycyrrhiza uralensis Fisch, the tubers of Aconitum Carmichaeli Ross, the rhizomes of Zingiber officinale Debx. and the bark of Cinnamomum cassia Nees instead of water. It was observed that in the verum group survival rate was significantly higher than in untreated control (Ma, unpublished data).

Analyses of the decoctions of the single herbs, indicated as TCM 1 to TCM 4 and TCM 5 for the mixture, were performed and different effects were observed (Figure 14 and Table 17).
Results

Figure 14. Effect of single drugs and TCM mixtures on HDAC activity (statistics: two-tailed t-test vs TCM5, ** = p < 0.0088, *** = p < 0.0001, n = 3)

<table>
<thead>
<tr>
<th>code</th>
<th>herb</th>
<th>concentration, mg/ml</th>
<th>inhibition, %</th>
<th>activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM1</td>
<td>Glycyrrhiza</td>
<td>2.1</td>
<td>17.1</td>
<td></td>
</tr>
<tr>
<td>TCM2</td>
<td>Zingiber</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCM3</td>
<td>Aconitum</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCM4</td>
<td>Cinnamomum</td>
<td>1.8</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td>TCM5</td>
<td>TCM mixture</td>
<td>2.2</td>
<td>36.5</td>
<td></td>
</tr>
</tbody>
</table>

TCM 1 showed an inhibitory effect whereas TCM 2 and TCM 3 had no effect on HDAC activity. In contrary a slightly activating effect of TCM 4 and a more pronounced one was observed for TCM 5.

D.2.6 Effect of Natural Compounds on HDAC1, HDAC2 and HDAC3 Activity in vitro

Due to HDAC inhibiting activity of aloines (aloin) and dianthrone (sennosides A and B) and the structural diversity of capsaicin, rutin, stachydrine and verbascoside they were further investigated. At concentrations of 50 µM the pure
Results

62 compounds were tested for an inhibitory effect in a homogeneous system with recombinant pure enzymes HDAC1, HDAC2 and HDAC3. Only sennoside A showed inhibition of 40% on isoform HDAC3 at a concentration of 50 µM. The extract of Leonuri herba and the fractions SPE F2, F3, F4 at concentration of 50 µg/ml were studied for their effect as well. The experiments were performed in triplicates. Appropriate blanks were subtracted from the values.

D.3 Bioactivity-Guided Fractionation of an Extract from Leonuri herba

To investigate the applicability of the adapted and validated assay for bioactivity-guided fractionations of plant extracts in addition to the study of different pure plant compounds, the optimized assay was further tested on extracts of Leonuri herba.

Leonuri herba, due to its traditional use against cardiac complaints was selected as an example. Extracts of different polarity were prepared. The extraction was performed with 9 g plant material (see C.2.4, page 20) and yielded CH₂Cl₂-, EtOAc-, MeOH- and H₂O extracts.

These extracts were tested at concentrations of 10 mg/ml for HDAC inhibition. The methanolic extract was the most active (Figure 15, page 63). Nevertheless, its activity was significantly lower than the one of 1 µM TSA.
Results

Figure 15. Effect of extracts from Leonuri herba on HDAC activity (statistics: two-tailed t-test vs positive control 1µM TSA, * = p < 0.026, ** = p < 0.051, *** = p < 0.0005, n = 3)

The composition of the extracts was investigated by TLC analyses. After detection with anisaldehyde sulfuric acid reagent in the dichloromethane extract mainly terpenoid compounds with Rf values 0.43, 0.47, 0.86 and 0.92 were detected. In the methanolic extract two pronounced green bands at Rf 0.33 and 0.52 were detected (Figure 16, page 64). Under UV 365 nm red fluorescent bands of chlorophyll were visible in the dichloromethane, ethyl acetate, and methanolic extracts with Rf values between 0.66 and 1.
Results

Figure 16. TLC of the different extracts from Leonuri herba, system 1 (page 23) and detection at day light after spraying with anisaldehyde sulfuric acid reagent and under UV 365 nm

Due to the amount of chlorophyll in the methanolic extract, these accompanying compounds had to be removed and the methanolic extract was further investigated after chlorophyll separation (see C.2.6., page 19). The chlorophyll removal was successfully performed in step I by partition between water and chloroform and subsequently water and ethyl acetate, in step II (C.2.6, page 19).

![TLC Image]

- MeOH extract 93.0 g
- CR CHCl\textsubscript{3} 23.4 g
- H\textsubscript{2}O phase
- CR EtOAc 1.7 g
- CR H\textsubscript{2}O 67.8 g
TLC analyses were used to confirm that the chlorophyll was mainly enriched in the chloroform phase (Figure 17). The intensive red colored zones with $R_f$ values of 0.9 to 1 confirmed that the partition with chloroform was effective for the removal of chlorophyll.

Figure 17. Chlorophyll separation steps of the MeOH extract, system 1 (C.3.3, page 23) and the detection under UV 365 nm after spraying with NEU reagent.

MeOH is original methanolic extract obtained by extraction of the drug with methanol, CR CHCl$_3$ are the combined chloroform phases after double partition of the methanolic extract with water and chloroform, CR EtOAc ethyl acetate phases after double partition with water and ethyl acetate, the remaining aqueous phase was indicated CR H$_2$O.
Results

The extracts MeOH, CR CHCl$_3$, CR EtOAc and CR H$_2$O obtained after chlorophyll removal were additionally analyzed by HPLC, see Figures 18-21, pages 66-68.

Figure 18. HPLC of the methanolic extract from the drug (MeOH), system 1 (page 24), gradient program 1 (page 24), detection at 254 nm
Results

Figure 19. HPLC of CR CHCl₃, system 1 (page 24), gradient program 1 (page 24), detection at 254 nm

Figure 20. HPLC of CR EtOAc, system 1 (page 24), gradient program 1 (page 24), detection at 254 nm
The HPLC analyses confirmed that by liquid partition of the methanolic extract between water and chloroform and water and ethyl acetate, the phenolic compounds were enriched in the aqueous phase. Some apolar compounds e.g. oily matters, terpenes and chlorophyll were removed from the methanolic extract.

D.4 Identification of Compounds in Fraction CR H₂O from Leonuri herba

TLC was used to identify the phenolic compounds in fraction CR H₂O from Leonuri herba. Rutin, caffeic acid, chlorogenic acid and hyperoside were compared with the compounds in the extracts. It was possible to confirm that the two orange zones in the extract as rutin (Rᵢ= 0.38) and hyperoside (Rᵢ= 0.56). The whitish fluorescent zone with a Rᵢ value of 0.40 revealed chlorogenic acid, Figure 22, page 69.
Results

Figure 22. TLC analysis of fractions CR H_2O, F2 and F3 with phenolic acids and flavonoids, system 2 (page 22), detection under UV 365 after spraying with NEU reagent

Standards rutin, caffeic acid (CA), chlorogenic acid (CCIA), rosmarinic acid (RA) and hyperoside (Hyp) were used to elucidate the composition of fractions F2, F3 and CR H_2O.

Further, by TLC analysis verbascoside (VS) was confirmed in fraction CR H_2O with R_t value 0.54 (Figure 23, page 70).
Results

Figure 23. TLC analysis of fraction CR H₂O with verbascoside (VS), system 2 (page 22), detection under UV 365 nm after spraying with NEU reagent

Fraction CR H₂O additionally contained glucose (Glu) with a Rᵢ value of 0.83, fructose (Fru) with a Rᵢ value of 0.83 and rhamnose (Rha) with a Rᵢ value of 0.9. Mannose (Mann) was not detected (Figure 24).

Figure 24. Sugar analysis by TLC of fraction CR H₂O, system 3 (page 23), detection at day light after spraying with anisaldehyde sulfuric acid reagent
D.5 Analysis of the SPE Fractions

After chlorophyll removal from the methanolic extract fraction CR H₂O was submitted to fractionation by SPE and five fractions F1 to F5 were obtained.

D.5.1 HDAC Assay

The fractions F1, F2, F3, F4 and F5 obtained by SPE were investigated for their effect on HDAC activity at concentrations of 1 mg/ml. The fraction F3 showed the highest potential to inhibit HDAC activity with 76% inhibition at 1 mg/ml. The effect was comparable to 1 µM TSA. Weak activity was determined for fraction F4, whereas fractions F1, F2 and F5 remained without effect (Figure 25).

Figure 25. Effect of SPE fractions on HDAC activity (statistics: two-tailed t-test vs positive control 1µM TSA, ** = p < 0.0017, *** = p < 0.0007, n = 3)

D.5.2 TLC Fingerprinting

In order to get detailed information about the chemical composition of the fractions they were characterized by TLC (Figure 26, page 73)
Results

In TLC fingerprinting of the SPE fractions focusing on phenolics the original fraction CR H2O showed three major whitish blue fluorescent compounds with Rf values 0.64, 0.89 and 0.98 under UV 365 nm after spraying with NEU reagent. Two of these compounds were enriched in fraction F2. Detection at day light after spaying with anisaldehyde sulfuric acid reagent revealed high amounts of polar compounds in CR H2O. In F1 three major compounds with Rf values of 0.72 (blue zone), 0.78 (green zone) and 0.98 (blue-green zone) were observed under UV 365. Detection at day light after spaying with anisaldehyde sulfuric acid reagent showed high amounts of polar compounds with Rf < 0.23 in F1, presumably sugars. Besides the two fluorescent major compounds with Rf values 0.64 and 0.89 F2 additionally contained various compounds of medium polarity (Rf= 0.24, 0.32, 0.41, 0.55 and 0.67). Three characteristic orange fluorescent zones were detected in fraction F3, presumably flavonoid compounds with Rf values 0.67, 0.78 and 0.81. Day light detection with the anisaldehyde sulfuric acid reagent showed a complex mixture of polar compounds. Fractions F4 and F5 contained mainly apolar substances.
Figure 26. TLC fingerprint of SPE fractions. System 1 (page 22) and detection under UV 365 nm after spraying with NEU reagent and at day light after spraying with anisaldehyde sulfuric acid reagent

**D.6 Separation of F3 by CPC**

Centrifugal Partition Chromatography is excellently suitable for the separation of flavonoid mixtures (Green, 2007). To avoid any loss of active compounds this method was chosen for the further fractionation of F3. For this purpose 86.0 mg F3 were dissolved in ethyl acetate-water and separated (see C.3.1, page 21). Fractions were collected, each fifth fraction was characterized by TLC and according to the similarity in chemical composition the fractions were pooled. The combined fractions of CPC1 were named from A1 to U1. The yields are presented in Table 18, page 74 and the TLC analyses of the combined fractions are given in Figure 27, page 75.
Results

Table 18: Yields of CPC₁ fractions

<table>
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</tbody>
</table>
Results

Figure 27. TLC of CPC fractions, system 1 (page 22), detection under UV 365 nm after spraying with NEU reagent and at daylight after spraying with anisaldehyde sulfuric acid reagent.
Results

Fraction C\textsubscript{1} revealed a major greenish compound under UV 365 nm, which appeared pale greenish after detection with anisaldehyde sulfuric acid reagent at day light with R\textsubscript{f} value 0.78. Fraction F\textsubscript{1} showed two yellowish fluorescent zones under UV 365 nm with R\textsubscript{f} values 0.67 and 0.72, which after spraying with anisaldehyde sulfuric acid reagent at day light appeared pale greenish. Fractions G\textsubscript{1} showed a blueish fluorescent compound under UV 365 nm with R\textsubscript{f} value 0.83, which after anisaldehyde sulfuric acid reagent was indicated as a dark green zone. A compound with similar characteristics was detected in fraction M\textsubscript{1}. Additionally in fraction G\textsubscript{1} a second blueish fluorescent compound was visible under UV 365 nm (R\textsubscript{f} value 0.68), which was hardly detectable with anisaldehyde sulfuric acid reagent at day light. Fraction I\textsubscript{1} revealed a major greenish fluorescent compound under UV 365 nm with a R\textsubscript{f} value of 0.59, which had a greenish color after spraying with anisaldehyde sulfuric acid reagent at day light observation. Fraction O\textsubscript{1} showed diverse green zones under UV 365 nm, the detection with anisaldehyde sulfuric acid reagent at day light revealed three major compounds with R\textsubscript{f} values 0.11, 0.24, 0.43. In fraction P\textsubscript{1} an intensive greenish fluorescent compound with R\textsubscript{f} value 0.59 was detected under UV 365 nm. Fraction Q\textsubscript{1} contained a major whitish fluorescent compound with R\textsubscript{f} value of 0.77. Fraction R\textsubscript{1} showed one major compound with orange fluorescence and a R\textsubscript{f} value of 0.48, at day light after applying anisaldehyde sulfuric acid reagent these substances in R\textsubscript{1} and Q\textsubscript{1} showed greenish color. Fraction S\textsubscript{1} contained as well one major compound with a R\textsubscript{f} value of 0.48, but under UV 365 nm it showed white fluorescence and after spraying with anisaldehyde sulfuric acid reagent revealed a dark green zone. Fraction T\textsubscript{1} was a mixture of highly whitish fluorescent compounds, the major of them had a R\textsubscript{f} of approximately 0.42, the same compound appeared in fraction U\textsubscript{1}. The orange band with R\textsubscript{f} = 0.3 in fraction T\textsubscript{1} with R\textsubscript{f} = 0.48 in fraction R\textsubscript{1} were identified as rutin and hyperoside, respectively. The strong whitish fluorescent compound in fractions T\textsubscript{1}, and U\textsubscript{1} was chlorogenic acid (R\textsubscript{f} = 0.42) (Figure 22, page 69). Isochlorogenic acid with R\textsubscript{f} = 0.77 was tentatively identified in fraction Q\textsubscript{1} (Wagner and Bladt, 1996).
Results

Fractions O₁ to U₁ were additionally analyzed by HPLC and as well their effect on HDAC activity was monitored. The most potent fractions were R₁ and T₁, showing HDAC inhibition of appr. 90% and 87% at concentrations of 5.5 mg/ml and 5.7 mg/ml, respectively (Figure 28), which at these concentrations were not significantly different from 1 µM TSA.

The effect of fractions A₁ to N₁ could not be monitored in the assay due to problems with solubility in the assay buffer.

Figure 28. Effect of CPC₁ fractions on HDAC activity (statistics: two tailed t-test vs positive control 1 µM TSA, ns = p = 0.15 (R) and 0.06 (T), ** = p = 0.04, *** = p < 0.0001, n = 3)

Due to the strongest inhibitory effect of fractions R₁ and T₁, further investigations were performed with these fractions.

HPLC analysis of fraction R₁ showed two major peaks at Rᵣ 11.39 and 11.82 with identical UV spectra with maxima at 256 nm and 354 nm (Figure 29, page 78).
Results

Figure 29. HPLC analysis of fraction R₁ and UV spectra of the two major compounds. System 1 (page 24), gradient program 1 (page 24), detection at 254 nm

UV spectra at R₁ 11.39 and 11.82
In order to collect more material for further analyses of the fractions R and T a second separation of 80 mg F3 by CPC was performed. Based on TLC analyses of each fifth fraction those with similar profile were pooled. The combined fractions of CPC\textsubscript{2} were named again from A\textsubscript{2} to U\textsubscript{2}. The yields are presented in Table 19.

Table 19: Fractions collected by CPC\textsubscript{2}

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Detailed analyses of fractions R\textsubscript{1} and R\textsubscript{2} were performed by TLC. Two intensive fluorescent orange zones were detected with R\textsubscript{f} values of 0.60 and 0.62 (Figure 30, page 80). The use of authentic standard rutin (R\textsubscript{f}= 0.62) confirmed the more apolar zone as rutin, whereas the more polar one was an unknown compound.
Figure 30. TLC of the subfractions 82 and 83 of fraction R (CPC$_{1,2}$), system 2 (page 22), detection under 365 nm after spraying with NEU reagent

Fractions R$_1$ and R$_2$ were additionally analyzed by HPLC and each of them showed two compounds, the unknown (R$_t$ = 11.39) and rutin (R$_t$ = 11.89) at different concentrations.

HPLC analysis of subfraction 82$_2$ revealed a higher concentration of the unknown compound (Figure 31, page 81). In subfraction 83$_2$ the rutin concentration was higher (Figure 32, page 82).
Results

Figure 31. HPLC analysis of subfraction 82 and UV spectra of the two major compounds. System 1 (page 24), gradient program 1 (page 24), detection at 254 nm

UV spectra at Rₜ 11.39 and 11.82
Results

Figure 32. HPLC analysis of subfraction 83₂ and the UV spectra of its two major peaks. System 1 (page 24), gradient program 1 (page 24), detection at 254 nm UV spectra at Rₜ 11.39 and 11.89

As a second potent fraction T was further analyzed. T₁ was a pool of subfractions 87, 88 and 89 from CPC₁ (Table 18, page 74) and T₂ of subfraction 87, 88 and
Results

89 from CPC₂ (Table 19, page 79). HPLC analysis showed one major compound with Rₜ 13.79 and a UV maximum at 330 nm (Figure 33).

Figure 33. HPLC analysis of fraction T and UV spectrum. System 1 (page 24), gradient program 1 (page 24), detection at 254 nm

The total yield of T (T₁ + T₂) was 31.4 mg. Due to the similarity in the composition of fractions T and U were combined to fraction “TU” with a total amount of 38.9 mg. This sample was loaded on a Sephadex LH 20 column and eluted with water. 80 fractions with a volume of 2 ml were collected and lyophilized.
D.7 Column Chromatography - Preparative Scale

As the amount of fraction TU was too low to isolate the active compound, a higher amount of fraction CR H₂O extract (60 g) had to be fractionated. Due to the very high cost of SPE in such a scale column chromatography on Sephadex LH 20 (SCC) was the method of choice. The extract was fractionated in two portions of 30 g, each. The elution was started with water and continued with increasing portions methanol at a rate of 40 ml/h. F₁ was used to indicate fractions eluted with water, F₂ with 20% methanol, F₃ with 40% methanol, F₄ with 80% and F₅ with 100% methanol. The fractions were analyzed, according to the similar composition pooled and the yields are presented in Table 20.

Table 20: SCC fractions after separation on a Sephadex LH 20 column

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</table>
Results

The SCC F2, 3, 4 and 5 fractions were studied at a concentration 1 mg/ml for their modulatory effect on the HDAC activity. The results are presented in Figure 34.

Figure 34. Effect of SCC F2, 3, 4 and 5 fractions on the HDAC activity, n = 2

Fraction SCC F2 showed a modulatory effect on HDAC activity from 50% to 70%. Fractions SCC F3a and SCC F3c showed inhibition of 65% and 77%, respectively, whereas the rest of SCC F3 fractions showed approximately 50% inhibition. Generally all SCC F4 fractions showed strong inhibitory effect between 70% and 75%, due to lack of time the detailed investigation was not performed. SCC F5 fractions demonstrated a slight inhibitory effect on the HDAC activity or remained without effect.
In order to investigate the chemical composition of several SCC fractions SCC F2e, SCC F3a, SCC F3b, SCC F3c, SCC F3d and SCC F3e, they were further characterized by TLC (Figure 35).

Figure 35. SCC selected fractions, system 1 (page 22), detection at day light after spraying with anisaldehyde sulfuric acid reagent and under UV 365 nm after spraying with NEU reagent.

Fraction SCCF 3a showed a major green fluorescent zone with $R_f$ value 0.30, additionally compounds at low concentrations with $R_f$ values of 0.22, 0.26, 0.38 and 0.61 were detected under UV 365 nm and spraying with NEU reagent. The TLC plate after spraying with anisaldehyde reagent and detection at day light revealed one major compound with a $R_f$ value of 0.3. In fraction SCC F3b several green and two blue fluorescent compounds with $R_f$ values of 0.22, 0.26, 0.30, 0.44, 0.52 and 0.61 (green fluorescent), 0.34 and 0.38 (blue fluorescent) were
Results observed. The observation at day light revealed four greenish compounds with \( R_f \) values 0.22, 0.30, 0.34 and 0.38. SCC F3c and SCC F3d showed almost identical profiles, three green fluorescent zones with \( R_f \) values 0.26, 0.56, and 0.67 which were detected as green zones as well at day light after spraying with anisaldehyde sulfuric acid reagent. SCC F3e showed green (\( R_f \) = 0.16, 0.19, 0.26, 0.30 and 0.34) and blue (\( R_f \) = 0.43 and 0.49) fluorescent zones and one orange band (\( R_f \) = 0.56). Fraction SCC F2e contained one major compound with a \( R_f \) value of 0.30, detected with both systems. Due to its potential to modulate HDAC activity fraction SCC F3c was used for further isolation of a pure compound.

D.8 Semi-Preparative HPLC

In order to isolate and characterize the major compound in fraction SCC F3c a semi-preparative HPLC was performed, according to the C.3.5, page 25.

60 mg of SCC F3c were separated by HPLC on a Nucleosil column eluting with 40% MeOH and increasing the solvent polarity from 40% to 95% MeOH within 20 minutes. 33 fractions with volume of 17 ml were collected (Figure 36, page 88). Fractions 7 and 8 were pooled, lyophilized and indicated as SPH L fraction.
Results

Figure 36. HPLC of SCC F3c and UV spectrum of SPH L fraction, gradient program 4 (page 26), detection at 215 nm

The UV spectrum of SPH L which showed two maxima 197 and 331 nm, respectively. HPLC analysis of SPH L showed a high purity of the compound (Figure 37, page 89) which was further analyzed by LC-MS (Figure 38, page 90) and finally characterized by NMR (Figures 39-40, pages 91-92).

The $^1$H- and $^{13}$C NMR shifts were in excellent correlation with those of lavandulifolioside (Calis at al., 1992). This compound has been described in
Results

Leonurus cardiaca before (Milkowska-Leyck et al, 2002). The MS results confirmed the structure by the molecular ion of 755.4 in the negative mode and the fragments with a molecular weight of 593.4, 461.3, 315.2 and 135.1 m/z are representing signals for the caffeoyl, arabinose, rhamnose and glucose moieties of lavandulifolioside, respectively (Figure 38, page 90). Thus, the structure was unambiguously elucidated as lavandulifolioside. The yield of lavandulifolioside was 33 mg.

Figure 37. HPLC of SPH L fraction and its UV spectrum, system 2 (page 24), gradient 2 (page 24), detection at 254 nm

UV spectrum at $R_t$ 11.74
Results

Figure 38. LC-MS analysis of lavandulifolioside

* The LC-MS measurements were performed by Dr. Martin Zehl, Department for Pharmacognosy, University of Vienna.
Figure 39. $^1$H NMR analysis of lavandulifolioside
Results

Figure 40. $^{13}$C NMR analysis of lavandulifolioside

![Chemical structure of lavandulifolioside]

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* The NMR measurements were performed by Ass. Prof. Hanspeter Kaehlig, Institut of Organic Chemistry, University of Vienna.
The influence of lavandulifolioside on HDAC activity was studied and an IC_{50} of 220 µM was determined.

Although lavandulifolioside is not a highly potent HDAC inhibitor, these results impressively confirmed the applicability of the assay for the successful and efficient bioactivity-guided isolation of HDAC modulators from plant origin.

To sum up, the used chromatographic methods (TLC and HPLC) revealed rutin, hyperoside, chlorogenic and isochlorogenic acids, verbascoside and lavandulifolioside as major phenolic compounds and as well the sugars glucose, fructose and rhamnose. Further phenolic acids and flavonoids were not identified due to the lack of respective standards.

D.9 Influence of Natural Compounds on HeLa Cells

Proliferation

Several compounds e.g. aloin, capsaicin, rutin, stachydrine, sennosides A and B, verbascoside, as well fractions CR H_{2}O, SPE F2, F3 and F4, which have shown some effects in the enzymatic assay, were further studied for a modulatory effect in a cell culture model by cell imaging. Based on the results from the screening obtained by the HDAC assay further analyses by image-based screening and Western blot were performed to identify the possible mechanism of their action.

HeLa cells were used for the image-based screening and Western blot analyses.

D.9.1 Cell Imaging

To study the modulating effects of natural substances on HDAC activity and on cell division process, the influence of small molecules was investigated in a cell model suitable for characterization the cytoskeletal phenotype after treatment with various compounds.
Results

A clear molecular and cell biological understanding of cytokinesis should result in the development of the new therapeutic strategies for treating a number of diseases, including cancer (Barr and Gruneberg, 2007). Based on the hypothesis that the failure of cell division results in the formation of genetically unstable cells which may go on to form malignant tumors, it was of interest to investigate the cell division process and which step in the cell division process is influenced by those natural compounds, which had inhibited HDAC activity in the enzyme assay. For this purpose HeLa cells were cultivated (C.10.2, page 38) and compounds of interest (aloin, capsaicin, rutin, sennoside A, sennoside B, stachydrine, verbascoside) were added to the medium at three concentrations (25, 37.5 and 50 µM). The incubation period was 24 and 48 hours. Fractions CR H₂O, SPE F2, F3, and F4 from Leonuri herba were analyzed at concentrations of 20, 30 and 40 µg/ml. DMSO was used as a control at a concentration of 0.1%.

The fluorescence labelling for the actin cytoskeleton was performed with FITC-conjugated phalloidin (green dye). The tubulin cytoskeleton in fixed cells was processed for tubulin immunofluorescence with TRITC-conjugated rhodamine (red dye). The nucleus was stained with DAPI (blue dye). The cell images are presented in the Figures 41-50, pages 95–103, respectively for 24 and 48 hours.
Figure 41. Influence of aloin on HeLa cell growth, cell cycle and cell division after cultivation of 24 (left) and 48 hours (right)

After 24 hours aloin did show strong influence on cell growth and the mechanism of cell cycle in HeLa cells as compared with the control DMSO. After 48 hours of incubation the actin filaments were thickened and in the dividing cells the contraction ring was brighter. This fact indicated that aloin disturbed the cell cycle, although the mechanism of action is still unclear.
Results

Figure 42. Influence of capsaicin on HeLa cell growth, cell cycle and cell division after 24 (left) and 48 hours (right)

<table>
<thead>
<tr>
<th>Concentration</th>
<th>50 µM</th>
<th>37.5 µM</th>
<th>50 µM</th>
<th>37.5 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 µM</td>
<td></td>
<td></td>
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</tbody>
</table>

Capsaicin at all concentrations strongly influenced the cell growth. The cell cycle mechanism was disturbed as showed at all concentrations after 24 and 48 hours as well. It was characteristic that the microfilaments changed their morphology, the tubulin bundles were very condensed and actin fibrils were loosening. Generally, after of 24 hours incubation the cells were in senescence and by further cultivation (48 hours) with capsaicin cell death was observed.
Results

Figure 43. Influence of rutin on HeLa cell growth, cell cycle and cell division after 24 (left) and 48 hours (right)

<table>
<thead>
<tr>
<th>Concentration</th>
<th>50 µM</th>
<th>37.5 µM</th>
<th>50 µM</th>
<th>37.5 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µM</td>
<td>control</td>
<td>25 µM</td>
<td>control</td>
<td></td>
</tr>
</tbody>
</table>

Rutin at a concentration 50 µM affected the cell growth after 24 hours incubation, the HeLa cells continued to divide although the process was decelerated. At lower concentrations 25 and 37.5 µM the effect was as well observed but not that intense.

After 48 hours incubation with rutin the cells showed elongated actin filaments. The tubulin was concentrated around the nuclei compared with the control DMSO.
Results

Figure 44. Influence of sennosides A and B on HeLa cell growth, cell cycle and cell division after 24 (left) and 48 hours (right)

Sennoside A

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Control</th>
<th>25 µM</th>
<th>50 µM</th>
<th>37.5 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sennoside A</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>50 µM</td>
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<tr>
<td>37.5 µM</td>
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<tr>
<td>37.5 µM</td>
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</table>

Due to the influence of sennosides A and B the tubulin microtubuli had changed their morphology, they were thicker and more condensed. The cell division after
Results

24 and 48 hours incubation at all concentrations was disturbed in comparison with the control DMSO.

Figure 45. Influence of stachydrine on HeLa cell growth, cell cycle and cell division after 24 (left) and 48 hours (right)

<table>
<thead>
<tr>
<th>50 µM</th>
<th>37.5 µM</th>
<th>50 µM</th>
<th>37.5 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µM</td>
<td>control</td>
<td>25 µM</td>
<td>control</td>
</tr>
</tbody>
</table>

Stachydrine at all concentrations strongly influenced the cell growth and the cell morphology. Substantially changes were observed in particular at a concentration of 50 µM after 24 and 48 hours cultivation. As expected the stachydrine induced apoptosis in the HeLa cells (Loh et al., 2009).
Verbascoside at all concentrations and incubation of either 24 or 48 hours strongly influenced the cell division. Typically the daughter cells were not fully separated, which was caused by incompletion of the last step of cell division (scission). The tubulin in all treated cells showed changed localization around the nucleus, was thicker and more condensed and led to accumulation of large multinuclear cells.
Results

Figure 47. Influence of fraction CR H$_2$O from Leonuri herba on HeLa cell growth, cell cycle and cell division after 24 (left) and 48 hours (right)

<table>
<thead>
<tr>
<th>Concentration</th>
<th>40 µg/ml</th>
<th>30 µg/ml</th>
<th>40 µg/ml</th>
<th>30 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>40 µg/ml</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>30 µg/ml</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>20 µg/ml</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>Control</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
</tr>
<tr>
<td>48 hours</td>
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<tr>
<td>40 µg/ml</td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
</tr>
<tr>
<td>30 µg/ml</td>
<td><img src="image21.png" alt="Image" /></td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
<tr>
<td>20 µg/ml</td>
<td><img src="image25.png" alt="Image" /></td>
<td><img src="image26.png" alt="Image" /></td>
<td><img src="image27.png" alt="Image" /></td>
<td><img src="image28.png" alt="Image" /></td>
</tr>
<tr>
<td>Control</td>
<td><img src="image29.png" alt="Image" /></td>
<td><img src="image30.png" alt="Image" /></td>
<td><img src="image31.png" alt="Image" /></td>
<td><img src="image32.png" alt="Image" /></td>
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</tbody>
</table>

The CR H$_2$O extract at a concentration of 40 µg/ml after 24 hours strongly affected the cell growth.

After 48 hours incubation at a concentration of 40 µg/ml large multinuclear cells were observed. It was clearly visible that the tubulin morphology and tubulin polymerization were disturbed as compared to the control.
Results

Figure 48. Influence of fraction SPE F2 on HeLa cell growth, cell cycle and cell division after 24 (left) and 48 hours (right)

<table>
<thead>
<tr>
<th>40 µg/ml</th>
<th>30 µg/ml</th>
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The influence of fraction SPE F2 at a concentration of 20 µg/ml after 24 and 48 hours incubation was the strongest. Big multinuclear cells and as well many apoptotic cells were observed. The concentrations of 30 µg/ml and 40 µg/ml influenced the cell morphology as well the tubulin distribution around the nucleus, which was concentrated as compared to the control.
Results

Figure 49. Influence of fraction SPE F3 on HeLa cell growth, cell cycle and cell division after 24 (left) and 48 hours (right)

40 µg/ml       30 µg/ml     40 µg/ml     30 µg/ml

20 µg/ml       control     20 µg/ml     control

The influence of fraction SPE F3 at all concentrations and both incubation times was not strong. Cell growth and cell division were not disturbed as compared with the control DMSO.

Figure 50. Influence of fraction SPE F4 on HeLa cell growth, cell cycle and cell division after 24 (left) and 48 hours (right)

40 µg/ml       30 µg/ml     40 µg/ml     30 µg/ml

20 µg/ml       control     20 µg/ml     control
Results

Fraction SPE F4 at all concentrations strongly influenced the cell division similar to the effect caused by verbascoside and stachydrine. The daughter cells were not fully separated. F4 caused cell senescence at a concentration of 40 µg/ml and 48 hours incubation.

The effects of the tested compounds, the fractions CR H₂O, SPE F2 and F4 of Leonuri herba after incubation of 48h were more pronounced as compared with the influence after 24h incubation. The cell cycle was affected, the cell morphology was changed and the pattern of the actin and tubulin microfibrili was interfered by polymerization in tubulin bundles and untypical actin structures. Fraction CR H₂O and the fractions SPE F2 and F4 after 48h had a massive effect on the cell division. The mechanism of action is still unknown. Further investigations are needed but could not be performed within the time frame of this thesis.

D.9.2 Western Blot Analyses

Western blot analyses of endogenous protein levels were performed using the nuclear extract to study the acetylated state of histones H3 (Figure 51, page 105) and H4 (Figure 52, page 106) and the whole cell lysate for characterization of expressed protein level of p21 (Figure 53, page 107) and acetylated tubulin (Figure 54, page 108).

The Western blot experiments were used to study the influence on the acetylated state of histones (H3 and H4) and the tubulin by the modulators (sennosides A and B, lavandulifolioside, TSA and DMSO), which have shown some effect on HDAC activity.

Protein p21 is a regulator of the cell fate which after stimulation leads to apoptosis. Thus, the activation of p21 was observed in HeLa cells after treatment with the selected compounds. The mechanism of action is unclear although it is
Results

proved that altering the function, the regulation and the stability of HDACs influences strongly the cell fate.

The experiments were done by cell stimulation with the tested compounds for 1, 2, 4, 6, 8, 20 and 24 hours. Then, the cells were lysed and the amounts of acetylated H3 (histone 3), acetylated H4 (histone 4), acetylated tubulin and p21 were characterized.

Tubulin was used as a loading control (Figure 55, page 109). All experiments were carried out twice.

Figure 51. Western blot analyses of the acetylated state of histone 3 (AcH3). Blank presents untreated HeLa cells compared to DMSO, TSA, lavandulifolioside (LA), sennosides A and B (SA and SB) treated HeLa cells

It was obvious that DMSO is a very active compound, which had a strong influence on the acetylated state of histone 3. After 6 hours treatment the level of acetylated histone was doubled and continued to increase rapidly. As expected, the influence of TSA was very strong. After achieving a maximum in hour 6, the level of acetylated histone 3 was almost constant up to the 24th hour. In the blank
Results

close a slow increase up to the 6<sup>th</sup> hour in the acetylated state of histone 3 was observed, which then dropped as expected, when the cells underwent cell division. Sennosides A and B did not influence the protein expression of acetylated histone 3, whereas the lavandulifolioside showed a clear tendency to increase the acetylated state of histone 3 within 24 hours.

Figure 52. Western blot analyses of the acetylated state of histone 4 (AcH4). Blank presents untreated HeLa cells compared to DMSO, TSA, lavandulifolioside (LA), sennosides A and B (SA and SB) treated HeLa cells.

Comparably to the acetylated state of histone 3, DMSO showed high potency on the expression level of the acetylated histone 4 after 6 hours of a treatment. The influence of TSA as well was strong. After achieving a maximum after 6 hours treatment the acetylated histone 4 level remained almost constant up to the 24<sup>th</sup> hour. The blank showed the same effect on the acetylated state of histone 4 as on the one of histone 3. The sennosides A and B remained without effect on the protein expression of acetylated histone 4, whereas the lavandulifolioside again showed a tendency to influence positively the acetylated state of histone 4.
Figure 53. Western blot analyses monitoring the expression of p21. Blank presents untreated HeLa cells compared to DMSO, TSA, sennosides A and B (SA and SB) treated HeLa cells

The expression of p21 in HeLa cells, which were treated with DMSO, increased rapidly up to 4th hour, then dropped until 8th hour to the initial level and stayed constant to the hour 20. DMSO at the beginning of the treatment triggered the cells into apoptosis. The cells, which survived continued proliferating so that the expression level of p21 was constant.

For TSA and sennoside B the expression level of p21 after the hour 8 was constant, whereas the level after treatment with sennoside A after 8 hours continued to increase as compared to the control.
Figure 54. Western blot analyses monitoring the acetylated state of tubulin. Blank presents untreated HeLa cells compared to DMSO, TSA, sennosides A and B (SA and SB) treated HeLa cells.

The acetylated tubulin level after treatment with DMSO, increased rapidly up to 8\textsuperscript{th} hour, and then stayed constant to the 20\textsuperscript{th} hour. For TSA and sennoside B the expression level of acetylated tubulin after the hour 8 was constant, whereas the level of acetylated tubulin after treatment with sennoside A was higher until 4\textsuperscript{th} hour and then continued to decrease as compared to the control.
Results

Figure 55. Western blot analyses monitoring the tubulin level. Blank presents untreated HeLa cells compared to DMSO, TSA, sennosides A and B (SA and SB) treated HeLa cells.

The tubulin level was used as a loading control in for the Western blot analyses. The level of expressed tubulin was approximately the same in all samples, as expected.

**D.10 Sennosides A and B and their Influence on HDAC Activity and HeLa Cells Proliferation**

The sennosides A and B *in vitro* had shown a potential to modulate the HDAC activity at micromolar concentrations (D.2.1, page 55) and additional information about the mechanism of action on the acetylated state of tubulin was collected by cell imaging (page 98) and Western blot analyses (D.9.2, page 104). As well it was shown in another study (Tran et al., 2007) that the modulation in the tubulin acetylation induced changes in microtubules dynamics and altered the acetylated state of α-tubulin.
In order to clarify the mechanism of action of the sennosides A and B, they were tested with the recombinant enzymes HDAC 1, 2, 3 and 6 and the influence on the proliferation rate of HeLa cells was studied.

D.10.1 Sennosides and HDAC Activity

Preliminary measurements on HDAC inhibition had showed an IC$_{50}$ value 180 µM for sennoside A, whereas the IC$_{50}$ value of sennoside B was 220 µM using HeLa nuclear extract as HDAC source. To test the activity of these compounds more detailed, measurements were performed using HDAC1, 2 and 3 recombinant enzymes in vitro. The effect of sennoside A was strong. At a concentration of 50 µM it showed an inhibition of 40% of the enzyme activity of HDAC3. It did not affect the activity of HDAC1 and HDAC2 at the tested concentration. Sennoside B at a concentration of 50 µM did not modulate the activity of any of the recombinant enzymes.

As the cell imaging data had shown that the sennosides affect the structure of tubulin, the influence of the sennosides on the HDAC6 activity was studied as well. HDAC6 is predominantly located in cytoplasm and has the potential to deacetylate tubulin and microtubules (Zhang et al., 2003, Tran et al., 2007) and influences on HDAC6 activity alter the cell migration (Tran et al., 2007).

The measurement in the HDAC assay showed no effect of sennoside B on the activity of HDAC6, whereas sennoside A was inhibiting HDAC6 with an IC$_{50}$ of 95 µM (Figure 56, page 111).
Figure 56. Influence of sennoside A on the HDAC6 activity (statistics: one-way ANOVA, no significant difference)

![SA and HDAC6 activity graph](image)

Although the performed analyses (HDAC assay and cell imaging) showed strong influence of sennoside A on the activity of HDAC3, HDAC6 and acetylated tubulin, the mechanism of its action is still unclear.

**D.10.2 Sennosides and HeLa Cells Proliferation**

In order to study the effect of the sennosides A and B on the proliferation rate of HeLa cells, a crystal violet staining was carried out. As demonstrated in Figure 57, page 112, HeLa cells treated with sennoside A and B at three different concentrations (1, 10 and 100 µM) for 48 hours were not affected as determined by cell counting. A similar result was obtained by treating the cells for 72 hours with sennoside B and performing the crystal violet assay (Figure 58, page 112). However, after treatment with sennoside A for 72 hours the cell proliferation was affected in a concentration dependent manner (1 to 100 µM).
Results

Figure 57. Influence of different concentrations of sennosides A and B (1, 10 and 100 µM) on the proliferation of HeLa cells after 48h incubation measured by crystal violet assay

![Graph showing proliferation percentages for different concentrations of sennosides A and B](image)

$n = 2$

Figure 58. Influence of different concentrations of sennosides A and B (1, 10 and 100 µM) on the proliferation of HeLa cells after 72h incubation measured by crystal violet assay

![Graph showing proliferation percentages for different concentrations of sennosides A and B](image)

$n = 2$
Results

These results were confirmed by use of the resazurin assay. The inhibition of the proliferation rate of HeLa cells by sennoside A occurred after 72 hours in a concentration dependent manner.

D.11 Immunoprecipitation (IP) of HDAC5

HDAC5 belongs to class II HDAC enzymes and is responsible for cardiac development and growth through different pathways. The suppression of the hypertrophic growth of cardiomyocytes by HDAC5 was investigated in a mouse model (Chang et al., 2005). Due to its implementation in the regulation of cardiac myocytes HDAC5 was of interest for our study and should be isolated by immunoprecipitation.

An immunoprecipitation study was performed to explore the content of HDAC5 in the VSMC nuclear extract and as well as to obtain pure native HDAC5 enzyme, for the further characterization of the enzyme kinetics. For this aim A-Sepharose beads, an HDAC5 antibody and VSMC nuclear extract were used (see C. 8 page 35). The results are presented in Figure 59.

Figure 59. HDAC activity of the VSMC nuclear extract and products from immunoprecipitation
Results

The activity of VSMC nuclear extract was assumed as 100% and compared to the activity of all IP products. The supernatant obtained after centrifugation of the A-Sepharose beads after addition of 100 µl lysis buffer was named SN1. SN2 was obtained after the second treatment of the A-Sepharose beads with lysis buffer. Immunoprecipitation product 1 (IP1) was designated as first pellet product between the HDAC5 from the VSMC nuclear extract and the A-sepharose beads, whereas with IP2 was indicated the second pellet product after the second centrifugation step. As expected IP1 and IP2 did not show any significant activity (10.5% and 7.5%, respectively), in contrary, SN1 possessed 65% higher activity than the VSMC nuclear extract.

Further, IP products were analyzed by gel electrophoresis. Commassie staining and Western blot were performed in order to determine a presence of HDAC 5 in the nuclear extract and IP products, Figure 60.

Figure 60. Analysis of the VSMC nuclear extracts and the IP products by Commassie staining (left blot) and Western blot (right blot)
Results

1 protein marker (Biorad, 20 - 250 kDa). 2 mixture of VSMC nuclear extract, anti-HDAC5 and A-sepharose beads, 3 SN1, 4 SN2 and 5 VSMC nuclear extract.

The molecular weight of the pure HDAC5 is around 120 kDa. In the Commassie staining blot 2 revealed an intense blue zone with a molecular weight of around 120 kDa and proved that the HDAC5 enzyme was completely bound on the A-sepharose beads with HDAC5 antibody. A small amount was detected in SN1 product as well. Thus, the method can be used for the enrichment and the purification of HDAC5 enzyme.

In conclusion, it was possible by IP procedure to achieve a high concentration of the HDAC5 (comparison between band 2 and 5 in the Commassie staining blot). The disadvantage of the IP procedure was that the enzyme was obtained in a diluted solution and instable.
E Discussion

E.1 An in vitro Assay on HDAC Modulation and its Application for Bioactivity-Guided Fractionation

Protein acetylation and deacetylation are major intracellular modification systems and important factors contributing to the regulation of the cell fate.

The treatment of diseases can be implemented at various levels of signaling pathways. It is essential for targeted drug discovery to understand the overall pathways and to have adequate assay and cell based models in which these pathways can be studied and dissected. Validated assay- and cell-systems are indispensable in preliminary screening.

The methods and the models applied in this study aimed on the screening of plant extracts, fractions and natural compounds for HDAC modulation. The enzymatic HDAC assay represents a model for fast answers in the search for modulators of HDAC activity. A fluorimetric enzyme assay was validated to investigate HDAC inhibition by different natural compounds in nuclear extracts of HUVECs, VSMCs and HeLa cells. The investigation involved screening of numerous natural products.

This work presents for the first time the application of a fluorimetric assay for the investigation of HDAC modulators in complex extracts from plant origin. A fast enzymatic method for the determination of HDAC activity and the identification of potent natural product-based inhibitors of HDAC activity was established.

The optimized test system combines high sensitivity, speed and reproducibility for the investigation of plant extracts of medium to high polarity. Our modified
Discussion

HDAC assay is well suited for medium to HTS efforts in the screening of plant extracts, fractions and natural compounds for HDAC modulators.

Cultivation of HeLa cells was the most frequently used for the preparation of the HDAC source. The cells rapidly produced large quantities of biomass. The quality, yield and composition of the HDAC source were very homogeneous and constant in all nuclear extracts prepared for the experiments.

Due to use of total nuclear extracts of HeLa cells the assay is not completely specific to study the effects on the enzyme activity of class I and class II HDAC family. Uncharacterized cofactors are present in the total nuclear extract, which can contribute to lysine hydrolysis and/or affect the properties of the test compounds. Nevertheless, this system mimics the natural environment of the enzyme and assures its activity and stability.

An ε-acetylated lysine-based substrate C-terminally coupled with 4-methylcoumarine-7-amide (MCA) was synthesized by the group of PhD Mazitchek, General Hospital of Massachusetts, Boston, US. The binding affinity of the MCA was investigated in detail. As the HeLa nuclear extract contains both class I and II HDACs, the binding affinity of the substrate was determined for the recombinant enzymes HDAC3 and HDAC6. $K_{d\text{HDAC3}}$ was 2 ± 0.2 and $K_{d\text{HDAC6}}$ 4.6 ± 0.2 nM, respectively. This substrate is suitable for screenings, in which an incubation time of minimum 60 minutes is important for complete turnover (Mazitchek et al., 2008). Other studies have shown that many factors affect the specificity of the enzyme and substrate. A site specificity is dependent on the presence of cofactors and the nature of acetyl group, cleaved from the substrate. Furthermore, additional substrate phosphorylation or influence of corepressor binding on the enzyme activity leads to strong influence of the deacetylation (Riester et al., 2004, 2007, Bradner et al. 2010).

Our study confirmed that the used substrate is suitable for screening in the search of HDAC modulators with HeLa nuclear extract. Additionally, experiments
Discussion

were performed with the recombinant HDAC1, 2, 3 and 6 and nuclear extracts from HeLa, HUVEC, VSMC, MCF7 and HL-60 cells (not all results are shown) and have also shown the suitability of this substrate.

Individual recombinant purified enzymes and acetylated histones should be used instead of nuclear extract and MCA-coupled peptide for more detailed mechanistic studies in the search of selective HDAC inhibitors.

A further advantage of the assay is the use of small reaction volumes, which makes it a very cost effective method. The consumption of reagents and analytes is very little.

E.2 Effect of Extracts of Leonuri herba on HDAC Activity

In a first pilot experiment with an extract of Leonuri herba the applicability of the assay for the bioactivity-guided fractionation of complex extracts was successfully demonstrated.

However, special caution has to be taken when working with highly autofluorescent, lipophilic or tannin rich extracts.

Our work has shown that coumarins and similar derivatives strongly interfere with the measurements due to autofluorescence. In the examination of extracts or fractions presumably containing such compounds a general check for blue fluorescent bands by TLC is highly recommended.

Extracts containing tannins may lead to precipitation of the proteins in the assay and to false positive results. Thus, detannification is indispensable before tests on HDAC activity.
Discussion

For very apolar extracts and fractions other assays have to be used due to insufficient solubility of such mixtures in DMSO and reaction buffer.

To sum up, the presented results proved the applicability of a fluorimetric assay for a rapid and effective primary screening of complex extracts and fractions from plant origin. It is a well-suited tool for such an approach.

E.3 Effect of Sennosides on HDAC Activity

Information about the inhibitory potency of the dianthrone sennosides A and B on HDAC activity was not available. Our results showed that sennoside A inhibits HeLa nuclear extract possibly by interfering with HDAC6. In this study we determined the IC$_{50}$ of 95 µM sennoside A for HDAC6.
In the used cell system sennoside A led to interaction with tubulin and in a concentration dependent manner decreased the proliferation rate of HeLa cells. However, the mechanism of inhibition by sennoside A is still unclear.

Nevertheless, despite the advantages of different culture models, it is clear that in vivo studies and species extrapolation is necessary.
Conclusion Remarks

F Conclusion Remarks

HDACs are involved in regulation of diverse cellular processes by altering the protein structure and function and by influencing the cell fate by modulation of nonhistone proteins and transcriptional factors.

The ability of HDAC inhibitors to modulate transcriptional activity and to alter various cellular functions, such as cell differentiation, cell cycle progression, apoptosis, cytoskeletal modifications and angiogenesis, built by HDAC research as a very important field. By targeting these key components of tumor proliferation, HDACi will play an important role as anti-cancer drugs. A major reason for their key role in the oncology is that they are able to improve the efficacy of already existing agents. The synergisms of HDACi with classic chemotherapeutic agents and angiogenesis inhibitors have shown positive influence on the treatments. This mechanism is still under investigation (Emanuele et al., 2008).

The efficacy of HDACi depends on the degree of suppression of cancer cell proliferation, survival, migration, invasion, metastasis and angiogenesis by modulation of the gene regulation in cancer cells. HDACs regulate these processes by single and multicomplex pathways in complex mechanism of actions, which are not elucidated yet (Mottet and Castronovo, 2008).

A hypothesis that HDAC inhibitors could be beneficial in multiple cancers and be applicable alongside a broad range of therapeutics is still a target.

Over the past few years, some HDAC inhibitors have entered the clinical studies. For example, HDAC inhibitors of the group of hydroxymates are used in phase I (PCI24781, LAQ824, SB939 and JNJ-16241199), phase II (panobinostat, belinostat and ITF2357) and phase III (SAHA) clinical trials. The group of short chain fatty acids (butyrate and valproate), cyclic tetrapeptides (depsipeptide) and
Conclusion Remarks

the benzamides (MS-275, MGCD0130 and N-acetyldinaline) are already in phase II clinical trials (Emanuele et al., 2008, Takai and Narahara, 2010). The HDACi FK228 is already used for the treatment of peripheral T-cell lymphoma, cutaneous T-cell lymphoma, chronic lymphocytic leukemia and androgen independent prostate cancer. SAHA, an orally bioavailable HDACi, is implemented against solid tumors, leukemia and lymphomas (Marks and Breslow, 2007).

Adverse cardiologic and hematological effects of first generation inhibitors are observed and the need for new candidates, selective inhibitors with higher efficacy and safety is still a challenge in this research field.
Future research is expected to lead to a better understanding of the molecular targets of HDACs and to facilitate the development of more potent and specific inhibitors of these enzymes. Selective inhibitors of the HDACs are currently lacking and the mechanism of action is still unknown. The aim can be the discovery of small molecules that specifically inhibit proteins in the HDAC pathway. Optimization and characterization could turn the small molecules into potential anti-cancer drugs. Using specific inhibitors in cancer model systems will bring better understanding of how HDAC modulations contribute to tumor development and progression.

In broader terms, the assay can be modified and used to find inhibitors of virtually signaling pathways where HDACs are involved. This could be a useful approach to study the role of HDAC in the regulation of gene expression and to target other biological pathways involved in cancer.

A further challenge is to learn the nature of the combinatorial HDAC-protein-DNA interaction and to understand how these interactions vary in different cell types and tissues, in order to target a specific region of the body by disease treatment.
H  Literature Citations


Literature Citations


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Literature Citations


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I.1 Abbreviations

Ac - acetylation
ADP - adenosine diphosphate
AMC - 7-amino 4-methylcoumarin
appr. – approximately
APS – ammonium persulphate
Ara – arabinose
ATP - adenosine triphosphate
b.w. - body weight
C-source - carbon source
CA - caffeic acid
CB – cytoskeletal buffer
CBS – cytoskeletal buffer with 1M saccharose
CNS - central nervous system
CCIA – chlorogenic acid
CVD – cardiovascular disease
DMEM - Dulbecco’s modified Eagle medium
DMSO - dimethyl sulfoxide
DNA - deoxyribonucleic acid
DTT - 1-4 dithiothreitol
ECL – enhanced chemiluminescence
EDTA - ethylenediaminetetraacetic acid
EGTA - ethylenglycol-bis(2-aminoethyl)-N,N,N’,N’-tetraacetic acid
EtOAc - ethyl acetate
EtOH - ethanol
FOXO - forkhead box O, transcription factor
Appendix

Glu - glucose
H1, H2A, H2B, H3, H4 - histones
HAT - histone acetyltransferase
HDAC - histone deacetylase
HDACi - histone deacetylase inhibitor
HDACm - histone deacetylase modulator
HeLa - cervical cancer cells from Henrietta Lacks
HEPES - 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
Hyp - hyperoside
HUVECs - human umbilical vein endothelial cells
HTS – high-throughput screening
IC\textsubscript{50} - the half maximum inhibitory concentration
K – lysine
LA - lavandulifolioside
Lc - \textit{Leonurus cardiaca} L.
MCA – substrate C-terminally coupled with 4-methyl-coumarine-7-amide
MeOH - methanol
MES – 2-(N-morpholino)ethanesulfonic acid
MgCl\textsubscript{2} - magnesium dichloride
NAD - nicotinamide adenine dinucleotide
NF-kB - nuclear factor kappa-light-chain-enhancer of activated B-cells
PAA – polyacrylamide
PAGE - polyacrylamide gel electrophoresis
p21 – cell cycle regulatory protein 21
p53 - tumor suppressor protein 53
PBS - phosphate buffered saline
PMSF - phenylmethylsulfonylfluoride
PTMs - posttranslational modifications
R\textsuperscript{2} - linear correlation factor
RA – rosmarinic acid
Rha - rhamnose
RFU - relative fluorescence units
rpm - rotations per minute
RT - room temperature
RV - reservoir volume
SA - sennoside A
SB - sennoside B
SCC – Sephadex LH-20 column chromatography
SDS – sodium dodecyl sulphate
SIRT - sirtuin
SPE - solid phase extraction
TCM - traditional Chinese medicine
TEMED - N,N,N',N'-tetramethylethylenediamine
Tris-HCl - tris(hydroxymethyl)aminomethane
TSA - trichostatin A
TX – triton X-100
VS - verbascoside
VSMCs - vascular smooth muscle cells

### I.2 Alphabetical List of Companies

Abcam, Cambridge, UK
BD Biosciences Pharmingen San Diego, CA, USA
Beckman Coulter Fullerton, CA, USA
BIO-RAD Laboratories Hercules, CA, USA
Calbiochem La Jolla, CA, USA
Carl Roth Karlsruhe, Germany
Fluka Buchs, Switzerland
Fujifilm Tokyo, Japan
Graph Pad Software, Inc. San Diego, CA, USA
Appendix

Invitrogen Carlsbad, CA, USA
Lonza Group Ltd. Basel, Switzerland
New England Biolabs Beverly, MA, USA
Olympus Europa GmbH Hamburg, Germany
Roche Diagnostics GmbH Basel, Switzerland
Sanova Pharma Vienna, Austria
Santa Cruz Santa Cruz, CA, USA
Sigma Aldrich St. Louis, MO, USA
Tecan Mannedorf, Switzerland
Upstate Charlottesville, VA, USA
Supplements

1. NSC31965  2. NSC34781  3. NSC78331

4. NSC96573  5. NSC137222  6. NSC162709

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20. NSC618567

21. NSC618689

22. NSC618800

23. NSC623869

24. NSC623876

25. NSC630366

26. NSC640959

27. NSC668820
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I would like to dedicate this work to my unborn son David, who was extremely active during the thesis writing and stimulated me to keep on going…

Vienna, June, 2010
J.2 Curriculum Vitae

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01.10.2006 - date PhD candidate, University of Vienna, Austria  

01.10.2003 - 18.05.2006 Master of Biotechnology, University of Natural Resources and Applied Science, Vienna, Austria  

15.09.1997 - 30.06.2001 Bachelor of Biotechnology, University of Food Technologies in Plovdiv, Bulgaria  
Thesis: “Enrichment of the isoflavon content in Soya-germ flour”

**Work experience**

01.06.2006 – 21.09.2010 Full-time assistant, University of Vienna, Department of Pharmacognosy, Vienna

09.06.2008 – 27.08.2008 Guest researcher in the laboratory of Prof. U. Eggert at Harvard Medical School and Dana-Farber Cancer Institute, Boston, MA

15.06.2001 – 01.06.2006 Research technician, University of Natural Resources and Applied Life Science, Division of Food Biotechnology, Vienna
Supplements

Projects:

01.11. 2005 - 31.05.2006  Project: “New separation strategies for isolation of secondary plant components”
02.01.2005 - 31.10.2005  Various Industrial Projects
01.06.2003 - 31.08.2003  Industrial project: “Waste water cleaning in Ottakringer brewery production”
02.01.2003 - 31.05.2003  Project: “Procedure and technology for the separation and isolation of secondary plant components by the example of grape pomace”
02.09.2002 - 01.11.2002  Industrial project: “Production of bakery-yeast”
15.06.2001 - 15.09.2001  Erasmus Project: “Production of isoflavones”

Publications


Hypericum adenotrichum Spach endemic to Western Turkey. Oncology Reports 22, 845-852, 2009.


Talks

Search for Histone Deacetylase Modulators in Plant Kingdom, presented at the International PhD Meeting of German Pharmaceutical Society, November 18\textsuperscript{th} to 21\textsuperscript{st} 2009, Pichlarn, Austria.

Influence of Leonurus cardiaca on the Histone Deacetylase Activity, presented at the 57th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, YRW August 16\textsuperscript{th} to 20\textsuperscript{th} 2009, Geneva, Switzerland.

Validation of a Fluorimetric Assay for Histone Deacetylase Inhibitors from Plant Origin, presented at the 21\textsuperscript{st} Scientific Congress of the Austrian Pharmaceutical Society, April 16\textsuperscript{th} to 18\textsuperscript{th} 2009, Vienna, Austria.

Screening for Inhibitory Effect of Natural Substances on Class I/II HDACs, presented at the 5\textsuperscript{th} Course on Epigenetics March 11\textsuperscript{th} to 18\textsuperscript{th} 2009, Paris, France.

Fluorimetric Assay of Histone Deacetylase Inhibitors of Plant Origin, presented at the PSNA Annual Meeting July 21\textsuperscript{st} to 25\textsuperscript{th} 2007, Donald Danforth Plant Science Center, St. Louis, Missouri, USA.
**Posters**

Extracts of *Leonurus cardiaca* L. influence the Histone Deacetylase Activity, presented at the 57th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research August 16th to 20th 2009, Geneva, Switzerland.

**Fluorimetric Assay of Histone Deacetylase Activity**, presented at the FASEB Summer Conference Histone Deacetylases and Reversible Acetylation in Signaling and Disease August 9th to 14th 2009, Lucca, Italy.

**Natural Product-based Screening Histone Deacetylase Inhibitors**, presented at the 55th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research September 2nd to 6th 2007, Graz, Austria.

**Fluorimetric Assay of Histone Deacetylase Inhibitors of Plant Origin**, presented at the PSNA Annual Meeting July 21st to 25th 2007, Donald Danforth Plant Science Center, St. Louis, Missouri, USA.
Abstract

Cardiovascular diseases (CVD) today are the one of the leading causes of death. Recent studies suggest a key role of histone deacetylases (HDACs) in the control of pathological cardiac remodeling. Thus, the main focus of our study was to investigate the effect of natural products on HDAC activity.

For the bioactivity-guided screening for HDAC modulators from plant sources a fluorimetric assay was extensively validated and applied for the first time to complex extracts and fractions.

Leonuri herba, a drug used in European traditional medicine against cardiac complaints, served as an example for a bioactivity-guided approach. Extracts of different polarity were tested in the established assay and fractionated by different chromatographic methods under consideration of their HDAC inhibiting activity. Lavandulifolioside was isolated as active compound and its structure established by various spectrometric/spectroscopic methods. Although for the substance an IC$_{50}$ value in the micromolar range (220µM) was determined only, this study showed impressively that the established assay is an adequate tool in the bioactivity-guided search for HDAC modulators in plant extracts, as the system combines high sensitivity, speed and effectiveness.

For some active plant secondary metabolites e.g. sennoside A the underlying molecular mechanism were studied in more detail. Although the mechanism remained unclear and further investigations on the molecular mechanism of action are needed, these findings increased the understanding of interactions between HDACs and some natural products.
Abstrakt

Herz-Kreislauf-Erkrankungen (CVD) stellen heute die führende Todesursache dar. Neuere Studien zeigen dass, Histoneacetetylases (HDACs) eine Schlüsselrolle bei der Steuerung pathologischer Prozesse des Herzens spielen dürften. Der Schwerpunkt der vorliegenden Arbeit war daher die Wirkung von Naturstoffen auf die HDAC-Aktivität.

Für das aktivitäts-gelieitete Screening nach HDAC-Modulatoren pflanzlicher Herkunft wurde ein fluorimetrischer Assay umfangreich validiert und erstmalig an komplexe Extrakte und Fraktionen angewendet.

Leonuri herba, eine Arzneidroge der traditionellen europäischen Medizin gegen Herzbeschwerden, diente als Beispiel für einen aktivitätsgeleiteten Untersuchungsansatz. Extrakte unterschiedlicher Polarität wurden mit dem etablierten Assay getestet und mittels unterschiedlicher chromatographischer Techniken unter Berücksichtigung ihrer HDAC-hemmenden Wirkungen fraktioniert. Lavandulifolioside konnte als aktive Substanz isoliert werden und die Strukturklärung erfolgte mittels verschiedener spektrometrischer/spektroskopischer Methoden. Obwohl für die Substanz nur ein IC₅₀ Wert im mikromolaren Bereich (220µM) bestimmt wurde, zeigte die Untersuchung eindrucksvoll, dass der etablierte Assay eine geeignete Methode für die aktivitäts-gelieitete Suche nach HDAC Modulatoren in Pflanzenextrakten darstellt und entsprechende Empfindlichkeit mit schnellen Analysen und überzeugender Effektivität kombiniert.

Für einige aktive pflanzliche Sekundärmetaboliten z.B. Sennoside A wurden auch zugrundeliegende molekulare Mechanismen detaillierter untersucht. Obwohl die Mechanismen nicht geklärt werden konnten und weitere Studien hinsichtlich der molekularen Wirkungsmechanismen erforderlich sind, tragen die Ergebnisse zu einem besseren Verständnis der Wechselwirkungen zwischen HDACs und den untersuchten Naturstoffen bei.