Synthesis of new, biologically active protoflavone derivatives

Summary of PhD Thesis

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Introduction

Protoflavones represent a unique class of natural flavonoids with a non-aromatic B-ring and a hydroxyl group at C-1'. The first protoflavone was isolated from the *Equisetum arvense* and it is called protogenkwanin 4'-glucoside. Protoflavonoids have been mainly reported from plant species ferns but not only. Such compounds have also been isolated from *Apium graveolens* and *Piper carniconnectivum*. The best studied protoflavone derivative is protoapigenone that was first isolated from the fern *Thelypteris torresiana in* 2005. Protoflavones are biologically active compounds. Protoapigenone has strong antitumor activity *in vitro* and *in vivo*. Together with a synthetic analog, WYC0209, it was found that it can affect the ATR signaling pathway, and sensitize cancer cells to DNA damaging chemotherapeutics. Moreover, protoapigenone was also found to have antiviral activity against the Epsten-Barr virus. The first total synthetic procedure of protoflavones was reported by Lin et al. in 2007. Protoapigenone and several synthetic protoflavones were synthetized as potential antitumor agents. Trihydroxyacetophenone (diprotected with Methoxymethyl) was used as starting material and 4-benzyloxybenzaldehyde was added to the reaction mixture, and, after removal of the benzyl protecting group by catalytic hydrogenation, oxidative de-aromatization was performed by PIFA. Finally, protoapigenone was successfully obtained by removing the methoxymethyl protecting group. The procedure was very long, resulting in a low final isolated yield. Protoapigenone can, however, also be synthetized directly from apigenin. That was reported by Attila Hunyadi in 2011. This method allowed a fast and economic synthesis of up to the gram scale, representing a breakthrough in studying the bioactivity of this interesting flavonoid**.** At the beginning of my PhD studies I had the chance to be partially involved in this work, which then served as the head-start for my studies.

Objectives

The following objectives were set up for this work.

1. As a primary aim of the work, to prepare new A-ring modified synthetic protoflavones and their 1′-O-alkoxy derivatives,
2. to perform an in vitro and in silico study on the potential formation of protoapigenone upon ROS scavenging by apigenin,
3. to test the cytotoxicity of the newly prepared compounds and to extend related structure-activity relationships, with a strong focus on multi-drug resistant cancer, and
4. to search for other potential bioactivities of these compounds, not or not directly related to the cytotoxic effect.

Materials and methods

Starting materials

Commercially available 4′-hydroxyflavones (4′-hydroxy-6-methylflavone, 4′-hydroxy-6-methoxyflavone, 4′-hydroxy-β-naphthoflavone) were purchased from Indofine Chemical Company, Inc. (Hillsborough USA). Chemicals were obtained from Aldrich, Inc. (USA).

Synthesis of protoflavone derivatives

Semi-synthesis

Protoflavone 1′-*O*-alkyl ethers were synthesized from apigenin, genkwanin, 4′-hydroxy-6-methylflavone, 4′-hydroxy-6-methoxyflavone and 4′-hydroxy-β-naphthoflavone by using a one step synthesis method. The oxidative de-aromatization was performed by a common hypervalent iodine reagent, [*bis*(trifluoroacetoxy)iodo]benzene (PIFA) in acetonitrile in the presence of water or the alcohol to be coupled at position C-1′.

Total-synthesis

C-6 modified protoflavone derivatives were synthetized from hydroxyacetophenone and 4-ethyl- and 4-pentylphenol as starting materials. Total synthesis was achieved in 4-6 steps by using different synthetic methods. (Fries-rearrangement reaction, Claisen-Schmidt condensation, Suzuki coupling, debenzylation and oxidative de-aromatization.)

Structure elucidation

Structure elucidation was carried out by means of nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS). NMR spectra were obtained on a Varian Gemini-2000 200 MHz or Bruker Avance DRX-500. Mass spectra were taken on an API 2000 triple-quadrupole (Ab Sciex, USA) or LCMS-IT-TOF (Shimadzu, Japan) with and ESI interface.

In silico studies on the formation of protoapigenone from apigenin

Calculations were achieved in the Gaussian09 software within the DFT (Density Functional Theory) formalism. For the phenolic group O-H bond dissociation enthalpies (BDE), were calculated as the difference between Fl-OH (the flavonoid) and Fl-O$^{\cdot}$+ H$^{\cdot}$ (the corresponding radicals formed after H-atom abstraction (HAT) from Fl-OH to the free radical. The effect of solvent was taken into account by using the integral-equation-formalism polarizable continuum model (IEF-PCM).

Experimental studies on the apigenin-protoapigenone transformation

Apigenin was dissolved in aqueous MeOH, and the pH was adjusted to pH=4 by using H_2SO_4. Iron catalyst ($FeSO_4 \cdot 7H_2O$) was added, followed by the slow addition of of 30% H_2O_2. The reaction mixture was purified by using SPE on C18 stationary phase, and investigated by HPLC.

Bioassays

Cytotoxicity

In the experiments on bioactivity, the compound were tested on four human cancer cell lines (HepG2, Hep3B, A549, MDA-MB-23) and on five non-MDR/MDR cell line pairs (including A431, $A431_{B1}$, $A431_{G2}$, MES-SA, MES-SA/Dx5, KB-3-1, KB-V1, L5178, $L5178_{B1}$, MCF-7, $MCF\text{-}7_{dox}$ cell lines)

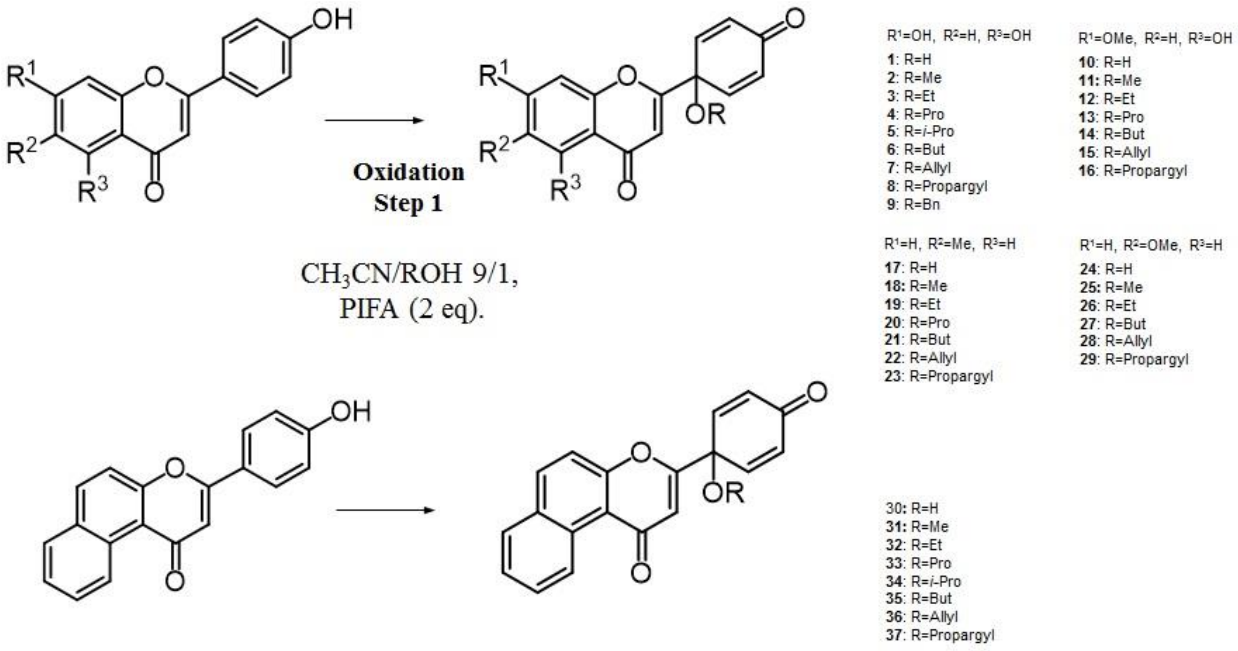
Xanthine Oxidase inhibition

XO inhibition activity were obtained by using commercially available XO activity assay kit (Sigma-Aldrich Ltd., USA), following the provided protocol. The 3D structure of the compounds was optimized prior to docking, by the Gaussian09 (Gaussian Inc., Wallingford, USA) software. Docking study was performed by using iGEMDOCK 2.1 (BioXGEM, Hsinchu, Taiwan) at default settings. Docking was validated by re-docking the "Que" residue into the macromolecule in mol2 format in order to allow flexible docking. Visualization of the ligand-residue interactions were achieved with Discovery Studio 3.

Results and discussion

Synthesis

Thirty-seven protoflavones and protoflavone 1′-*O*-alkyl ethers were synthesized from commercially available 4′-hydroxyflavones (apigenin, genkwanin, 4′-hydroxy-6-methylflavone, 4′-hydroxy-6-methoxyflavone and 4′-hydroxy-β-naphthoflavone) by utilizing PIFA-mediated oxidative de-aromatization.



R¹=OH, R²=H, R³=OH
1: R=H
2: R=Me
3: R=Et
4: R=Pro
5: R=*i*-Pro
6: R=But
7: R=Allyl
8: R=Propargyl
9: R=Bn

R¹=OMe, R²=H, R³=OH
10: R=H
11: R=Me
12: R=Et
13: R=Pro
14: R=But
15: R=Allyl
16: R=Propargyl

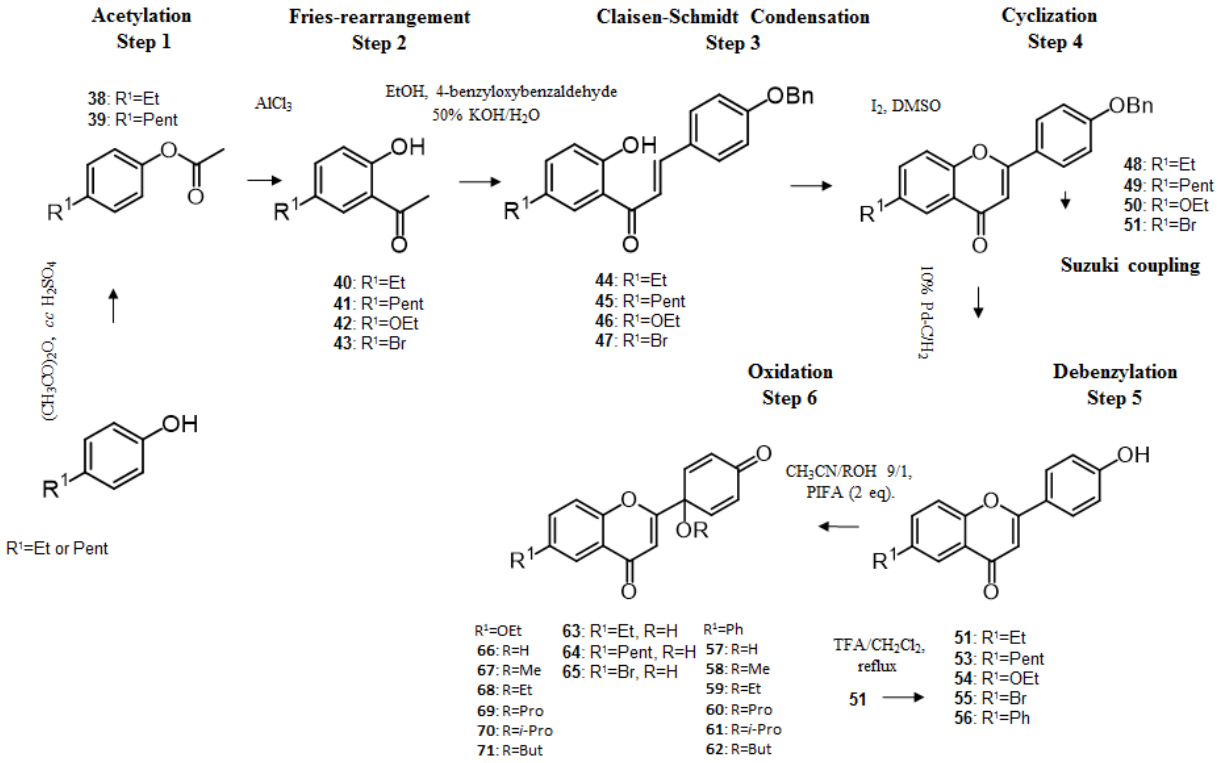
R¹=H, R²=Me, R³=H
17: R=H
18: R=Me
19: R=Et
20: R=Pro
21: R=But
22: R=Allyl
23: R=Propargyl

R¹=H, R²=OMe, R³=H
24: R=H
25: R=Me
26: R=Et
27: R=But
28: R=Allyl
29: R=Propargyl

30: R=H
31: R=Me
32: R=Et
33: R=Pro
34: R=*i*-Pro
35: R=But
36: R=Allyl
37: R=Propargyl

Fifteen protoflavones and protoflavone 1′-*O*-alkyl ethers were synthesized by using 4-6 step total synthetic method. In order to obtain starting materials (*i.e.* 5′-ethyl-2′-hydroxyacetophenone and 5′-pentyl-2′-hydroxyacetophenone for our 6-ethyl and 6-pentyl substituted target compounds, the appropriate *p*-substituted phenols were acetylated and subjected to Fries-rearrangement reaction under the condition of dry $AlCl_3$ in dichloromethane. The resulting 2′-hydroxyacetophenones and those commercially available with a 5′-ethoxy or -bromo substituent were utilized in Claisen-Schmidt condensation reactions with *p*-benzyloxybenzaldehyde to yield chalchones, which, after performing ring closure with iodine in DMSO, yielded the corresponding 6-substituted 4′-benzyloxyflavones. The 6-bromo substituted compound was subjected to Suzuki coupling in order to obtain the corresponding 6-phenylflavone. Debenzylation of the flavonoids obtained this way

and subsequent oxidative de-aromatization of the flavones with PIFA, as described above, allowed us to obtain the protoflavones with various substituents at positions C-6 and C-1′.



Anticancer activity

Cytotoxicity of the newly obtained compounds was tested on a panel of sensitive and multi-drug resistant cell lines. The ability of protoflavones to evade efflux-mediated MDR was confirmed both in ABCB1 and ABCG2 expressing cell lines, with the exception of protoapigenone, which was identified as an ABCG2 substrate. Moreover, MDR selective cytotoxicity was observed for most of the tested protoflavones in a breast cancer cell line adapted to doxorubicin (MCF-7_{Dox}) and SAR revealed importance of the A-ring substitution, while in the uterine sarcoma MES-SA/Dx5, another doxorubicin-selected cell line, only the 1′-OH containing compounds showed relevant selectivity. Studies on the mechanism for the MDR selectivity suggested the involvement of changes in the antioxidant defense of the cancer cells during the evolution of resistance.

Activity of selected compounds on xanthine

We performed a screening of some of our compounds for xanthine oxidase inhibitory activity. The genkwanin derivatives were inactive, whilst a weak inhibition was found for some of the naphthoflavone derivatives) and weak to moderate activity was observed for most of the protoapigenone analogs. However, protoapigenone 1′-*O*-propargyl ether was found to inhibit the enzyme almost completely at the tested concentration. The dose–effect curve was determined for this compound and compared to those of allopurinol and apigenin. Enzyme kinetic studies were also performed in order to investigate the inhibition mechanism of the propargyl ether derivative. The kinetic curve was found to be characteristic for substrate inhibition, and the data indicated that the compound is a competitive inhibitor of the enzyme. The binding mode of the propargyl ether derivative into the enzyme was investigated by *in silico* docking. *In silico* docking studies revealed a flip-flop orientation of this compound as compared to that of quercetin, and provided a reasonable explanation for the role of the propargyl side chain, fitting perfectly into the hydrophobic pocket formed by the Leu648, Phe649, Asp872, Leu873 and His875 residues.

The role of OH radical scavenging in the formation of protoapigenone from apigenin.

The possible formation of protoapigenone from apigenin was first studied *in silico*, within the DFT (Density Functional Theory) formalism. Bond dissociation enthalpies (BDEs) for the 4′-OH group of apigenin were calculated in the gas phase or by taking into account the effect of solvent. Electron spin density was calculated for the resulting phenoxyl radical in order to have an estimation on the position where an OH radical could possibly attack to this intermediate. Here we can see that position 1' is prefered. Based on the above *in silico* results, it can be stated that, even though the initiating hydrogen atom transfer requires a relatively large energy, such a transformation is indeed possible. In order to obtain experimental verification or disproof to this hypothesis, Fenton's reaction was performed on apigenin, and the resulting mixture was analyzed by RP-HPLC-DAD after pre-purification. Traces of protoapigenone were identified. By means of direct CE measurements on such mixtures, we could conclude that protoapigenone is likely a major bioactive metabolite of apigenin whenever such a scavenging event takes place. Furthermore, the possible reduction of protoapigenone to apigenin was studied by incubating it with reduced glutathione (GSH) for 24h. Protoapigenone could be reduced back to apigenin, which is evidence for the existence of an apigenin-protoapigenone-apigenin redox cycle.

Summary

- Fifty-two protoflavone derivatives including 50 new compounds were prepared
- The ability of protoflavones to evade efflux-mediated MDR was confirmed both in ABCB1 and ABCG2 expressing cell lines (except protoapigenone in ABCG2).
- MDR selective cytotoxicity was observed for most of the tested protoflavones in a breast cancer cell line (MCF-7_{Dox}).
- Protoapigenone 1′-*O*-propargyl ether was identified as an efficient competitive inhibitor of xanthine oxidase.
- *In silico* DFT calculations, and HPLC and CE analyses revealed the apigenin-protoapigenone transformation upon OH radical scavenging.

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The thesis is based on the following publications

I. Hunyadi A, Chuang DW, **Danko B**, Chiang MY, Lee CL, Wang HC, Wu CC, Chang FR, Wu YC; Direct Semi-synthesis of the Anticancer Lead-drug Protoapigenone from Apigenin, and Synthesis of Further New Cytotoxic Protoflavone Derivatives. *PLoS ONE* **2011**, Vol. 6(8), pp. e23922. **IF: 4.092**

II. **Danko B**, Martins A, Chuang DW, Wang HC, Amaral L, Molnar J, Chang FR , Wu YC, Hunyadi A; *In vitro* Cytotoxic Activity of Novel Protoflavone Analogs – Selectivity against a Multi-drug Resistant Cancer Cell Line. *Anticancer Res.* **2012**, Vol. 32, pp. 2863-2870. **IF: 1.725**

III. Poor M, Li Y, Kunsagi-Mate S, Varga Zs, Hunyadi A, **Dankó B**, Chang FG, Wu YC, Kőszegi T; Protoapigenone Derivatives: Albumin Binding Properties and Effects on HepG2 Cells. ***Journal of Photochemistry and Photobiology B-Biology 2013, Vol*** 124*, pp. 20-26.* **IF: 3.110**

IV. Hunyadi A, Martins A, **Danko B**, Chuang D-W, Trouillas P, Chang F-R, Wu Y-C, Falkay G; Discovery of The First Non-planar Flavonoid That Can Strongly Inhibit Xanthine Oxidase: Protoapigenone 1′-o-propargyl ether. *Tetrahedron Letters* **2013**, Vol 54(48), pp. 6529-6532. **IF: 2.397**

V. Hunyadi A, Martins A, **Danko B**, Chang FR, Wu YC; Protoflavones: a Class of Unusual Flavonoids As Promising Novel Anticancer Agents ***Phytochemistry Reviews, 2014, Vol*** 13***,*** *pp. 69-77.* **IF: 2.407**

VI. Stankovic T, **Danko B**, Martins A, Dragoj M, Stojkovic, Isakovic A, Wang H-C, Wu Y-C, Hunyadi A, Pesic M. Lower Antioxidative Capacity of Multidrug-Resistant Cancer Cells Confers Collateral Sensitivity to Protoflavone Derivatives. ***Cancer Chemotherapy and Pharmacology, 2015, Vol*** 76(3)***,*** *pp. 555-565.* **IF:2.824**

VII. **Danko B**, Toth S, Martins A, Vagvolgyi M, Kusz N, Molnar J, Chang FR, Wu YC, Szakacs G, Hunyadi A. Synthesis and SAR Study of Novel Anticancer Protoflavone Derivatives – Investigation of Cytotoxicity and Interaction with the ABCB1 and ABCG2 Multidrug Efflux Transporters. *ChemMedChem*, 2017, Vol. 12, pp 850-859. **IF=3.225**

Other Publications

VIII. Hunyadi A, **Danko B**, Boni, M, Militaru A, Alexandru T, Nastasa V, Andrei IR, Pascu M, Amaral L**;** Rapid, Laser-Induced Concersion of 20-Hydroxyecdysone and its Diacetonide – Experimental Set-up of a System for Photochemical Transformation of Bioactive Substances. *Anticancer Res.* **2012**, Vol 32(4), pp. 1291-1297. **IF: 1.725**

IX. Csupor D, Widowitz U, Blazsó G, Laczko-Zold E, Tatsimo J. S. N., Balogh A, Boros K, **Danko B**, Bauer R, Hohmann J; Anti-inflammatory Activities of Eleven *Centaurea* Species Occurring in the Carpathian Basin. *Phytotherapy Res.* 2013 Vol 27 pp540-544. **IF: 2.650**

X. Csupor D, Boros K, **Danko B**, Veres K, Szendrei K, Hohmann J; Rapid Identification of Sibutramine in Dietary Supplements Using a Stepwise Approach. ***Pharmazie 2013, Vol*** 68, *pp. 15-18.* **IF:1.006**

XI. Pascu ML, **Danko B**, Martins A, Jedlinszki N, Alexandru T, Nastasa V, Boni M, Militaru A, Andrei IR, Staicu A, Hunyadi A, Fanning S, Amaral L; Exposure of Chlorpromazine to 266nm Laser Beam Generates New Species with Antibacterial Properties: Contributions to a New Process for Drug Discovery. ***Plos One 2013, Vol*** 8(2), *pp. e55767.* **IF:4.490**

XII. Hunyadi A, Veres K, **Danko B**, Kele Z, Weber E, Hetenyi A, Zupko I, Hsieh TJ; In vitro Anti-diabetic Activity and Chemical Characterization of an Apolar Fraction of *Morus alba* Leaf Water Extract. ***Phytotherapy Research 2013, Vol*** 27(6) *pp. 847-851.* **IF: 2.086**

XIII. Armada AM, Alexandru T, Machado D, **Danko B**, Hunyadi A, Dinache A, Nastasa V, Boni M, Ramos J, Viveiros M, Molnar J, Pascu ML, Amaral L; The In Vitro Activity of Products Formed from Exposure of Chlorpromazine to a 266nm LASER Beam against Species of Mycobacteria of Human Interest. ***In Vivo 2013, Vol*** 27(5) *pp. 605-610.* **IF: 1.148**

XIV. Alexandru T, Armada A, **Danko B**, Hunyadi A, Militaru A, Boni M, Nastasa V, Martins A, Alexandru Viveiros M, Pascu ML, Molnar J, Amaral L; Biological Evaluation of Products Formed from the Irradiation of Chlorpromazine with a 266 nm Laser Beam. ***Biochemistry and Pharmacology 2013, Vol*** 2(1) *pp. 1-4.* **IF: -**

XV. Lai WC, **Danko B,** Csabi J, Kele Z, Chang FR, Pascu ML, Gáti T, Simon A, Amaral L, Tóth G, Hunyadi A. Rapid, Laser-Induced Conversion of 20-Hydroxyecdysone - a Follow-up Study on the Products Obtained *Steroids* **2014**, Vol 89, pp. 56-62. **IF: 2.639**

XVI. Lai WC, Wu YC, **Danko B**, Cheng YB, Hsieh TJ, Martins A, Hohmann J, Hunyadi A, Chang FR. Bioactive Constituents of *Cirsium japonicum var. australe*. *Journal of Natural Products* **2014**, Vol 77**,** pp. 1624-1631. **IF: 3.798**

Presentations related to the thesis

1. Hunyadi A, **Danko B**, Chuang DW, Chen SL, Martins A, Molnar J, Chang FR Wu YC; Microwave-assisted one-step synthesis and *in vitro* cytotoxic activity of protoapigenone and its 1'-O-alkyl derivatives. 25th Symposium on Natural Products, Nov. 6-7. 2010. Checheng, Taiwan,
2. **Danko B**, Lee JC, Chang FR, Wu YC, Hunyadi A; Synthesis and anti-HCV activity of 1'-*O*-alkyl protoflavone derivatives. Trends in Natural Products Research: A PSE Young Scientist's Meeting, June 12–15. 2011. Kolymvari, Crete, Greece
3. Hunyadi A, **Danko B**, Chuang DW, Chang FR, Wu YC, Falkay G;Strong inhibition of xanthine oxidase by a non-planar flavonoid, protoapigenone 1'-*O*-propargylether. 12th Eurasia Conference on Chemical Sciences, Apr. 16-21. 2012. Corfu, Greece
4. **Danko B**, Bayach I, Fabre G, Trouillas P, Hunyadi A; In silico study of the redox properties of apigenin and protoapigenone - a midterm STSM progress report. COST Action CM0804, joint cost meeting, November 5-6, 2012, Salerno, Italy
5. **Danko B**, Martins A, Chang FR, Wu YC, Hunyadi A; Total synthesis of new protoflavone derivatives. COST Action CM1106, 2nd Working Group Meeting, September 19 – 20 2013, Warsaw, Poland
6. **Danko B**, Martins A, Amaral L, Molnar J, Chang FR, Wu YC, Hunyadi A; Synthesis of new MDR selective protoflavone derivatives. COST Action CM1106, 3nd Working Group Meeting, 27 – 28 March 2014, Budapest, Hungary
7. Stankovic T, **Danko B**, Milosevic Z, Bankovic J, Martins A, Molnar J, Amaral L, Hunyadi A, Pesic M; Selectivity of protoflavone derivatives towards human multi-drug resistant cancer cell lines. COST Action CM1106 3rd Working Group Meeting, March 27 – 28 2014, Budapest, Hungary

8. **Danko B**, Martins A, Amaral L, Molnar J, Pesic M, Szakács G, Hunyadi A; Semi- and total-synthetic protoflavone derivatives as mdr selective anticancer agents 9th International Conference of Anticancer Research, 6 - 10 October 2014, Sithonia, Greece.
9. **Dankó B**, Jin-Ching Lee, Fang-Rong Chang, Yang-Chang Wu, Hunyadi Attila; P-8 Synthesis and anti-HCV activity of 1'-O-alkyl protoflavone derivatives XII. Magyar Gyógynövény Konferencia, 5-7 May 2011. Szeged.
10. Hunyadi A, **Dankó B**, Martins A, Amaral L, Molnár J; Protoflavonok előállítása és farmakológiai vizsgálata. Az MTA Alkaloidkémiai és Flavonoidkémiai munkabizottsága Előadóülése. 12-13 May 2014. Balatonalmádi.
11. **Dankó B**, Martins A, Chang FR, Wu YC, Hunyadi A. MDR szelektív rák ellenes hatással rendelkező protoflavon származékok előállítása. Congressus Pharmaceuticus Hungaricus XV, 10-12 April 2014 Budapest