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Natural antioxidants in hybrids for the treatment of neurodegenerative diseases: a successful strategy?

“The combination of natural antioxidants with other moieties can often lead to disadvantageous drug properties, such as high MW, and therefore low bioavailability, poor solubility, rapid metabolism and hampered blood–brain barrier penetration.”

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It is well established that oxidative stress is one of the characteristics of neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease [1]. Free radicals, that is, reactive oxygen species (ROS) and reactive nitrogen species, are damaging a variety of biomolecules like lipids, proteins and DNA. Such radicals are generated in the pathology of neurodegenerative diseases and eventually lead to neuronal cell death. They contribute to the overall pathophysiology and pathogenesis of these diseases, but are not their sole cause. In fact, it is unclear until now at which stage of the pathophysiological cascades oxidative stress occurs. Irrespective of the stage though, it is obvious that ROS enhance if not multiply the effects of the neuropathological cascades [1].

In AD, for example, pathological mechanisms mainly include hyperphosphorylation of T proteins, reduction of acetylcholine receptor levels, mitochondrial dysfunction, neuronal DNA damage, cerebral glucose hypometabolism, aggregation of β -amyloid peptides (A β) and oxidative stress [1,2]. With several mechanisms contributing to a complex pathological network, development of therapeutically applicable drugs is extremely challenging. A drug addressing only one biological target is probably not going to yield

any success in stopping neurodegenerative diseases. Therefore, multitarget or hybrid drugs – combining two or more pharmacologically active units – that are capable of modifying several biological targets, may overcome the shortcomings of a single-target drug. Many multitarget compounds have been developed in recent years [3]. A very significant number of those compounds contain – mostly natural – antioxidants to target oxidative stress, therefore counteract the formation of ROS and are claimed to hold the potential to interfere with the neuropathological cascades [3,4]. The term ‘antioxidant’ is actually kind of vague and unspecific in both the chemical and biological sense and more specific terms are desirable. The combination of natural antioxidants with other moieties can often lead to disadvantageous drug properties, such as high MW, and therefore low bioavailability, poor solubility, rapid metabolism and hampered blood–brain barrier penetration. Problems typical for natural antioxidants also include rapid CYP metabolism – often 3A4 – and CYP induction.

We want to shortly discuss both the physicochemical as well as the cell-based *in vitro* assays that have been applied for assessing the ‘antioxidant’ properties of multitarget compounds containing antioxidant

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moieties and discuss what the outcomes of these assays actually mean, before we report on some very recent *in vivo* studies.

“A tacrine–ferulic acid hybrid...showed reduction of oxidative stress markers as well as positive effects on learning and memory in transgenic Alzheimer’s disease mice”

***In vitro* cell-free assays**

Natural antioxidants belong to very diverse chemical classes including carotenoids, vitamins C and E, stilbene derivatives, curcuminoids, phenolic acids (such as cinnamic, caffeic and ferulic acids) and flavonoids (such as quercetin, rutin and genistein). Due to this structural diversity it is assumed that they exhibit different modes of action [4]. The cell-free assays used to assess antioxidant activity *in vitro* are fairly similar in what properties they determine, namely their ability to scavenge radicals. These assays include the DPPH assay, an ABTS scavenging assay, the NBT assay and the oxygen radical absorbance capacity assay [5]. The oxygen radical absorbance capacity assay, for example, provides quantification of the test compounds’ abilities to protect fluorescein from being oxidized by scavenging peroxy radicals. Tacrine–ferulic acid hybrids, for example, show even higher values than the natural antioxidant ferulic acid by itself [6,7].

The ability of molecules to scavenge radicals determined in these assays is a prerequisite and an indication for the occurrence of effects in cell-based assays or *in vivo*, but not more than that. For that reason, more advanced cell-based assays are indispensable for better prediction of ‘antioxidant’ and therapeutically desirable activities *in vivo*.

***In vitro* cell-based assays**

A variety of set-ups can be used *in vitro* to simulate disease-like conditions in cells, including oxidative stress, and to investigate the neuroprotective properties of test compounds. To quantify the protection against ROS-mediated apoptosis, cells are incubated with ROS-inducing or producing agents in the presence of the antioxidant compounds and the experimental read-out is the reduced cell viability determined in an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) or a chemically related assay.

Different cell lines are established that reflect the quite diverse origins of ROS. Mouse hippocampal HT-22 cells are used to study neuroprotective effects of hybrids toward ROS produced by glutamate-induced toxicity [8]. Presence of high glutamate concentrations in culture medium leads to glutathione depletion inside the cells. This causes ROS accumulation leading

to cell death by apoptosis. A β exposure can also induce oxidative damage in cells and lead to apoptosis due to A β -induced mitochondrial dysfunction [9]. Protection against A β -induced oxidative stress through ferulic–tacrine acid hybrids was shown in PC-12 cells [10], a pheochromocytoma cell line of the rat adrenal medulla. MC-65 and SH-SY5Y are human neuroblastoma cell lines and are also applied in such assays since they derive from neuronal tissue and are therefore very suitable to investigate neurodegenerative diseases. For example, SH-SY5Y cells were used to investigate neuroprotection of tacrine–melatonin hybrids against A β -induced toxicity [11]. This cell line also served to investigate ROS production induced by application of hydrogen peroxide and rotenone. Rotenone specifically inhibits mitochondrial complex I leading to increased ROS formation and mitochondrial-induced apoptosis [12], whereas hydrogen peroxide causes DNA damage and lipid peroxidation, being a source of nonselective reactive oxygen species [13].

Drawbacks & first *in vivo* data

Curcumin represents a prime example for a natural product for which numerous beneficial effects have been claimed and that has also been used in hybrid molecules. A remarkable review has been published recently [14], that discusses the positive results of numerous *in vitro* assays found for this compound and derivatives thereof and the article raises some questions: low solubility, instability due to the oxidation-prone phenolic and enolic hydroxyl groups, the Michael system and its fluorescent properties, respectively, are likely leading to false-positive results *in vitro* for curcumin and curcumin-derived compounds. Albeit to a lower extent, these false positives can also occur with other natural products that, for example, possess a Michael system.

Numerous multitarget compounds containing antioxidant moieties have been claimed to be lead structures for AD therapy after their investigation in one or more of the above described *in vitro* assays (cell-free and/or cell-based). Nevertheless, only *in vivo* studies giving access to information about bioavailability, blood–brain barrier penetration and *in vivo* neuroprotection plus antineuroinflammation, are really answering the question, whether such compounds hold true therapeutic potential. Only very recently first *in vivo* data has become available [15]. A melatonin–curcumin hybrid was applied to 4-month-old transgenic mice and *in vitro* results could be confirmed *in vivo* with regard to reduction of markers of oxidative stress on the molecular level [16], even though no behavioral effects have been reported. However, a tacrine–ferulic acid hybrid described above showed reduction of oxidative stress markers as well as positive effects on learning and memory in transgenic AD mice [10].

Summary

Taken together, multitarget (hybrid) compounds and other chemical compounds containing antioxidant moieties are prone to several disadvantages: metabolic instability, false-positive results in (some – nonsuitable) *in vitro* assays and unknown modes of action. There are also problems of transferring the relevance of *in vitro* data to an *in vivo* setup. Nevertheless, this does not mean that antioxidants are not versatile and promising components of hybrids, but only specific assays for assessing the compounds' activities are suitable. The methods deemed suitable have to be carefully selected on the basis of the chemical structure of the compounds under investigation. Positive results should not be overinterpreted. The

often unknown modes of action of natural antioxidants with regard to neuroprotection *in vitro* and *in vivo* still hamper the development of effective hybrid molecules.

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