# Organofluorine Hydrazone Derivatives as Multifunctional Anti-Alzheimer's Agents with CK2 Inhibitory and Antioxidant Features

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A set of novel hydrazone derivatives were synthesized and analyzed for their biological activities. The compounds were tested for their inhibitory effect on the phosphorylating activity of the protein kinase CK2, and their antioxidant activity was also determined in three commonly used assays. The hydrazones were evaluated for their radical scavenging against the DPPH, ABTS and peroxyl radicals. Several compounds have been identified as good antioxidants as well as potent protein kinase CK2 inhibitors. Most hydrazones containing a 4-N$(CH_3)_2$ residue or perfluorinated phenyl rings showed high activity in the radical-scavenging assays and possess nanomolar $IC_{50}$ values in the kinase assays.

## Introduction

Due to the ever-increasing number of patients affected by Alzheimer's disease (AD), the development of new therapeutics for AD is of high importance. AD is a complex disease, and identifying the related biochemical processes and signaling pathways significantly contributes to finding new therapeutic targets.[1,2] The major targets include protein kinases due to their involvement in many cellular processes and a plethora of signaling pathways. An additional advantage of kinases is that they are drugable, thus they are frequently considered therapeutic targets for AD drug development.[3]

Protein kinase CK2 was found in the AD affected brain as early as 1990 and has been proposed to play a role in the pathology of the disease.[4,5] The CK2 holoenzyme possesses two catalytic α subunits of slightly different sizes (39 and 45 kDa) and two regulatory β subunits (26 kDa), however, all subunits also exist in their monomeric forms in the cell.[6] In addition, the catalytic α subunit can be found in several different isoforms, the three most common ones are the α, α′ and α″.[7,8] CK2 is constitutively active and uses both adenosine triphosphate (ATP) and guanosine triphosphate (GTP) as phosphate donors.[5,9,10] This protein kinase has a broad spectrum. Up to date more than 350 of its protein substrates have been identified.[11] CK2 is known to phosphorylate tyrosine besides the serine and threonine residues, thus it is considered a dual-specificity kinase.[10] Although there is no broad agreement regarding the mechanisms of CK2 regulation, its potential role in pathogenesis associated with inflammation has been proposed.[12]

A wide range of compounds have been identified as inhibitors of CK2,[13,14] including 4,5,6,7-tetrabromo-1*H*-benzotriazole (TBB).[15] This compound is an ATP-competitive inhibitor that has been commonly applied as a molecular probe to identify the function of CK2. Silmitasertib (CX-4945) is another selective, ATP-competitive inhibitor that can modulate the activity of the α and α′ catalytic subunits of CK2.[16,17] It is the first CK2 inhibitor that has reached the level of clinical trials in 2010 for the treatment of various forms of cancer.[17]

In addition to its potential as an anticancer drug target, the role of CK2 has also been evaluated in the hippocampus and temporal cortex of AD patients in comparative studies. The effects of CK2 inhibitors on IL-6 and MCP-1 secretion have also been investigated in stimulated human primary astrocytes and U373 cells. These reports highlight the recent sentiment regarding a possible role of CK2 in neuroinflammation and thus, in the pathogenesis of AD. It has been proposed, that CK2 could serve a potential drug-able target to modulate inflammatory response in AD.[18]

In Alzheimer's disease therapy, the application of CK2 inhibitors could prove to be advantageous alleviating the effect of hyperphosphorylated tau and neurofibrillary tangles. In addition, the reduction of insulin receptor internalization and improvement of the Aβ disabled fast axonal transport could improve the condition of AD patients.[19] In our earlier publications we described the inhibition of various CK2 isoforms by natural and synthetic compounds such as halogenated benzimidazoles and flavonoids.[20–22]

Hydrazones, as largely nontoxic entities, have attracted significant attention in drug development. They are considered

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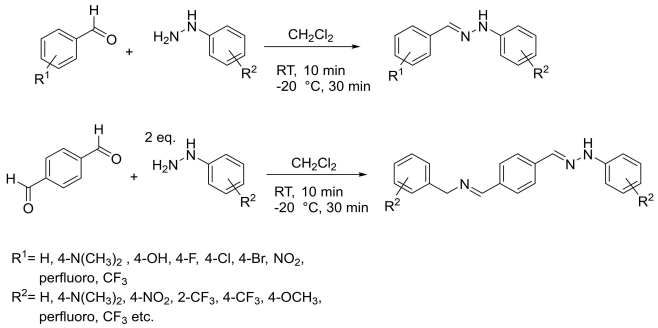
as part of hybrid compounds when designing more effective, often multitarget therapeutics.[23–26] Hydrazones exhibit a broad spectrum of biological activities and have been described in the treatment of tuberculosis,[27] malaria[28] or as antiviral[29,30] and anticancer agents.[31] Furthermore, hydrazones have also been identified as strong radical scavengers.[32,33] Their multitarget features have been combined as potential AD therapeutics; several hydrazones were found to be excellent inhibitors of amyloid beta fibril and oligomer formation as well as showed highly effective radical scavenging properties.[34]

Continuing our efforts in the development of multifunctional anti-AD agents, herein we describe the synthesis and evaluation of novel organofluorine hydrazones as protein kinase CK2 inhibitors and free radical scavengers.




**Scheme 1.** General synthesis of hydrazones.

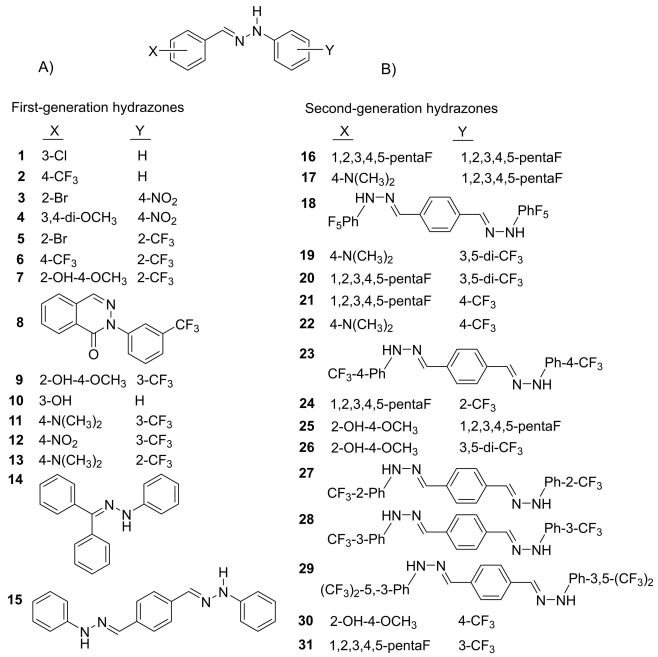



**Figure 1.** Structure of the A) first and B) second generation hydrazones used in this study.

## Results and Discussion

For this study, several novel hydrazone derivatives were designed and prepared (Scheme 1, Figure 1). Our earlier studies indicated that hydrazones are potent multitarget compounds to combat AD. They appeared to be excellent inhibitors of Aβ self-assembly, including both oligomer and fibril formation and also acted as effective radical scavengers.[34] In this study it was intended to observe how similar hydrazone derivatives would affect protein kinase CK2 activity that has been connected to the development of AD as well. The first generation of hydrazones (Figure 1A) included in this work were compounds possessing the hydrazone core unit and a variety of substituents. The major goal was to investigate the role of different substituents and identify compounds with CK2 inhibitory activities that potentially could be improved by further design.

Based on the preliminary data tabulated in Table 1, it was found that hydrazones that possessed one or more fluorine atom showed the highest potency. This is in agreement with our earlier findings with different scaffolds that the presence of multiple halogens, such as bromine or iodine atoms to benzimidazoles resulted in a significant increase in their inhibitory potential.[20] Combining the efficacy of the hydrazone scaffold with the beneficial effects of fluorine incorporation it was decided to design and synthesize compounds with gradually growing number of fluorine atoms (Figure 1B). In general, two types of fluorine incorporation was considered; the aromatic F, and the $CF_3$ groups. Both of these substituents exhibit high stability during a wide range of chemical and biological conditions,[35] thus by their application one can avoid the formation of highly toxic metabolites. The identity and purity of the new compounds were confirmed by gas chromatography-mass spectrometry (GC-MS) and NMR spectroscopy (see the Supporting Information). The structures of the compounds prepared for the current study are summarized in Figure 1.

**Table 1.** Inhibition of different CK2 isoforms by the first generation hydrazones (**1**–**15**).

| Compound | $IC_{50}$ [μM] CK2α | CK2α′ | CK2$\alpha_2\beta_2$ | CK2$\alpha'_2\beta_2$ |
|---|---|---|---|---|
| **1** | >50 | >50 | 17.8±1.4 | >50 |
| **2** | >50 | >50 | >50 | >50 |
| **3** | >50 | 9.8±0.8 | >50 | >50 |
| **4** | >50 | >50 | 16.5±1.2 | 12.1±1.1 |
| **5** | >50 | >50 | 15.4±1.2 | 16.6±1.8 |
| **6** | >50 | 10.8±0.6 | >50 | >50 |
| **7** | 22.4±2.5 | 8.4±0.6 | 13.8±1.1 | >50 |
| **8** | >50 | >50 | >50 | >50 |
| **9** | 25.1±2.8 | 1.2±0.1 | 10.3±0.9 | 15.2±1.6 |
| **10** | >50 | >50 | 15.5±1.0 | 15.1±1.6 |
| **11** | >50 | 7.3±0.5 | 14.7±1.2 | >50 |
| **12** | >50 | >50 | >50 | >50 |
| **13** | >50 | 1.7±0.1 | 14.4±1.4 | >50 |
| **14** | 14.6±1.7 | >50 | >50 | >50 |
| **15** | >50 | 13.2±0.9 | >50 | 13.6±1.4 |

$IC_{50}$ values ± standard deviation are expressed as the mean of three independent experiments.

In the first step we tested 15 hydrazone derivatives (Figure 1A) containing varying substituents. The inhibitory effect, described as $IC_{50}$, was determined by using a series of concentrations of inhibitors and using yeast acidic ribosomal protein P2B as phosphoacceptor. From these enzyme inhibition studies the activity of the compounds has been determined. The obtained results are summarized in Table 1. Analyzing the data in connection to the structural features of the molecules, it was observed that compounds with either the 2-OH–4-$OCH_3$ or 4-$N(CH_3)_2$ substituent exhibited an improved effect on CK2α′. Compounds **3**, **6**, **7**, **9**, **11** and **13** showed the best inhibitory effect towards CK2α′ subunit. Those compounds seem to be selective inhibitors for the CK2α′ subunit with $IC_{50}$ values ranging from 1 to 11 μM. Whereas inhibitors **1**–**6**, **10**–**13** and **15** were largely ineffective on CK2α, decreasing its activity only to 70% at a concentration of 50 μM, compounds **7** and **9** also possessed moderate inhibitory activity towards CK2α.

Afterwards, we analyzed the potential of these compounds towards both CK2 holoenzymes. Only one hydrazone (compound **9**) possessed activity against all four CK2 isoforms. Several compounds (**4**, **5**, **10**) were effective only towards the holoenzymes without influencing the enzymatic activities of the free catalytic subunits. Interestingly, compounds which showed no or only moderate effect towards monomeric CK2α possessed activity on its holoenzyme $CK2\alpha_2\beta_2$. Similar tendency was already reported in our former work with flavonoid compounds.[22] Compounds **1**, **4**, **5**, **7**, **9**, **10**, **11** and **13** possessed modest inhibitory effect ($IC_{50}$ = 10–20 μM) against $CK2\alpha_2\beta_2$. Some compounds (**3**, **6**, **7**, **11** and **13**) showing inhibitory influence on the monomeric CK2α′ subunit lost this activity towards the holoenzyme $CK2\alpha'_2\beta_2$. Within all tested compounds three (**2**, **8**, **12**) had no inhibitory effect on any of the four examined CK2 isoforms.

Based on the information obtained by screening the general 1st generation hydrazones as well as earlier evidence on the beneficial effect of halogen incorporation to other CK2 inhibitors a new set of hydrazones (noted as 2nd generation, Figure 1 B) were designed and synthesized as described above (Scheme 1). Sixteen hydrazones, fluorinated to various extents were included in these assays with the aim of further improving the inhibitory properties of the compounds. The preparation of fluorinated test compounds was limited to the addition of aromatic F and $CF_3$ residues based on the results from the first series as well as to ensure the high metabolic stability of these substituents. In addition to the general positive features of fluorine introduction to drug candidates, a major reason to replace other halogens was the high stability of these groups.[35] In earlier studies we tested several brominated, chlorinated and iodinated compounds, which usually possess higher cytotoxicity and are less stable.[20] The CK2 inhibition data obtained with the second generation hydrazones are tabulated in Table 2.

The addition of fluorine atoms led to highly selective inhibitors towards the CK2α′ subunit. As can be seen extensively fluorinated compounds are highly effective inhibitors. Derivatives **16**–**20** possessed $IC_{50}$ values in the 100–900 nM range. All of these compounds have at least one (often two) pentafluorophenyl ring highlighting its importance. The tendency observed in these experiments is that the addition of fluorine atoms improves the inhibitory activity of the compounds. It is worth noting that both compounds (**16**, **17**) that exhibit strong to excellent activity in the inhibition of all four forms of the enzyme, contain perfluorophenyl group on either side of the hydrazone core. Furthermore, only one of the six high activity compounds (**16**–**21**) does not contain the perfluorophenyl unit, emphasizing its importance in ensuring favorable molecular characteristics for inhibition of CK2. Interestingly, several compounds are active against the free CK2α′ subunit and the $CK2\alpha_2\beta_2$ holoenzyme. This is a similar mode as in case of natural flavonoids we tested previously.[22] The regulatory subunit affects the inhibitory activity leading to better inhibition of CK2α′ than the holoenzyme $CK2\alpha'_2\beta_2$, but increased inhibition for $CK2\alpha_2\beta_2$ than the free CK2α subunit.

**Table 2.** Inhibition of different CK2 isoforms by the second generation hydrazones (**16**–**31**).

| Compound | $IC_{50}$ [μM] CK2α | CK2α′ | $CK2\alpha_2\beta_2$ | $CK2\alpha'_2\beta_2$ |
|---|---|---|---|---|
| **16** | 18.9 ± 1.9 | 0.1 ± 0.01 | 32.5 ± 2.4 | 17.7 ± 1.2 |
| **17** | 21.2 ± 2.2 | 0.2 ± 0.1 | 34.3 ± 2.6 | 18.5 ± 1.3 |
| **18** | 16.4 ± 1.4 | 0.9 ± 0.04 | > 50 | 18.7 ± 1.3 |
| **19** | > 50 | 0.2 ± 0.01 | 39.1 ± 2.6 | 19.2 ± 1.4 |
| **20** | > 50 | 0.1 ± 0.01 | 37.3 ± 2.5 | 15.1 ± 1.1 |
| **21** | 14.8 ± 1.1 | 19.8 ± 1.8 | > 50 | 25.7 ± 1.9 |
| **22** | > 50 | 16.8 ± 1.3 | 36.2 ± 2.3 | > 50 |
| **23** | > 50 | 1.3 ± 0.07 | > 50 | > 50 |
| **24** | > 50 | 13.2 ± 1.1 | > 50 | > 50 |
| **25** | > 50 | 11.4 ± 1.0 | 38.2 ± 2.7 | > 50 |
| **26** | > 50 | 14.9 ± 1.3 | > 50 | 31.4 ± 2.7 |
| **27** | > 50 | 34.7 ± 2.5 | 28.7 ± 1.9 | > 50 |
| **28** | > 50 | 12.2 ± 1.1 | > 50 | > 50 |
| **29** | > 50 | 17.3 ± 1.3 | 28.9 ± 2.1 | > 50 |
| **30** | > 50 | 13.6 ± 0.9 | 32.6 ± 2.3 | > 50 |
| **31** | > 50 | 8.2 ± 0.7 | > 50 | > 50 |

$IC_{50}$ values ± standard deviation are expressed as the mean of three independent experiments.

To evaluate the mechanism of the hydrazones in the inhibition of the CK2 activity different ATP concentrations (10, 20 and 40 μM) were evaluated in the reaction mixture. All inhibitors decreasing the catalytic activities acted via a purely ATP-competitive mode of inhibition. The lowest $K_i$ values were estimated for compounds **16** and **17** with 107 and 69 nM, respectively (Figure 2).

As it was shown for benzimidazoles and flavonoids[36,37] the efficacy of many ATP-binding site competitive inhibitors depends on hydrophobic amino acids, namely V66, M163, I174 and V67, M164, I175 in CK2α and CK2α′, respectively. Those amino acids are replaced with less bulky residues in most other protein kinases which give the pocket in CK2 its specific narrow characteristics. Table 3 summarizes the results obtained with the CK2α′ mutants V67A, I117A and I175A. The inhibitory effect of the hydrazone derivatives on all three tested mutants is decreased when compared to the wild-type. This proves that the compounds fit into the ATP-binding pocket and the hinge region.

As oxidative stress plays a significant role in the development of AD, incorporating antioxidant features into multitarget

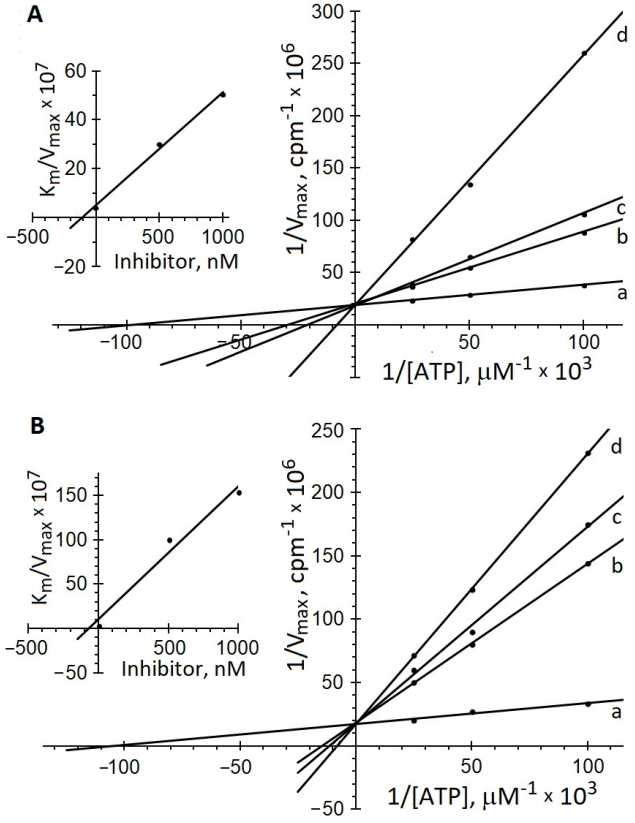



**Figure 2.** Inhibition of CK2α′ by compounds A) **16** and B) **17**. Lineweaver–Burk double reciprocal plots are shown, the insets refer to the $K_m/V_{max}$ vs. inhibitor concentration replot. Inhibitor concentration = a) 0, b) 500 nM, c) 1000 nM, and d) 5000 nM.

**Table 3.** $IC_{50}$ values for the inhibition of CK2α′ WT and mutants by selected second generation hydrazones.

| Compound | $IC_{50}$ [μM] CK2α′ wt | CK2α′V67A | CK2α′I117A | CK2α′I175A |
|---|---|---|---|---|
| **16** | 0.1 ± 0.01 | 18.4 ± 1.6 | 2.5 ± 0.1 | 15.1 ± 1.3 |
| **17** | 0.2 ± 0.01 | 12.6 ± 1.1 | 3.7 ± 0.2 | 11.8 ± 1.1 |
| **18** | 0.9 ± 0.04 | 15.3 ± 1.2 | 5.8 ± 0.2 | 25.7 ± 2.1 |
| **19** | 0.2 ± 0.01 | 11.9 ± 1.1 | 2.8 ± 0.2 | 13.4 ± 1.2 |
| **20** | 0.1 ± 0.01 | 8.1 ± 0.7 | 2.6 ± 0.1 | 5.2 ± 0.4 |
| **23** | 1.3 ± 0.07 | 15.7 ± 1.1 | 9.8 ± 0.8 | 40.8 ± 3.4 |

$IC_{50}$ values ± standard deviation are expressed as the mean of three independent experiments.

anti-AD compounds is highly desirable. Thus, in addition to the evaluation of the CK2 enzyme inhibitory potential, the antioxidant potential of the compounds was also determined using broadly accepted radical scavenging assays. The first and second sets of hydrazone derivatives were evaluated for their radical scavenging activity by the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and Oxygen Radical Absorbance Capacity (ORAC) assays.[38] The data are illustrated in Figure 3.

Several trends both for compounds with excellent radical scavenging activity and poor scavenging activity were observed. In all three assays, compounds with the 4-$N(CH_3)_2$ group

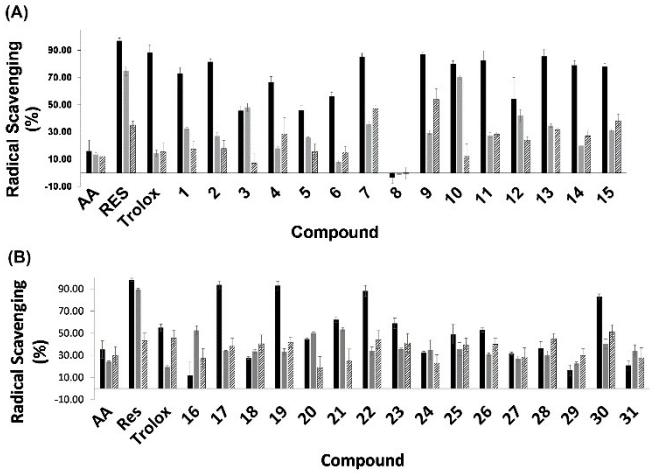



**Figure 3.** Radical scavenging potential of the hydrazone derivatives (**1**–**31**) used in this study, in comparison to known standards such as ascorbic acid (AA), resveratrol (Res) and Trolox. The activity was measured in ORAC (black), ABTS (gray) and DPPH (pattern) assays at 10 μM compound concentration. The values are shown as mean of % radical scavenging ± standard deviation, where the number of independent repeats is 3.

and compounds containing the 2-OH and 4-$OCH_3$ motifs showed high activity with recorded radical scavenging higher than that of ascorbic acid and Trolox. In the ORAC assay, the 4-$N(CH_3)_2$ containing compounds were of the highest activity ranging from 83% to 94% for compounds **11**, **13**, **17**, **19** and **22**, which is double of the potency of ascorbic acid and Trolox, and about the same as that of resveratrol. The next highest compound activity in the ORAC assays was for the hydrazone derivatives containing the 2-OH and 4-$OCH_3$ moiety, with the highest activity ranging from 49 to 87% for compounds **7**, **9**, **25**, **26** and **30**. In the DPPH assay, the 2-OH 4-$OCH_3$ and 4-$N(CH_3)_2$ containing compounds dominated, showing the highest levels of activity with compounds **7**, **9**, **22**, **28** and **30** ranging from 28 to 54%. In the ABTS assay, the highly fluorinated compounds (pentafluorophenyl rings) **16**, **18**, **20**, **21**, and **24** all showed high activity with radical scavenging percentages from 34 to 53%. The 2-OH and 4-$OCH_3$ containing compounds performed well with 30 to 41% radical scavenging as well as the 4-$N(CH_3)_2$ compounds ranging from 28 to 35% radical scavenging activity in the ABTS assay. Compound **8** with a bicyclic structure and no NH-group, revealed the importance of the hydrazone moiety for the radical scavenging activity of these compounds having a radical scavenging of less than zero percent in all three radical scavenging assays. Compounds with two hydrazone moieties did not show much improvement in radical scavenging activity with many of these compounds having limited potency. Overall the hydrazones had generally higher radical scavenging activity than the Trolox and ascorbic acid control compounds in all three of the radical scavenging assays. Several compounds such as **7**, **9**, **15**, **19**, **22**, **26**, **28** and **30** all had equal or higher activity than resveratrol in the DPPH assay. These compounds show promise as future candidates for the development of free radical scavenging agents.

The analysis of the combined results of CK2 inhibition data and the radical scavenging activities reveals important details about the structure-activity relationship of the compounds. Although the hydrazone scaffold showed overall promising behavior in both CK2 inhibition and radical scavenging, every compound that showed strong activity against all four isoforms of the enzyme (**9**, **16**, **17**) and moderate (**16**) to excellent (**9**, **17**) radical scavenging, had multiple F atoms in their structure, either in the form of a perfluorophenyl ring or a $CF_3$ group. This is also true for compounds that inhibited at least three isoforms of the enzyme (**7**, **18**–**21**) and showed moderate (**7**, **18**, **20**, **21**) to excellent (**7**, **19**) radical scavenging. Based on the data it is reasonable to propose that the incorporation of multiple fluorine atoms to the scaffold strongly improves the enzyme inhibitory potential, while it is somewhat neutral regarding the antioxidant features in vitro. In addition to these effects, these organofluorine hydrazones possess much better membrane transport properties in vivo. The radical scavenging potency is, however, enhanced by the presence of substituents that can back donate an electron pair to the system, such as $-N(CH_3)_2$, or OH and $OCH_3$, which increase the mobility of the N–H hydrogen of the hydrazone unit contributing to effective radical scavenging via the HAT mechanism.[23,38] Concerning the activities in all assays the best combination appears to be when one aromatic ring possesses the $-N(CH_3)_2$ substituents with a perfluorinated ring as a counterpart (**17**). Although based on a limited number of hydrazones, the structure-activity relationship is not absolutely clear, the activity data provide sufficient guidance for further structural refinement.

## Conclusions

In conclusion, the findings in this study corroborate our former results concerning the antioxidant features of hydrazone derivatives. To our knowledge this is the first report identifying hydrazones as inhibitors of the protein kinase CK2. Up to now they were known as inhibitors of other kinases, like RSK2, CDK4 and MARK4.[39–41]

The role of CK2 in the brain is not completely clear and still remains elusive. It was reported that CK2 subunits ($\alpha$, $\alpha'$, $\beta$) are differently distributed in the major brain regions.[42] Whereas CK2$\alpha$ is found at equal level in the different regions, the hippocampus and the prefrontal cortex contain significantly higher amounts of CK2$\alpha'$ when compared to other analyzed regions. It seems that CK2$\alpha'$ participates in cell migration and adhesion.[43] In AD patients, it was shown that CK2$\alpha/\alpha'$, and thereby enzyme activity, is significantly overexpressed in the hippocampus and temporal cortex when compared to the control group.[18]

Some of the new inhibitors, especially compounds **7**, **9**, **11**, **13**, and **16**–**20** are promising candidates for further structural refinement of the hydrazone scaffold to obtain more efficient multifunctional Alzheimer's agents, with **17** emerging as a likely lead compound.

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## Conflict of Interest

The authors declare no conflict of interest.

**Keywords:** Alzheimer's disease · antioxidants · hydrazones · organofluorine · protein kinase CK2 · radical scavenging

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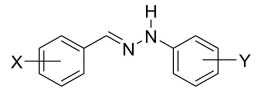


| X | Y | $IC_{50}$ CK2 inhibition | radical scavenging |
|---|---|---|---|
| 1,2,3,4,5-pentaF | 1,2,3,4,5-pentaF | 100 nM | up to 55% |
| 4-N($CH_3$)$_2$ | 1,2,3,4,5-pentaF | 200 nM | up to 90% |

*Prof. A. Baier*, Dr. A. Kokel, Dr. W. Horton, E. Gizińska, Dr. G. Pandey, Prof. R. Szyszka, Prof. B. Török, Prof. M. Török**

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**Seeing off CK2**: Organofluorine diarylhydrazones are effective multitarget compounds that alleviate the activity of protein kinase CK2 and show strong antioxidant effects in in vitro radical-scavenging assays. They contain a phenyl C–F bond and/or the $CF_3$ group. As both CK2 activity and oxidative stress appear to contribute to the development of Alzheimer's disease, the compounds are potential anti-AD agents.

**Organofluorine Hydrazone Derivatives as Multifunctional Anti-Alzheimer's Agents with CK2 Inhibitory and Antioxidant Features**