Final report to the project "Antioxidants as oxidative stress activated prodrugs: antitumor potential of an overlooked chemical space"

With the support of this project, **30 SCI articles** were published (cumulative **IF=114.43**), **five PhD theses** were delivered (4 students graduated *summa cum laude*, 1 thesis is currently under review), and the **PI** has delivered his **DSc thesis** for evaluation by the Hungarian Academy of Sciences.

The project aimed to utilize an antioxidant-inspired diversity-oriented synthetic strategy, based on biomimetic, or even biorelevant oxidative chemical transformations of small-molecule antioxidants to obtain new bioactive compounds with an enhanced potential against cancer. Theoretical background and the general idea behind the project were published as a review paper in a top-ranking journal (*Med Res Rev, 2019*).

The project resulted in the preparation of a **library of altogether 318 antioxidants and/or their metabolites and analogs** (not counting the expectably inactive intermediates of the multi-step syntheses), and bioactivity of these compounds were explored primarily against *in vitro* tumor models but also against various further pathologies to expand the scope of the project.

From the chemical point of view, the project involved natural product isolation and semi- and total synthesis, and it was proposed to follow three major directions: 1) *p*-quinols, 2) ecdysteroids, and 3) oxidized products of cinnamic acids and other phenolic antioxidants. Our most important results are briefly summarized below.

p-QUINOL DERIVATIVES

p-Quinol derivatives were inspired by the protoflavone skeleton that we have previously identified as an ROS-oxidized derivative of 4'-hydroxyflavones. Over the time frame of the project, the work was expanded towards several non-flavonoid type analogs of these oxidized antioxidant metabolites to explore relevant structure-activity relationships. As chemical strategy, we used hypervalent iodine-mediated oxidative de-aromatization of natural or synthetic *p*-phenolic compounds (leading to 15 dienone B-ring-containing protoflavones – *ChemMedChem, 2017*; 7 flavanone oximes – *Int J Mol Sci, 2019*; 14 protochalchones, 12 spiroethers, 16 spirolactones, 1 azaspiroquinone and 16 phenanthrene derivatives – *J Nat Prod, 2020*) or a C-C coupling reaction of *p*-quinone and a boronic acid (leading to further 22 compounds); from this work, three manuscripts are currently in preparation. Four triazol-coupled protoflavone-chalchone hybrid compounds were also prepared to explore the antitumor potential of combining ATR inhibitor fragment (protoflavone) with an oxidative stress inducer that interferes with mitochondrial membrane potential (chalchone or ferrocene). To also start exploring the miscellaneous pharmacology of protoflavones, 20 B-ring modified compounds were also prepared, in which the cytotoxic pharmacophore was eliminated (*ChemPlusChem, 2018*, *Int J Mol Sci, 2019*); from this work, two new antiviral agents (one against HIV, and another against EBV) were identified (*Int J Mol Sci, 2019*). The chemical diversity explored during this work is briefly summarized in Fig. 1.

Structure-activity relationships have been thoroughly evaluated and reported in the corresponding publications for each group of compounds. Considering the most potent compounds obtained within each compound group, a rough order of antitumor potential of these groups can be concluded as spirolactones and spiroethers ≈ protoflavone-chalchone hybrids > boronic acid-coupled quinones > protoflavones ≈ phenanthrene quinols > protochalchones.

Accordingly, a set of spirocyclic compounds and protoflavone-chalchone hybrids were identified as the most potent antitumor leads from this line of our work, and the best of these reached well below micromolar (ca. 2-300 nM) IC_{50} values on various cancer cell lines including multidrug resistant ones. Concerning the mechanism of action, investigation of the spirocyclic derivatives is currently ongoing, to this end, their expected ATR inhibitor activity has been confirmed.

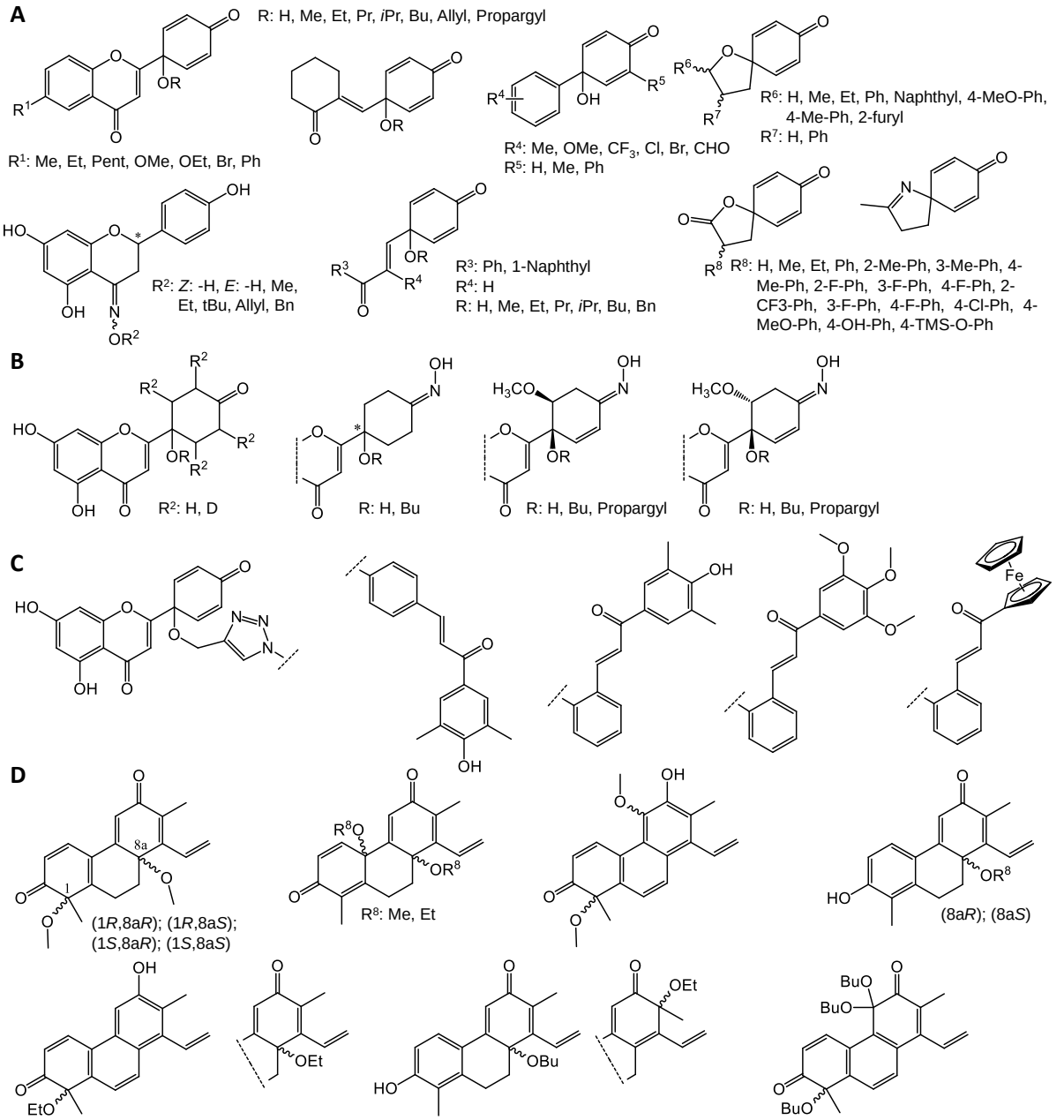

Figure 1. Cytotoxic flavonoids and *p*-quinol derivatives prepared during the project. **A**: flavonoids, protoflavonoids, and protochalchones, and *p*-quinol-inspired spirocycles, **B**: non-cytotoxic protoflavones, **C**: protoflavone-chalchone hybrids, **D**: phenanthrene derivatives obtained through diversity-oriented synthesis from juncuenin B. Most racemic compounds were obtained in enantiopure form through preparative chiral HPLC and stereospecific differences in the bioactivities were elaborated.

For the hybrid compounds (Fig. 1C), a novel approach was taken to use the Chou–Talalay method for an evaluation of the nature of interactions between relevant fragments of the hybrids. Experimental combination treatment with the fragments showed additive effects or slight/moderate synergism, while strong synergism was observed when the fragments were virtually combined into their hybrids, suggesting a relevant pharmacological benefit of the coupling. All hybrids were strong inhibitors of the ATR-mediated activation of Chk1, and they interfered with the redox balance of the cells leading to mitochondrial membrane depolarization. Additionally, they induced late apoptosis and primary necrosis in MDA-MB-231 and MCF-7 breast cancer cells, respectively. Our results demonstrated that coupling the ATR-dependent signaling inhibitor protoflavone with a pro-oxidant chalcone dramatically increases the antitumor activity compared with either fragment alone. Such compounds may offer an attractive novel strategy for the treatment of various cancers (*Antioxidants, 2020*).

Currently, we are working on new nitrogen-containing spirocycles (to introduce chemical properties of ATR inhibitors that are subjects of ongoing clinical trials, hence increasing the ATR inhibitory activity of our leads), and further protoflavone-based hybrid compounds (combining these ATR inhibitors with a p53 reactivator fragment to simultaneously target these two synergistic antitumor pathways).

ECDYSTEROIDS

Ecdysteroids were involved in this work based on their non-phenolic antioxidant properties, and on our previous observation on the increased bioactivity of certain autoxidized ecdysteroid metabolites as compared with their parent compound.

To obtain enough starting material for semi-synthesis and to further expand the chemical space of these chemically and pharmacologically versatile compounds, a large-scale natural product isolation procedure was performed from a commercial extract of *Cyanotis arachnoidea* (*J Chromatogr B, 2017*), worldwide used as a food supplement. Twenty-seven ecdysteroids, many of them with unusual, and/or highly oxidized chemical structures were obtained; their structures are shown in Fig. 2. Thanks to this approach, some of these otherwise rare minor ecdysteroids became available in up to the several grams scale. In a side-project using these compounds as analytical standards, we discovered that phytoecdysteroids are abundant in the blood of insectivorous wild passerine birds (*Sci Rep, 2019*).

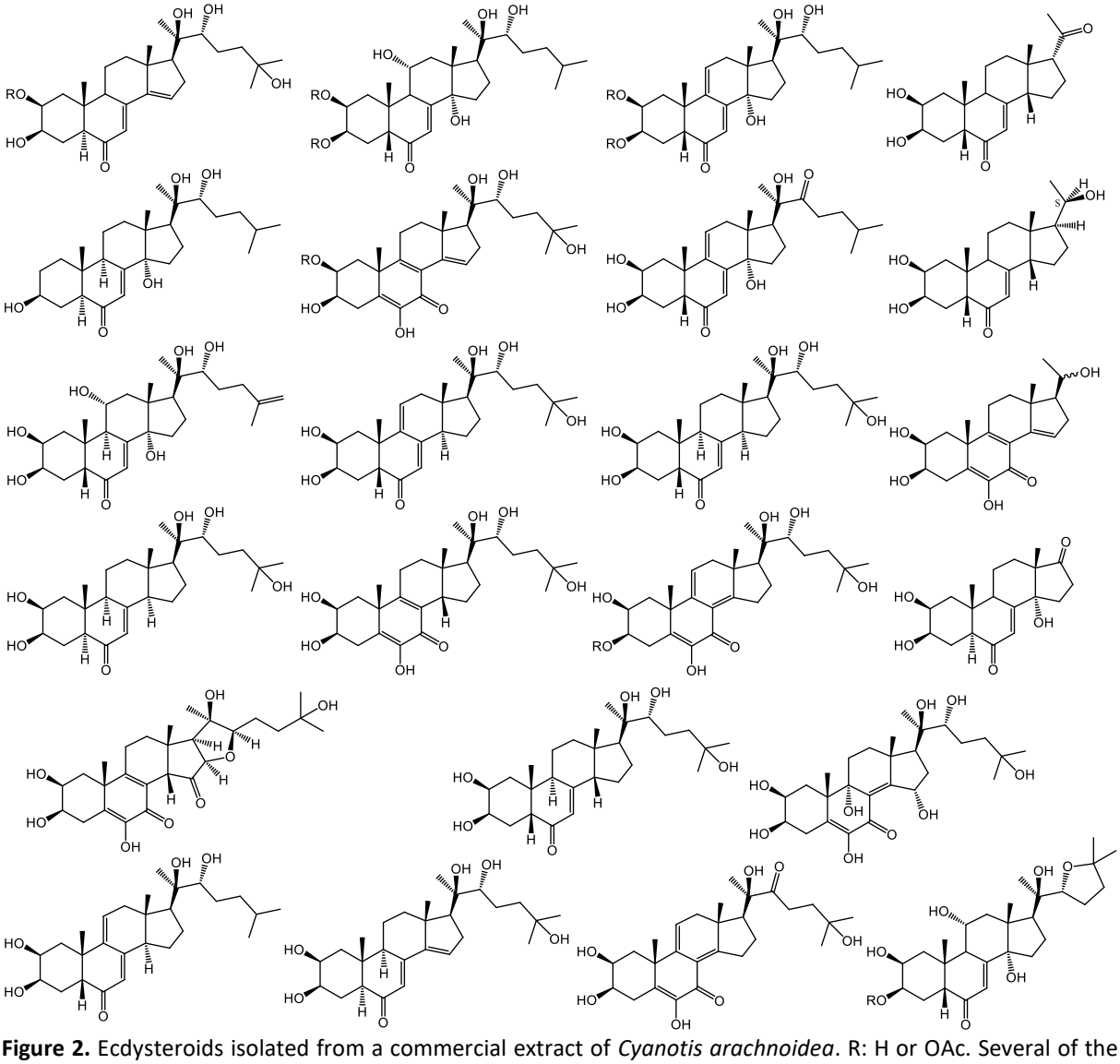

Figure 2. Ecdysteroids isolated from a commercial extract of *Cyanotis arachnoidea*. R: H or OAc. Several of the compounds were obtained in large enough quantities for further semi-synthetic transformations.

The semi-synthetic preparation of new ecdysteroid derivatives followed four main chemical approaches and their combinations: base-catalyzed autoxidation, oxidative side-chain cleavage, and introduction of F, S, or N heteroatoms were performed, and many of the compounds were functionalized with acetonide groups to assure their efficacy against tumor drug resistance (*MedChemComm, 2016*, *Magn Reson Chem, 2017*, *Molecules, 2017*, *Eur J Med Chem, 2018*, *Bioorg Chem, 2019, Bioorg Chem, 2020*). The chemical diversity explored in this work is briefly summarized in Fig. 3.

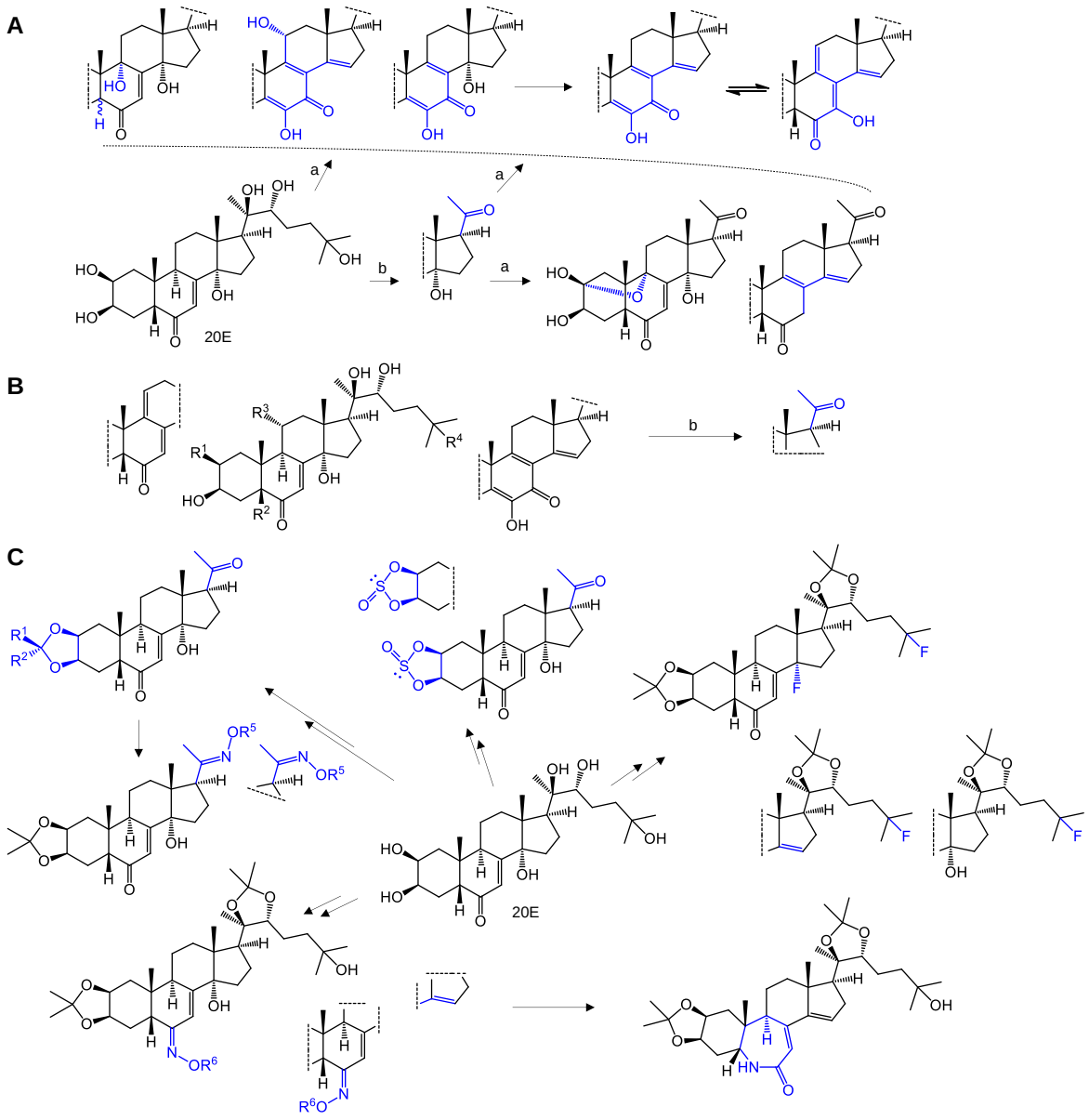

Figure 3. Ecdysteroids prepared from 20-hydroxyecdysone (20E) through base-catalyzed autoxidation (a) and/or oxidative side-chain cleavage (b) (**A**), from other phytoecdysteroids, obtained from the *C. arachnoidea* extract, through oxidative side-chain cleavage (**B**), or from 20E through various chemical approaches to introduce new heteroatoms (**C**); and most important structure-activity relationships observed (**D**). Structural changes as compared to the parent compound are highlighted in blue.

Rich SAR data were obtained for these compounds on various pharmacological models. As the most important findings, the followings can be highlighted.

1) Among the autoxidized derivatives, calonysterone was identified as a more potent cytoprotective agent than 20E, and calonysterone 2,3;20,22-diacetonide as an order of magnitude stronger cytotoxic agent than 20E 2,3;20,22-diacetonide. When evaluating the time dependency of autoxidation of 20E by using capillary electrophoresis (allowing direct injections of the alkaline reaction mixtures to the instrument), it was revealed that the intermediate leading to the rare natural product calonysterone is formed at around 80% yield, therefore with an appropriate reaction work-up calonysterone could be prepared at surprisingly high yields (*J Pharm Biomed Anal, 2017*). Optimization of this work-up is currently ongoing.

2) A synthetic method was developed for the scalable preparation of the sidechain-cleaved poststerone from 20E at around 80% isolated yield. Poststerone was identified as an active *in vivo* metabolite of 20E concerning its anabolic action in rats (*Phytomedicine, 2019*).

3) A set of natural and previously prepared semi-synthetic ecdysteroid derivatives were tested within the project's framework for their capacity to cross the blood-brain barrier (BBB) by using parallel artificial membrane permeability assay (PAMPA). Those demonstrating a favorable BBB penetration in this system were investigated as potential chemo-sensitizers on a CNS originated tumor cell line (SH-SY5Y neuroblastoma). Ecdysteroid dioxolanes showed an extremely strong sensitizing activity on this non-MDR cell line; in particular, 10μM of the most potent compound could increase the cytotoxicity of vincristine by three orders of magnitude (from IC_{50}=39.5nM to IC_{50}=56 pM) (*Eur J Pharm Sci, 2017*).

4) Concerning the S, F, or N atom-containing derivatives' antitumor potential, the most important findings are connected to the latter group. Evaluation of their antiproliferative and cytotoxic activity on several cancer cell lines revealed that a new, *t*-butyl substituted ecdysteroid 6-oxime ether exerts stronger antiproliferative effect on HeLa and MDA-MB-231 cells than cisplatin. The $\Delta^{14,15}$ *E*-oxime derivative exerted a substantially increased cytotoxic and P-gp inhibitory activities in a susceptible / multi-drug resistant (L5178/$L5178_{B1}$) cell line pair as compared to its parental compound. Clear SAR was observed for the compounds' activity as functional P-gp inhibitors, and many of them were identified as highly potent MDR-selective chemo-sensitizers. In particular, a novel $\Delta^{14,15}$ δ-lactam ecdysteroid derivative (Fig. 3, bottom right corner) was revealed as a most promising new lead compound with low intrinsic cytotoxicity, and strong ability to sensitize MDR and also non-MDR cancer cells towards doxorubicin without interfering with the efflux function of P-gp (*Eur J Med Chem, 2018*).

By using the chemical strategy introduced by the group of Couvreur (Université Paris-Saclay, France) and modified by the group of Passarella (University of Milan, Italy), we have prepared and evaluated several squalenoylated ecdysteroid-containing self-assembling nanoparticle pro-drugs (*ACS Med Chem Lett, 2018*, *Magn Reson Chem, 2018*, *Frontiers Pharmacol, 2020*); structures of these conjugates are shown in Fig. 4. According to the findings of the Couvreur group, such nanoparticles do not remain intact in the blood stream but get dissolved in lipoproteins, mainly LDL, and therefore they indirectly target LDL-receptor-rich tissues, such as solid tumors.

Some of these compounds, when combined into heteronanoparticles with squalenoylated doxorubicin, were found to exert potent antitumor action and to overcome the resistance of a doxorubicin-resistant cancer cell line ($A2780_{ADR}$). The *in vitro* cell uptake was demonstrated, and the involvement of an endosomal-mediated pathway was suggested (*ACS Med Chem Lett, 2018*). Our further studies into the efficacy of 11α-hydroxyecdysteroid conjugates, however, suggested that reaching the full antitumor potential of these compounds would require the replacement of the acid sensitive 2,3-acetonide group of ecdysteroids by an appropriate acid resistant lipophilic group (*Frontiers Pharmacol, 2020*).

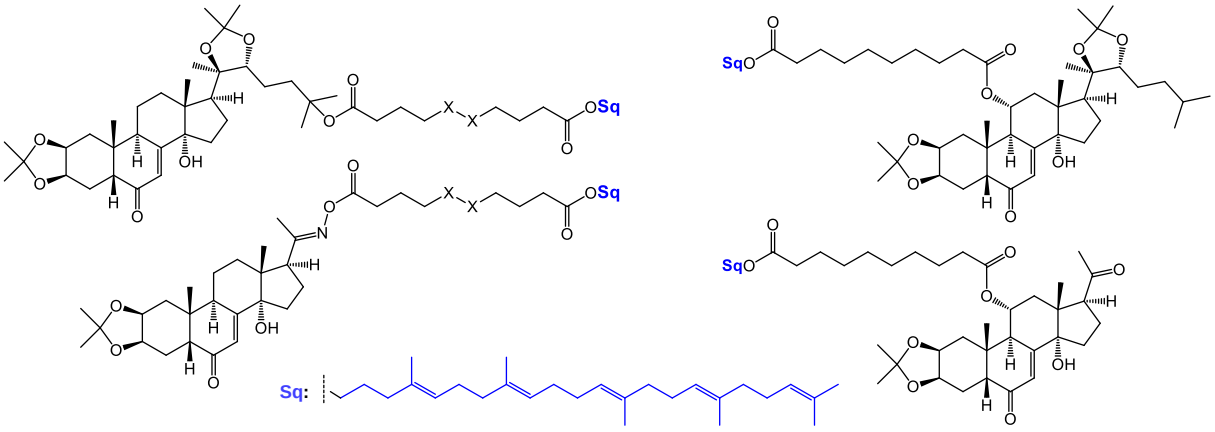

Figure 4. Squalenoylated ecdysteroid pro-drugs that form self-assembling nanoparticles in aqueous environment. Conjugates on the C-25 were efficient in overcoming multi-drug resistance of cancer cells.

CINNAMIC ACID DERIVATIVES AND OTHER PHENOLIC ANTIOXIDANTS

Two important proof-of-concept type studies were performed on hydroxycinnamic acid derivatives. Both studies suggest that the background hypothesis of the project, i.e. that the diversity-oriented synthesis using ROS/RNS to obtain oxidized antioxidant metabolite mixtures may serve as a valid natural product-based drug discovery strategy.

First, the processing of oxidized mixtures of *p*-coumaric acid methyl ester (pcm) revealed a new antitumor lead, graviquinone (GQ in Fig. 5). Graviquinone bypassed ABCB1-mediated resistance, induced DNA damage in lung carcinoma cells but exerted DNA protective activity in normal keratinocytes, and modulated DNA damage response in MCF-7 cells. The cytotoxic effect of pcm in MCF-7 cells was potentiated under H_2O_2-induced oxidative stress, and the formation of graviquinone was confirmed by Fenton's reaction on pcm. *In silico* density functional theory calculations suggested graviquinone as a kinetic product of pcm-scavenging $^{\cdot}OH$ radicals (Fig. 6). Our results therefore demonstrated the high pharmacological value of an in situ-formed, oxidative stress-related metabolite of an antioxidant, supporting (*J Med Chem, 2019*).

Second, pcm and methyl caffeate (cm) were subjected to oxidation by peroxynitrite ($ONOO^-$), a biologically relevant reactive nitrogen species (RNS), or by α,α'-azodiisobutyramidine dihydrochloride (AAPH) as a chemical model for reactive oxygen species, and the formation of potentially bioactive products was evaluated. A continuous flow system was developed to achieve reproducible in situ $ONOO^-$ formation. Reaction mixtures were tested for their cytotoxic effect on HeLa, SiHa, MCF-7 and MDA-MB-231 cells. As a most important finding, bioactivity-guided isolation from the reaction mixture of cm with AAPH produced two dimerization products, including a dihydrobenzofuran lignan that exerted strong antitumor activity in vitro, and has potent *in vivo* antimetastatic activity which was previously reported. This compound was also detected from the reaction between cm and $ONOO^-$. Furthermore, we found that pre-treatment of cancer cells with t-BHP, a well-known inducer of intracellular oxidative stress, modulated the cytotoxicity of cm in a way that coincided with the cells' sensitivity to this RONS scavenging-related metabolite. Altogether, our results provided direct evidence for the RONS-mediated transformation of an abundant dietary antioxidant into a potent antitumor agent (*Biomolecules, 2020*).

Chemical structures of the antioxidant metabolites obtained from hydroxycinnamic acid derivatives are shown in Fig. 5. As an example of studying RONS scavenging-related formation of certain metabolites, Fig. 6 shows the *in silico* results identifying graviquinone as a kinetic product from the $^{\cdot}OH$ radical scavenging by methyl-*p*-coumarate.

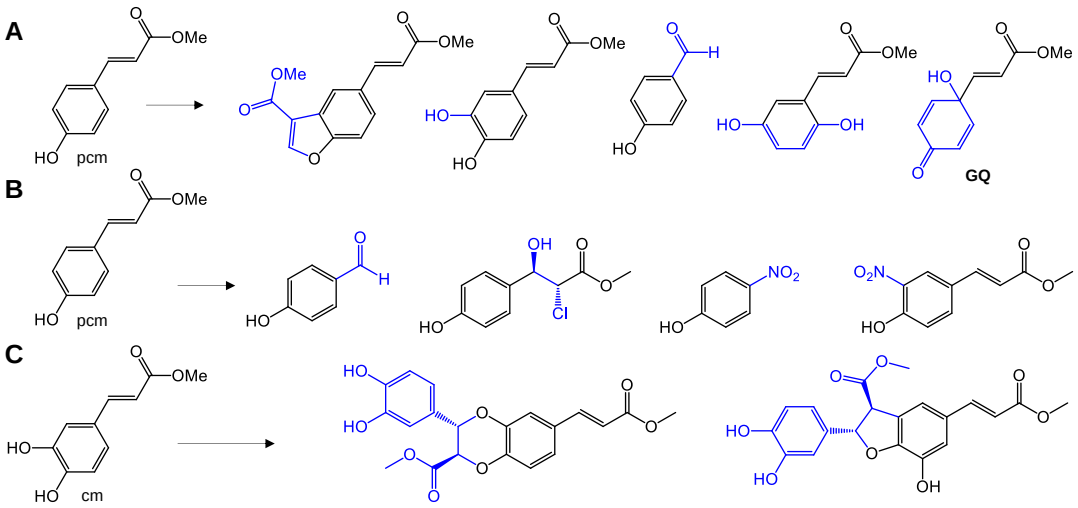

Figure 5. Metabolites obtained from oxidation of methyl hydroxycinnamates. A: PIFA-mediated oxidation of pcm, B: $ONOO^-$-mediated oxidation of pcm, C: AAPH-mediated oxidation of cm. The two antitumor leads (top right, GQ, and bottom right) were also identified from other reaction conditions, confirming their formation upon scavenging various types of biologically relevant ROS/RNS (*J Med Chem, 2019*; *Biomolecules, 2020*).

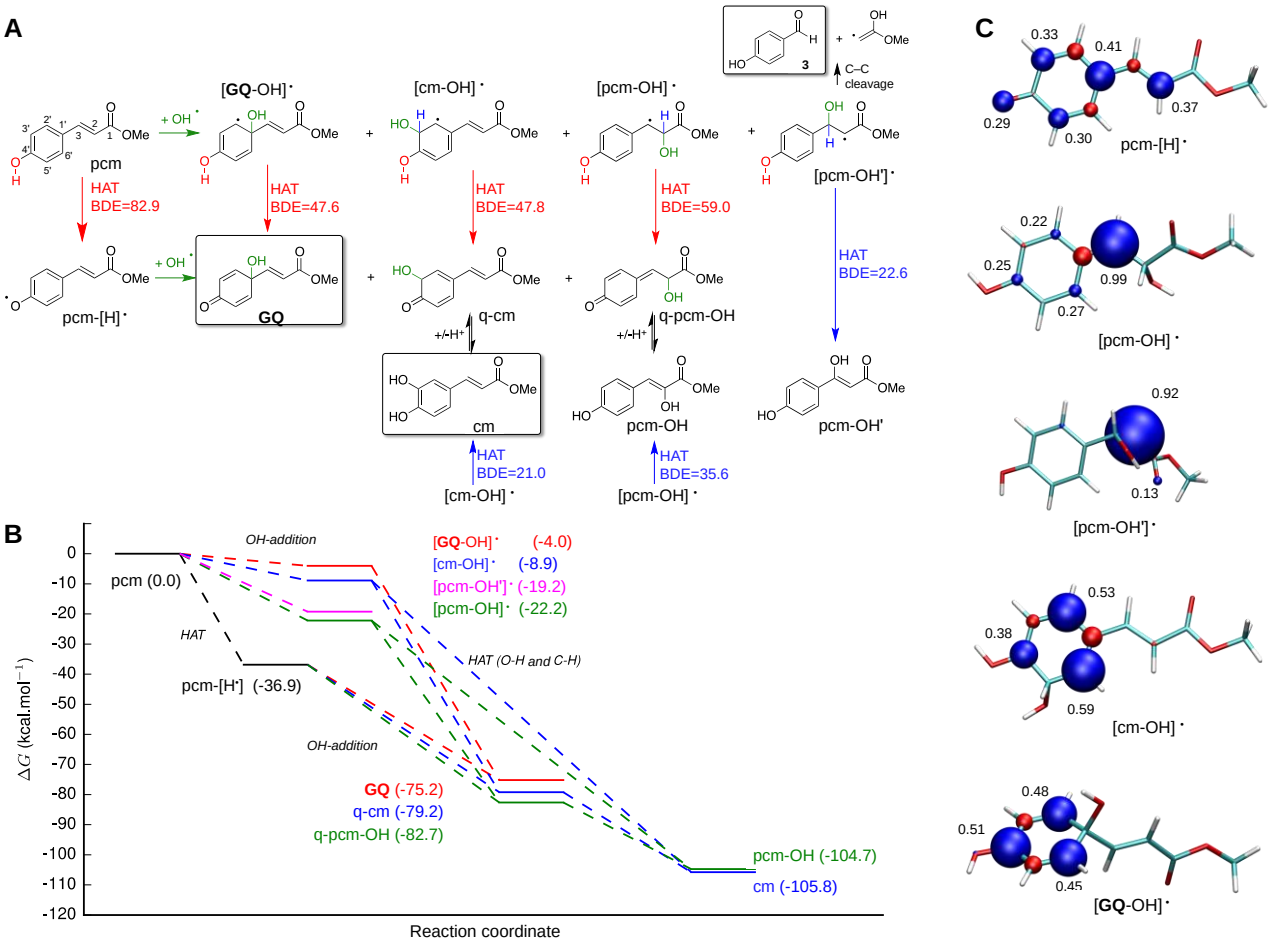

Figure 6. Reaction mechanism of ˙OH radical scavenging by pcm (**A**), thermodynamic analysis of the possible reaction routes (**B**), and spin density distribution of the radicals formed by the reaction between pcm and ˙OH radicals (**C**). Experimentally identified species are marked with a frame; **GQ**: the potent antitumor graviquinone that was identified as a kinetic product from ROS scavenging by pcm (*J Med Chem, 2019*).

Within this project, we isolated 11 natural antioxidants from the root bark of *Morus nigra*, including morusin as a major compound. Morusin was subjected to various biomimetic oxidative reactions, several of which resulted in the formation of neocyclomorusin. Catalytic hydrogenation was also performed to further increase variability. Structures of the isolated and semi-synthesized compounds are shown in Fig. 5.

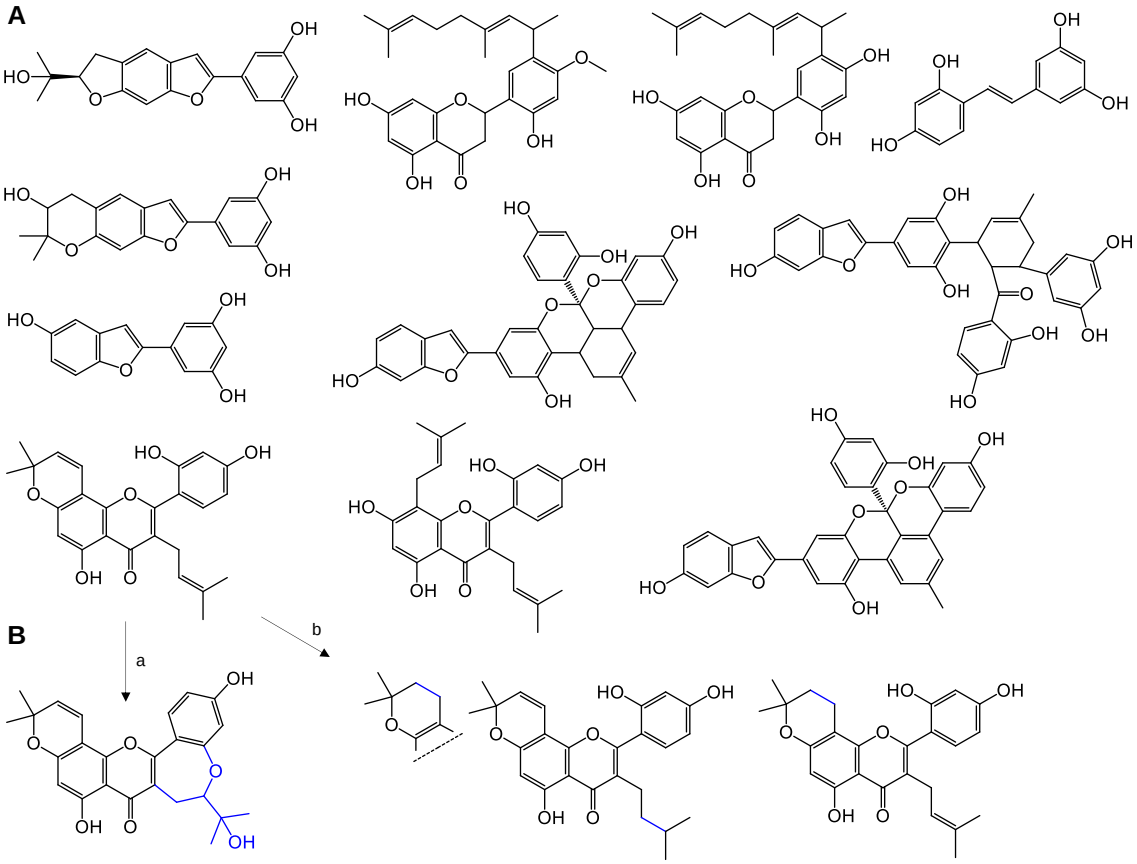

Figure 7. Phenolic antioxidants obtained from the root bark of *Morus nigra* (**A**), and compounds obtained from the oxidative (a) and reductive (b) semi-synthetic transformation of morusin (**B**).

In connection with their antitumor potential, selected compounds from *M. nigra* were studied for their potential to modulate isoforms of the sarco-endoplasmic Ca^{2+}-ATPase (SERCA). The Diels-Alder adduct Albanol A was identified as a SERCA inhibitor that may have a possible antitumor application (*ACS Med Chem Lett, 2020*).

Moracin O (Fig. 7, upper left corner) was identified as a more potent smooth muscle relaxant than papaverine (*Molecules, 2019*).

Several of our compounds obtained from *M. nigra* were involved in an international collaborative project aiming to target sirtuins, important epigenetic enzymes involved in the pathomechanism of various chronic diseases including cancer. In this work, a quercetin dimer, obtained by the silver-catalyzed biomimetic oxidation of quercetin, was revealed as a SIRT6 inhibitor stronger than its parent antioxidant (*Biomed Pharmacother, 2019*).

Neocyclomorusin, the oxidized metabolite of morusin (Fig. 6, bottom left corner) was a weaker antiproliferative agent but a significantly stronger inhibitor of Pgp-mediated efflux in multi-drug resistant cancer cells than morusin, while the reduced metabolites showed 2-3-folds increased antiproliferative activity as compared to their parent compound (*manuscript in preparation*).

Several further natural antioxidants, including 6-gingerol, 6-shogaol, resveratrol, and magnolol were involved in diversity-oriented oxidative transformations by various biomimetic or biorelevant chemical models of ROS/RNS. To this end the oxidized metabolite mixtures of these compounds did not show antitumor potential but some of them demonstrated bioactivities relevant to cytoprotective effect, therefore, they are going to serve as a starting point to independent future studies on the potential protective effects of oxidative stress-related antioxidant metabolites.

SUMMARY

From the chemical point of view, the project involved natural product isolation (*Cyanotis arachnoidea* and *Morus nigra*), and semi- and total synthetic approaches, and this work resulted in the successful preparation of a significant number of chemically diverse antioxidant-inspired new compounds. Many of these compounds represent highly unusual chemical structures.

From the pharmacological point of view, extensive bioactivity studies were performed on the newly prepared compounds' anticancer potential, including screening on various pharmacological models and mechanism of action studies in several cases. Rich SAR data were obtained, and several highly promising new antitumor leads were identified in each group of compounds. As it was originally expected from the possible chemical-pharmacological complexity of the project, this includes some compounds with bioactivities that are not related to cancer, accordingly, several side-projects were raised some of which allowed the initiation of independent new research directions. Altogether, it can be concluded that a diversity-oriented chemical strategy utilizing oxidative transformations on small-molecule antioxidants has a high potential to discover new bioactive compounds.

Finally, concerning the biological relevance of our starting hypothesis, using *in vitro* chemical and cellular models and *in silico* calculations, we gathered a significant number of direct and indirect proofs demonstrating that several of the potent antitumor compounds identified in this study are indeed formed upon ROS and/or RNS scavenging in a biological environment. Further, we have indirect proofs for this phenomenon not only to exist, but to also manifest in the complex bioactivity profile when a biological system under oxidative stress is treated with an antioxidant able to scavenge reactive oxygen or nitrogen species.