Finding bioactive compounds in plant extracts by HPLC-coupled assays: novel approaches to natural product-based drug discovery

Ph.D. Thesis

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Introduction

Mother Nature has been considered both historically and statistically as the most prolific source of drugs in human medicine: the vast majority of drugs used today is natural products (NPs), NP derivatives or synthetic mimetics related to NPs. Nevertheless, over the past two decades, in parallel with the expansion of high-throughput screening (HTS) and large compound libraries prepared by parallel synthesis, the practice of NP-based drug discovery has been gradually de-emphasized by the pharmaceutical industry. This paradigm shift can be traced back to a number of scientific as well as economical reasons. However, one of the main reasons argued against the NP-based hit generation (i.e., screening mixtures), that it is difficult to harmonize with the modern HTS-based approach: sequential and iterative application of the bioassay, the separation (fractionation/isolation) and structure elucidation steps lead necessarily to an inherently slow workflow. Moreover, the process of bioactivity-guided fractionation is further burdened by the "re-isolation" of already known compounds (dereplication).

The above outlined issues, however, could be efficiently tackled by the synergistic melding of the three crucial processes, resulting in a "coupled" (profiling) analytical approach, comprising the high resolution of the complex mixture, the reliable detection and correlation of the biological response to each constituent, and finally the rapid dereplication of the bioactives. Depending on the studied biological phenomenon (e.g., enzyme inhibition, receptor binding, free radical scavenging activity), and the type of coupling (e.g., at-line, off-line, on-line), a number of innovative methods with different terms have been implemented in drug discovery practice. It must be noted here, that the enormous development in separation technology and instrumental analysis, which took place in recent years, was essential to the birth of this area. Owing to the appearance of robust ion sources and ultra-efficient chromatographic stationary phases and instruments, the high-performance liquid chromatography coupled to mass spectrometry (HPLC-

MS) systems have opened a new dimension in the NP-mixture analysis both in terms of resolution and speed. In addition, the huge improvement in the sensitivity of nuclear magnetic resonance spectroscopy (NMR), and the expansion of the increasingly richer chemical and biological databases have also contributed significantly to more efficient dereplication processes.

This PhD work is a contribution focusing on the implementation of the above discussed methodological approach in two distinctive, but therapeutically highly relevant NP research areas. First, to identify antioxidants in plant extracts, *in vitro* radical scavenging assays were coupled off-line to HPLC methods. Second, to narrow the gap between NP research and the demand of early stage central nervous system drug discovery, the applicability of the parallel artificial membrane permeability assay specific for the blood-brain barrier (PAMPA-BBB) to NPs and plant extracts was thoroughly studied.

Objectives

The primary aim of our work was to design, adopt and/or validate, and perform screenings of the plant extract library (N=4400) of Gedeon Richter Plc. The screening assays included a cytotoxicity, an antioxidant (2,2-diphenyl-1-picrylhydrazyl radical, DPPH), and a blood-brain barrier permeability screening campaign. As the plant extract hits emerged from the latter two screenings proved to be complex mixtures of secondary plant metabolites, we attempted to couple the bioassays of particular screens to HPLC-based analytical procedures. While doing this, our motivation was to shorten the bioassay-guided isolation route of the active principle(s), particularly in the lead compound identification and dereplication step. Moreover, by mean of the presented case studies, we have endeavored to contribute to the phytochemical and pharmacological characterization of the investigated plant species.

The specific aims were the followings:

1. To screen the plant extract library for cytotoxic activity and for antioxidant activity, and to analyze the dependence of these activities of plant extracts on the type (polarity) of the solvent extraction procedure.

2.a. To analyze and prioritize (dereplicate) the resulted non-cytotoxic and antioxidant plant extract hits by LC-MS, and to develop a LC-MS method that can be coupled with the DPPH assay in order to effectively identify the radical scavenger constituents in one antioxidant plant extract hit, namely in the methanolic extract of *Artemisia gmelinii*.

2.b. To isolate and elucidate the chemical structure of the most active radical scavenger compounds in the methanolic extract of *Artemisia gmelinii*.

3.a. To adopt and validate the pyrogallol red bleaching test for HPLC in order to screen and characterize chemical constituents with peroxynitrite ($ONOO^-$) scavenging activity in alcoholic extracts of *Salvia* species, since *Salvia* extracts proved to be predominant among the antioxidant hits.

3.b. To demonstrate the performance of the developed HPLC-based $ONOO^-$ scavenging assay on the methanolic extract of *Salvia miltiorrhiza* Bunge.

4.a. To investigate the applicability of the PAMPA-BBB assay for NPs and plant metabolites, and to screen the plant extract library for NP compounds with high brain penetration propensity.

4.b. To couple the PAMPA-BBB assay to NMR experiments and to demonstrate the feasibility of this type of coupling on BBB+ plant extract hits (exemplified by the extracts of *Tanacetum parthenium*, *Vinca major*, *Salvia officinalis*, and *Corydalis cava*).

Materials and Methods

Plant material

The plant extract library of Gedeon Richter Plc. was assembled mainly between 1999 and 2001, in the course of a contractual cooperation with major Institutes of Botany, Pharmacognosy and Medicinal Plant Research in Hungary (Vácrátót, Budapest, Szeged, Budakalász). This cooperation resulted in 4400 randomly collected individual extracts, originated from ca. 500 drugs of 300 plant species endemic or to-grow in the Carpathian Basin. Based on the solvent extraction procedure, the apolar subset (N=1996) comprised extracts prepared with chloroform or in some case with petroleum ether, whereas samples originated from aqueous alcoholic extraction were considered as the polar subset (N=2404) of the library.

Plant extracts used in detailed hit profiling case studies were the followings:

- HPLC-based off-line DPPH scavenging: *Artemisia gmelinii* Webb. ex Stechm. (Asteraceae): aerial parts, Vácrátót, crude apolar ($CHCl_3$-MeOH 9:1) and polar extracts (70% MeOH), fractionated on silica gel.
- HPLC-based off-line $ONOO^-$ scavenging: *Salvia miltiorrhiza* Bunge (Lamiaceae): herb, Vácrátót, crude polar extract (70% MeOH).
- Blood-brain barrier permeability screening by PAMPA-BBB: *Tanacetum parthenium* (L.) Sch. Bip. (Asteraceae), aerial parts, Debrecen, cold CH_2Cl_2-MeOH (9:1);
  Corydalis cava Schweig. & Kört. (Papaveraceae), tubers, Dobogókő, prefractionated weakly basic alkaloid fraction;
  Salvia officinalis L. (Lamiaceae), leaves, Vácrátót, crude polar extracts (70% MeOH), fractionated on polyamide gel;
  Vinca major L. (Apocynaceae), aerial parts, Vácrátót, crude apolar ($CHCl_3$-MeOH 9:1) and polar extracts (70% MeOH), fractionated on silica gel.

Instrumentation

High performance liquid chromatography – mass spectrometry: All experiments were performed on an Agilent 1200 liquid chromatography system, coupled with an Agilent 6410 triple quadrupole mass spectrometer (QQQ-MS) equipped with an ESI source. MassHunter B.04.01 was used for data acquisition, and for qualitative and quantitative analysis. All analyses were carried out at 40 °C on an Ascentis Express C_{18} column (50 × 3.0 mm, 2.7 μm) in optimized gradient elution modes.

Nuclear magnetic resonance spectroscopy: All NMR measurements were performed on a Varian 800 MHz spectrometer equipped with a $^{1}H\{^{13}C/^{15}N\}$ Triple Resonance ^{13}C Enhanced Salt Tolerant Cold Probe operating at 800 MHz for ^{1}H and 201 MHz for ^{13}C. All pulse sequences were taken from the VNMRJ-3.1 or 3.2 pulse sequence library without modification.

Citotoxicity screening campaign

Cytotoxicity screening of the full plant extract library was performed by the fluorescent measurement of resazurin reduction (Promega) on an immortalized chinese hamster ovarian (CHO) cell line. The assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Plant extract plates were screened in a test concentration of 400 μg/mL with 1% DMSO present. Samples with activity above 20% were considered as cytotoxic.

Antioxidant activity screening campaign

The antioxidant activity screening of the full plant extract library was performed in microplate format according to the spectrophotometric DPPH method. Test samples were incubated with 60 μM ethanolic solution of DPPH for 30 min at RT. Screening concentration was set to the value of 66.7 μg/mL. Plant extracts with activity above 80% were considered as primary hits. IC_{50} values of these hits were measured in a secondary experiment.

HPLC-based off-line DPPH scavenging assay

The modified DPPH-spiking method of Tang et al. (2008) was applied to characterize the active constituents in the methanolic extract of *A. gmelinii*. Briefly, 0.5 mL of *A. gmelinii* methanolic extract dissolved in DMSO and 0.5 mL of 1.5 mM DPPH stock solution were mixed and allowed to react for 30 min. The reaction mixture was then filtered and analyzed with an optimized HPLC-MS method. Ethanol was added to the extract to provide the unreacted control sample. After comparing the chromatograms of the reaction mixture with the one of the control sample, the most active two compounds of the extract were isolated with targeted preparative HPLC and analyzed extensively by HRMS and NMR.

HPLC-based off-line peroxynitrite scavenging assay

The pyrogallol red bleaching test used for peroxynitrite ($ONOO^-$) scavenging activity measurement was adopted for HPLC by an extensive analysis of a phenolic model mixture representative for alcoholic extracts of *Salvia* species (N=17, 600 μM for each). HPLC conditions, such as eluent pH (1.90), injection volume (6 μL) and detection wavelength (470 nm) were carefully optimized for the selective detection of the test substrate pyrogallol red. Degradation kinetics of phenolic compounds in mixture were assessed after the reaction with increasing concentration of $ONOO^-$ (0.5-10 mM) and compared with individual scavenging activitites (IC_{50}). Finally, the developed assay was demonstrated on the methanolic extract of *S. miltiorrhiza*.

Blood-brain barrier permeability screening and related methods

PAMPA-BBB experiments were performed according to standard a protocol: Millipore 96-well sandwich plate system, 0.01 M phosphate buffered saline (PBS) at pH 7.4 in the donor as well as in the acceptor phase, BBB-specific porcine brain lipid dissolved in n-dodecane, incubation at 37 °C for 4 hrs. Validation of the PAMPA-BBB assay was performed by the effective permeability (P_e, cm/s) measurement of a NP (N=23) and NP-like drugs (20)

test set. The non-cytotoxic subset of the plant extract library (N=1760) was screened with the same protocol in a test concentration of 1.0 mg/mL with 10% DMSO as co-solvent in donor wells by an UV-vis reader (240-400 nm). In the case of the four BBB+ extracts studied (*T. parthenium*, *C. cava*, *S. officinalis*, *V. major*) PAMPA-BBB experiments were repeated in deutero PBS buffer, starting from the most concentrated samples (50 mg/mL) with 10% co-solvent. The resulting acceptor solutions were collected and 1-1 mL from each sample was subjected directly to NMR analysis.

Quantification of reference compounds, NP and NP-like drugs, and dereplication and characterization (by the chromatographic retention factor and MW) of BBB+ plant extracts (hits) were performed by optimized LC-MS methods. Co-solvent content (DMSO, MeOH) of PAMPA-BBB-derived acceptor solutions was quantified by gas chromatography-flame ionization detector.

Results and Discussion

1. The cytotoxicity and the antioxidant screening of the plant extract library were successfully designed and performed. It was found that more than the half of the library (57%) possessed cytotoxic activity, whereas the percent of samples with considerable antioxidant activity was only 5.7%. Moreover, we demonstrated that both the cytotoxic and the antioxidant activity were significantly dependent on the type of the solvent extraction procedure: samples extracted with chloroform were twice as likely to show citotoxicity than samples originated from methanolic extraction, whereas radical scavenging activity was more pronounced among samples extracted with methanol.
2. Afterwards, in course of a case study, a DPPH-HPLC method was developed for the antioxidant-activity guided phytochemical investigation of a methanolic plant extract hit, namely *Artemisia gmelinii*.

   Novel methodological findings: It was demonstrated that the off-line coupling of the DPPH assay with the LC method enabled the rapid and reliable identification of the free radical scavenger molecules in the mixture. This proved to be a key advantage, since after the dereplication of 6 major constituents by LC-MS, the preparative HPLC purification was only targeted toward the isolation of the two most active compounds (3,5-*O*-dicaffeoylquinic acid and ethyl-3,5-*O*-dicaffeoylquinate). It must be noted, however, that the structure elucidation of the isolated dicaffeoylquinic acids (DCQAs) turned out to be an unexpectedly difficult task due to literature ambiguities.

   Novel phytochemical and pharmacological findings: Out of the eight identified compounds six, namely chlorogenic acid, 4-*O*-caffeoylquinic acid, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, and the two DCQAs were first described in *A. gmelinii*. Moreover, based on the high degree of phytochemical similitude with major hepatoprotective compounds of artichoke, it was speculated that the identified

phenylpropanoids and flavonoids may confirm by their antioxidant potential the ethnopharmacological usage of *A. gmelinii* in inflammatory liver conditions.

3. Next, the same off-line coupling concept was realized in the case of another radical species. The colorimetric pyrogallol red bleaching assay used for $ONOO^-$ scavenging assessment was successfully adopted for HPLC.

   Novel methodological findings: We proved the theory by a thorough validation study that upon reaction with $ONOO^-$, the peak areas of compounds in complex mixtures with radical scavenging potential will significantly and proportionally decrease or disappear in the chromatograms. Aspecific degradation of carnosol represented the inherent limitation of the assay. Thus, it was demonstrated that the developed assay enabled simultaneously and reliably the rapid chemical characterization and the $ONOO^-$ scavenging activity profiling of alcoholic extracts of *Salvia* species.

   Novel phytochemical and pharmacological findings: By studying the $ONOO^-$ scavenging activity of 17 phenoloid marker compounds of the genus *Salvia*, it was found that gallic acid, caffeic acid, rosmarinic acid, salvianolic acid B, quercetin, salvianolic acid A, and kaempferol possessed outstanding activity against $ONOO^-$. Moreover, application of the developed assay on the methanolic extract of *S. miltiorrhiza* revealed that basically the phenylpropanoid constituents were responsible for the significant $ONOO^-$ scavenging activity of this widely used medicinal herb.

4. In the second part of our work, to narrow the gap between NP research and the demand of early stage CNS drug discovery, applicability of the PAMPA-BBB assay to NPs and plant extracts was thoroughly demonstrated.

   Novel methodological findings: It was found that the PAMPA-BBB assay preserved its predictive power in the case of NPs and provided high phytochemical selectivity, which enabled its use as a unique

filtering tool in terms of selecting brain penetrable compounds from plant extracts. We took advantage of the single mechanism-based (passive diffusion) as well as the *in vitro* nature of the PAMPA-BBB assay: it was demonstrated that simple modifications in the assay design (i.e., performed in deuterated milieu with an elevated dose of multicomponent extracts) allowed the direct (at-line) use of PAMPA-BBB filtered samples in a dereplication process, as performed by NMR and LC-MS. Finally, it was concluded that the developed PAMPA-BBB/LC-MS/NMR cascade is worthy of being integrated in a HTS-based, NP-utilizing CNS drug discovery environment.

Novel phytochemical and pharmacological findings: By studying the effective BBB permeability profile of major phytochemical compound classes, it was revealed that, in accordance with basic concepts, glycosides and carboxylic acids were unable to pass across lipid bilayers by passive diffusion, whereas representative compounds of flavonoids (as aglycones), alkaloids, terpenes and coumarins showed moderate or considerable BBB permeability potential. Moreover, our work provided important *in vitro* evidences regarding the brain penetrability of the pharmacologically active constituents of *T. parthenium*, *V. major*, *S. officinalis*, and *C. cava*.

Conclusions

Today's drug discovery relies basically on the HTS-based approach and operates at an accelerated pace. To meet the demand of this industrial environment, innovative methods focusing on the improvement and acceleration of the NP-based lead generation have emerged and been implemented recently. Among those, HPLC-coupled assays used for both chemical and biological profiling of complex NP extracts have shown potential to significantly increase the efficiency of or even to fully substitute time-consuming bioactivity-guided fractionation procedures. The essence and simultaneously the greatest challenge in the development of such HPLC-based profiling assays, beyond instrumentation issues, is the valid and efficient interfacing of biological data with chemo-analytical information.

Based on the resolution and multi-dimensional information content provided by the presented HPLC-based profiling assays, we conclude that the marriage of advanced separation and spectroscopic techniques to robust bioassays is a viable and powerful approach to NP-based lead generation. In addition, the developed profiling assays could be superior for the quality, efficacy and safety assessment of medicinal plant extracts and herbal formulas. Finally, we believe that these novel approaches will be gradually naturalized also in the mainstream drug discovery.

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