PART V
CHAPTER 7

General discussion & Summary
GENERAL DISCUSSION

Despite considerable efforts to halt or reverse the symptoms of AD, there is currently no effective disease-modifying treatment or cure available. Present pharmacological interventions produce moderate symptomatic benefits but fail to stop disease progression. Acetylcholine esterase inhibitors, which increase the neuronal acetylcholine amounts, and antagonists of the N-methyl-D-aspartate receptor (NMDAR), which reduce a neuronal overstimulation caused by the neurotransmitter glutamic acid, are currently used for treatment of AD [1,2]. Unfortunately, the benefit for the patient is poor and limited to early clinical stages of AD [3,4].

The discovery of a new disease-modifying treatment or cure for AD has been unsuccessful so far. While the systemic use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of developing AD, these drugs were not able to slow down disease progression [5,6]. Unfortunately, clinical trials with anti-inflammatory drugs have failed, most likely because not all components of the inflammatory response in AD are detrimental [7].

Drug development efforts have long been focussed on the modulation of Aβ, by either limiting its production or by facilitating its clearance from the brain [8]. Due to the complexity of these processes the success was limited. Recent efforts in drug target discovery address the potential of inhibiting protein kinases such as Gsk3β (involved in tau phosphorylation and Aβ production) and Cdk5 (mediator of tau hyper-phosphorylation) for the treatment of AD (for reviews see [9–15]). Also, kinases playing a role in neuroinflammation and synaptic plasticity (like Src and p38) are currently under investigation as drug targets for AD. The general involvement of protein kinases in AD pathogenesis and the possibility to inhibit or enhance kinase activity using small molecules that can easily pass the blood-brain barrier, validates an investigation into the role of protein kinases in AD and might reveal new attractive drug targets. In order to get more insight into the role of protein kinases in neuroinflammation and synaptic transmission, an extensive literature search was performed, which resulted in the identification of 66 protein kinases that are involved in either or both of these processes (Chapter 1, Table 1). Several of these protein kinases are currently under investigation as potential drug targets.

Mapping of protein kinase activity during AD pathogenesis

Most of the currently known protein kinases that play a role in AD have been identified based on changes in protein levels in AD brain tissue compared to non-demented controls, often in a small number of cases. Of special interest is the hippocampus, since this part is affected at early (pre-clinical) stages of AD. However, it is essential to probe the biological variations of the samples across all stages of AD to obtain information about the overall number and type of protein kinases that play a role during AD disease progression.
Investigating protein kinase activity, as opposed to determining the presence of a protein kinase, is an unbiased and more direct approach to monitor global changes that occur in human brain tissue (and fluid). In order to measure protein kinase activity, we used a peptide microarray that contains peptides with potential phosphorylation sites. Protein kinases that are present in brain tissue extracts will phosphorylate these peptides in the presence of ATP [16–18]. Kinase activity profiling using peptide microarrays has proven to be a robust and fast method that supports high throughput and requires low amounts of protein (below 600 ng) per assay, which makes it feasible to perform several experiments with as little as 1 mm$^3$ of brain tissue. The result is a map of phosphorylated peptides that can be used to reveal proteins (including kinases) and signalling pathways involved in AD [17,19]. Databases containing information about kinase-substrate relations such as Phosphosite or UniProt provide information about the kinases that might be responsible for the phosphorylation [20]. Furthermore, *ex vivo* pharmacology of kinases can be performed by determining kinase activity in the presence of specific kinase inhibitors.

A cohort of 100 human *post mortem* hippocampal brain tissue representing all stages of AD (Braak stages 0–VI) was analysed, an overall decrease of protein kinase activity was observed at each of the different stages [18]. We discovered aberrant activities already at Braak I and II, suggesting that aberrant kinase activity already occurs before clinical symptoms of AD are apparent. Most interestingly, we identified protein kinases that have previously not yet been associated with AD such as FRK, FES and PTK6/BRK. The FRK protein shows considerable amino acid similarity with the tau kinase Fyn (60 %), though its function is not known. Although no link has been reported between FES and AD, it is known that FES plays a role in the regulation of the actin cytoskeleton, microtubule assembly, cell differentiation and neuritic outgrowth. PTK6/BRK promotes cell survival in breast, colon and skin cancers [21]. In addition, using our approach, we confirmed the involvement of several proteins already implicated in AD (Src, Gsk3β, p38 MAPK), indicating the validity of this technique. The current status of members of the Src family of protein kinases, Gsk3β and p38 MAPK as targets for the treatment of AD is discussed in the following section.

**Src-family kinases**

The Src family of protein kinases (SFKs) has received a lot of attention due to the ability of several members to phosphorylate the tau protein [22] and their participation in long-term potentiation (LTP) [23]. Src kinase activity is decreased during the early stages of AD and potentially contributes to the loss of synaptic plasticity [18]. In contrast, increased activity of Src-kinase family members was suggested to be an underlying mechanism of amyloid-dependent microgliosis in AD [24,25]. While studies about the role of Src itself in AD are limited, Fyn is one of the most extensively studied tau-
kinases. Fyn is upregulated in AD-neurons [26], where it phosphorylates tau at Tyr18. Fyn and pTau are co-localized in neurofibrillary tangles in AD [27]. Fyn knockout mice display impaired short- and long-term memory after contextual fear conditioning which is further strengthening the importance of Fyn in memory formation [28].

An inhibitor that targets Src and Abi is dasatinib, which decreases amyloid associated microgliosis in APP/PS1 transgenic AD mice [24]. As microgliosis is part of the neuroinflammatory response, Dhawa et al. suggested that Src inhibition might be a valid anti-inflammatory approach for AD [24]. Phase II studies for dasatinib in patients with metastatic castration-resistant prostate cancer (CRPC) [29] and chronic-phase myeloid leukemia [30] provide reasonable tolerability and treatment-related adverse events were moderate [31]. However, dasatinib has not yet been tested in patients with neurodegenerative diseases.

Recently, a clinical phase Ib study was completed for saracatinib (24 AD subjects) [32]. Saracatinib (AZD0530) is a small molecular inhibitor of Src-kinase family, blocking Src, Fyn, Yes and Lyn with 2-10 nM potency. At a 10- to 100-fold higher concentration saracatinib also inhibits Abl-family kinases [33]. The drug was generally well tolerated. Despite rescued memory deficits in transgenic mouse models [34], one month treatment with saracatinib had no significant effect on clinical efficacy or on the regional cerebral glucose levels of the participants [32]. A larger phase IIa trial has recently started (https://clinicaltrials.gov; NCT02167256).

**Gsk3β**

Increased Gsk3β activity in the brain of AD patients correlates well with an increase in neuronal death [10]. Gsk3β might contribute to AD neuropathology by regulating the phosphorylation of tau [35] and promoting neuroinflammation [36]. In addition, Gsk3β is directly involved in synaptic plasticity [37] and the inhibition of LTP caused by Aβ oligomers [38]. Hence, Gsk3β inhibition may be beneficial in models of neuroinflammation and AD [39]. The activation of three signalling pathways is known to inhibit Gsk3β activity [39,40], namely the PI3K–PKB pathway, the classical MAPK cascade and the mTOR pathway, where p70 S6 kinase (S6K) inhibits GSK3 [40].

A phase II clinical trial for AD with the Gsk3β inhibitor tideglusib was recently completed [41]. While treatment with tideglusib results in reduced tau hyper-phosphorylation, Aβ deposition, neuronal loss, markers of inflammation and neuritic dystrophies in several animal models [42–45], no clinical benefit was observed in mild to moderate AD patients after 26 weeks (short term) administration of tideglusib [41].
**p38 MAPK**

The p38 MAPK is a serine/threonine kinase related to ERK and JNK kinases. p38 MAPKα is expressed in neurons, where it is involved in memory formation and synaptic plasticity [46]. p38 also plays a role in acute and chronic neurodegeneration due to its ability to regulate the release and activation of pro-inflammatory cytokines like IL-1β and TNFα [47–49]. In a transgenic mice model for AD (APP751), an increased inflammatory response is observed hand in hand with an upregulation of p38 activity [50]. Exposure of cultured primary rat microglia and human THP1 monocytes to Aβ stimulates p38 MAPK phosphorylation and thereby the production of pro-inflammatory cytokines [51]. Hence, p38 MAPKα has gained interest as a potential therapeutic target for a wide range of CNS disorders [48]

A study in APP751 mice revealed that the selective p38 MAPK inhibitor SD282 is able to reduce the activation of microglia [52]. In addition, p38 MAPKα antagonists improve cognitive functions in the AD model (APP/PS1 mice [53] and adult wild type Fischer rats [54]). These results led to a phase Ila clinical trial with a BBB penetrant selective p38 MAPKα antagonist (VX-745) by EIP Pharma LLC (www.eippharma.com). VX-745 is a highly selective and potent inhibitor of the α isoform of the protein kinase p38 MAPK and is expected to reduce the Aβ plaque load and inflammation in the brain of AD patients [55].

**Protein kinase CK2**

CK2 protein kinase, originally identified in one of our kinase profiling studies [17], is involved in neuroinflammation and functions downstream of p38 MAPK (Figure 1). Levels of CK2 are increased in the temporal cortex and hippocampus of AD patients compared to non-demented controls [56]. Co-localisation studies show that the presence of CK2 in astrocytes is associated with amyloid deposits, suggesting an involvement of CK2 in the neuroinflammatory response. While a neuroinflammatory response in AD brains is considered primarily beneficial, e.g. removing Aβ aggregates from the brain, chronic neuroinflammation is thought to be harmful due to the constant excess production of pro-inflammatory cytokines, prostaglandins and reactive oxygen species that exacerbate Aβ deposition and induce neuronal dysfuction [57]. So far all clinical trials with AD patients using anti-inflammatory drugs have failed, indicating the need for new anti-inflammatory treatments [7].

Interestingly, CK2 overexpression has been implicated in a number of different cancers including head and neck [58], colorectal [59], renal [60], lung [61], leukemias [62] and prostate cancer [63]. Reports suggest that downregulation of CK2 activity with specific inhibitors, like CX-4945, could reduce cancer cell viability and induce apoptosis
CX-4945, is currently in clinical trials for the treatment of cancer and has shown generally mild to moderate side effects [64,66]. We found that treatment of human primary astrocytes with CX-4945 leads to significant inhibition of the IL-1β/TNF-α mediated secretion of IL-6 and MCP-1, suggesting that CK2 is a potential target to modulate the neuroinflammatory response in AD (Figure 1) [56]. Whether CX-4945 is able to pass the BBB in order to reduce neuroinflammation in the brain needs to be resolved.

Figure 1 - Potential role of p38 MAPK and CK2 in neuroinflammation. Arrows indicate enzyme-substrate relationships. Stress-induced activation of p38 MAPK subsequently leads to the activation of CK2 and phosphorylation of p53 at Ser392 [67]. Tumor necrosis factor-α or interleukin 1 activate NF-κB through phosphorylation of the p65 subunit, increasing its transcriptional activity [68]. CK2 also phosphorylates IκB, leading to its degradation which appears to be relevant in the aberrant activation of NF-κB in breast cancer cells [69]. The inhibition of CK2 by CX-4945 downregulates PI3K/Akt by de-phosphorylation of Akt [70]. Adapted from [65,71].

EphA4 and EphA/B-kinase family
The erythropoietin-producing hepatocellular (Eph) receptor tyrosine kinases (RTK) form the largest of the 20 subfamilies of human receptor kinases. Eph receptors and their ligands, the so-called ephrins, contribute to the aberrant synaptic functions associated with neurodegeneration. We report an altered distribution of EphA4 RTK in human hippocampal brain tissue already at early stages of AD (Braak stage II) compared to non-demented controls, while the expression levels and activity remain unchanged ([72] and chapter 5 of the thesis). These results indicate that the reduced availability of EphA4 is likely to contribute to synaptic dysfunction that occurs early in AD.
In rodents, EphA4 is present in the hippocampus where it plays a role in adult synaptic plasticity and learning [73,74]. A series of studies of the adult rodent hippocampus suggests that EphA4 is present on the dendritic spines of neurons where it regulates spine morphology by interacting with its ephrin-A3 ligand that is expressed in astrocytes [75]. Aberrant EphA4/ephrin-A3 signalling leads to perturbed morphology of dendritic spines and defects in LTP and memory formation [76]. Others found decreased EphB2 and EphA4 expression levels in hAβPPase-ind mice, which co-occurred with the onset of memory decline [77]. In wild type mice, EphA4 mediates Abl activation, which is a key signalling event causing synaptic damage and LTP blockage induced by Aβ oligomers [78,79]. It has been hypothesized that the activation of c-Abl during early stages of AD might induce downstream phosphorylation of the tau kinases CDK5 and Gsk3β resulting in the hyper-phosphorylation of tau and the formation of neurofibrillary tangles [80].

The complexity of Eph receptor/ephrin signalling mechanisms certainly poses a challenge for effective therapeutic targeting. Reversing EphB2 depletion in a mouse model of AD was shown to rescue cognitive functions and might be a promising therapeutic strategy [81]. However, few follow up studies have been reported. Numerous EphA4 inhibitors are currently under investigation for their potential to serve as drug targets (for reviews see [82–86]).

**Protein kinase activity profiling as a clinical biomarker for AD diagnosis**

AD is a heterogeneous disease in which a large number of molecular mechanisms are changed [87]. Finding biomarkers that reflect this heterogeneity will be a big step forward for the clinical diagnosis and prognosis for AD and possibly improve the quality of life for the patients. Since brain tissue is rather inaccessible, we considered the possibility of protein kinase profiling of CSF as a novel biomarker for use in the clinic. In chapter 6 we report proof of this concept by showing that protein kinase activity is present in human CSF and that the activity profiles of AD and controls are distinguishable. Elucidating changes in the activity of extracellular protein kinases in the CSF, is also expected to provide insights into intracellular molecular pathways and mechanisms such as synaptic transmission and neuroinflammation [88]. In addition, the activation state of kinase-driven signalling networks contains valuable information concerning both disease pathogenesis and potential therapeutic target selection [20].

To further establish kinase profiling of CSF as a clinical biomarker for AD, the following considerations should be taken into account for sample selection in the validation phase. *Post mortem* confirmation of the clinical diagnosis at the time of death should be available for all cases used for profiling, since the composition of the CSF reflects brain...
pathology. For patients with subjective memory complaints (SMC) but normal CSF biomarkers (Aβ42, pTau, t'au) included in the analysis, a follow-up check-up of at least 2 years showing normal biomarker values should be available. CSF samples should be taken regularly, e.g. annually since patients can progress to later stages of AD.

The current guidelines for the standardisation of CSF collection protocols are based on the initial consensus guideline of the BioMS consortium [89] and listed elsewhere [90,91]. Currently, clinical protocols for CSF collection are optimized for detection of protein levels and not for enzymatic activity measurements. Routinely, CSF is kept at room temperature for up to an hour before processing. In general, protein kinases are more stable at lower temperature. Therefore, I recommend that the collected CSF is placed on ice as soon as possible and is processed at a low temperature. Adapting the current protocols in the clinic will be a valuable and important issue for future biomarker discovery research.

Technical issues regarding the pre-analytical stability of CSF for biomarker testing should also be taken into account. Pre-analytical handling is known to account for 60 % of total laboratory errors [92]. Factors such as sample collection mode [93], type of test tubes [94,95], tube/plate adsorption, freeze/thaw cycles [89] and length of storage should be carefully considered [91,96,97]. Therefore, I recommend that patient-related factors and variation in the CSF collection process as well as variation in assay performance should be carefully documented and should be included in the data analysis. Given the heterogeneous nature of the disease, it is very likely that combinations of biomarkers will prove to be the most useful for disease diagnosis (presence vs. absence of AD pathology) and prognosis (prediction of cognitive decline) [98].

A translational approach to identifying AD disease mechanisms, drug targets and novel biomarkers

In order to identify new drug targets and novel biomarkers for AD a translational approach is paramount. Based on our experience, we propose the following setup. The selection of well-characterized brain tissue samples (and CSF) is the first important step. The following recommendations for sample selection should be taken into account. Staging of AD pathology should be performed according to the modified assessment of Braak and Alafuzoff [99]. Cases with and without clinical neurological disease diagnosis should be processed identically. Patients with co-morbidities like Parkinson’s disease or Lewy-body disease need to be excluded from AD studies. The availability of information about post mortem delay (hours), clinical diagnosis, age, sex, pH of the CSF and Braak score for NFT allows to determine possible confounding factors (Chapter 2 – 6). For protein kinase activity profiling of freshly frozen tissue using peptide microarrays
(PamGene) Standard Operating Procedures (SOP) and Good Laboratory Practices (GLP) were established. Performing the data analysis in a team of neuroscientists, (bio)statisticians, (bio)informaticians, neuro-pathologists and clinical neurologists allows application of advanced statistical methods without losing the connection to biological interpretation. The outcome of the in silico data analysis should be followed by a verification step in which the same brain tissue is analysed by Western blotting (WB) and immunohistochemical (IHC). Figure 2 shows the various steps in our established research pipeline. Further validation can take place by studying the effect of specific kinase inhibitors on the brain tissue lysate to validate the involvement of a particular kinase or to study the effect of such a drug in a large cohort of tissues of various Braak stages.

In order to establish whether a protein is a potential drug target we find it is essential not only to demonstrate that the protein presence or protein kinase activity is changed in human brain tissue but that there is a correlation between the function of the protein and AD pathogenesis. Therefore, we recommend that human primary cell cultures (astrocytes and microglia) are used for in vitro cell assays. To establish a functional link, we use specific kinase activators and/or inhibitors and analyse the effect on read outs that represent aspects of AD pathogenesis e.g. activation of proteins in the pathway downstream of the inhibited kinase or the production of pro inflammatory cytokines [19,56]. These in vitro cell assays using either human cell lines or primary cell cultures are extremely useful since they can be utilized to test new potential drugs for AD.
FUTURE STRATEGIES & FINAL REMARKS

For complex multifactorial diseases such as AD it is unlikely that the disease can be controlled effectively by using one selective protein kinase inhibitor. Therefore, multitarget drug discovery (MTDD) e.g. either the combination of two or more compounds selective for a single target with an additive or synergistic effect, or the development of agents which are able to address multiple targets simultaneously, seems like a promising approach [64,100]. The MTDD strategy can be applied to CK2 as an AD drug target as a combination of CX-4945 with other inhibitors led to synergistic effects in cell death induction for the treatment of haematological malignancies [65].

For testing novel protein kinase inhibitors to assess the role of particular kinases in tau hyper-phosphorylation and neurofibrillary degeneration animal models have been developed that reproduce tau-related pathology [101]. Extensive testing of potential AD drugs in animals is cost- and time-intensive and not in line with the aim to reduce animal testing. But even more important, the effects of a drug in AD animals are often not reproducible in humans, not only because there are significant differences between a rodent and a human brain, but also because the disease symptoms in rodents may be induced by one genetic defect, in contrast to the multifactorial cause in humans.

Based on the result presented in this thesis we recommend that AD drug candidates that are designed to inhibit protein kinases are tested directly on human brain tissue using the peptide microarray kinase profiling assay. It is cheaper, faster, requires only small amounts of brain tissue (µg) and will give a more accurate depiction of the potential effect of the inhibitor in humans, especially when the drug is tested in a substantial number of samples (allowing to probe the heterogeneity) and when testing is combined with in vitro functional (primary) human cell assays (Rosenberger et al., in preparation).
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SUMMARY
Recent studies suggest that the pathogenesis of Alzheimer’s disease (AD) might be a result of neuroinflammation in response to stress, which is followed by alterations in neuronal physiology and is accompanied by synaptic loss, neuronal dysfunction and subsequent neuronal death. AD is characterised by a long pre-clinical phase (20-30 years), during which significant brain pathology manifests itself. Neuroinflammation and synaptic failure are considered early events in the course of AD and are hence attractive targets for the prevention and pharmacological intervention to slow down or halt disease progression. Substantial evidence implicates aberrant protein kinase signalling in AD. An increase in kinase activity results in hyper-phosphorylation of the tau protein and the formation of toxic aggregates (tangles) in neuronal cells, while the extracellular amyloid beta protein (Aβ) is phosphorylated and forms extracellular insoluble deposits (plaques). In order to determine the (potential) involvement of protein kinases in neuroinflammation and synaptic changes that occur during AD pathogenesis, the literature between 2000 and 2016 was searched. The NCBI PubMed database was used for data mining with each of the 523 known protein kinases in combination with the search term ‘Alzheimer’s disease’ and either ‘neuroinflammation’ or ‘synaptic changes’ as input. This approach resulted in a list of 66 protein kinases implicated in neuroinflammation and/or synaptic changes in AD (Chapter 1). To shine light onto early events in AD pathogenesis and the role of protein kinases, protein kinase profiling of 100 hippocampal post mortem brain tissue lysates of patients with AD and non-demented controls (Braak stages 0-VI) was performed using a peptide-based microarray platform. The results show an overall decrease of protein kinase activity, which correlates well with disease progression. Protein kinase activity already decreases at pre-clinical stages of AD pathology (Braak I-II). Bioinformatics analysis (STRING) in combination with pathway analysis allowed the identification of the Ephrin-receptor A1 (EphA1) kinase, a risk gene for AD, and sarcoma tyrosine kinase (Src), which is involved in memory formation. In addition, protein kinases that have not previously been associated with AD were identified such as protein tyrosine kinase 6 (PTK6/BRK), feline sarcoma oncogene kinase (FES) and fyn-associated tyrosine kinase (FRK) (Chapter 2). Even though protein kinase CK2 (former casein kinase II) is one of the best-studied kinases, its involvement in AD is far from clear. We find increased levels of CK2 in the hippocampus and temporal cortex of AD patients compared to non-demented controls. CK2 immunoreactivity is specifically increased in astrocytes that are associated with amyloid deposits, one of the hallmarks of AD. The function of CK2 was investigated using human U373 astrocytoma cells and human primary adult (HPA) astrocytes. Stimulation with IL-1β or TNF-α results in the secretion of pro-inflammatory cytokines MCP-1 and IL-6. The CK2 inhibitor CX-4945 shows a dose-dependent reduction of IL-1β or TNF-α secretion. These results suggest that CK2 might be considered a potential drug target for the modulation of neuroinflammation in AD (Chapter 3). Activation of signalling
pathways through chronic neuroinflammation is thought to lead to the activation of tau kinases, tau dysfunction followed by the dysfunction of synapses. The aberrant signalling at synapses and the subsequent loss of neurons occur early in AD and correlate with the cognitive decline observed in patients with mild cognitive impairment. EphA4, a member of the Eph receptor family, which we previously identified (Chapter 1+2) had been linked to memory loss in a transgenic mouse model for AD. These results inspired an investigation into the amount and localization of EphA4 in human hippocampal tissue of AD and non-demented control cases. We found that the total amount of EphA4 is the same in AD compared to controls. In contrast, immunohistochemical analysis showed that the localization of EphA4 immunoreactivity is altered as EphA4 is found in plaque-like structures in AD cases and co-localizes with neuritic plaques, one of the hallmarks of AD pathology. This re-distribution is already apparent at early stages (Braak stage II) (Chapter 4). In order to test whether in addition to the observed re-localization the kinase activity is affected, EphA4 activity in human hippocampal brain tissue lysates derived from AD and non-demented control cases was compared. A new kinase immunodepletion assay (KID) was developed and used in combination with kinase activity profiling. Employing KID made it possible to selectively deplete EphA4. Interestingly, protein kinase activity of EphA4 remains unchanged in AD as compared to controls, revealing that the re-distribution of EphA4 happens independently of its kinase activity (Chapter 5). The need for biomarkers that reflect early stages of AD pathology led us to explore the potential of using protein kinase activity profiling as a clinical biomarker for AD. We show that serine/threonine protein kinase activity is present in clinical CSF and that the activity is significantly lower in AD compared to controls (p-value < .05). Kinases that are differentially active in CSF include members of the CaMK family and the AGC kinase group (Chapter 6). In Part V, Chapter 7 the findings of this thesis are discussed. The lack of medical treatments for AD and the increasing prevalence due to the ageing population has raised efforts in the search for new therapeutic approaches which could lead to new effective drug targets. Protein kinases regulate key phosphorylation pathways and thereby control most aspects of cell life. Aberrant phosphorylation plays a role in many diseases e.g. cancer and neurodegeneration. Protein kinases are therefore key players as therapeutic targets. The regulation of protein kinase activity by small ligand molecules seems very promising for future clinical use. A few examples of protein kinases involved in early processes in AD pathophysiology such as neuroinflammation and synaptic changes are presented. Several protein kinase inhibitors are currently in clinical trials for the treatment of AD (e.g. Gsk3β, p38 MAPK, Src). Testing the effect of selective kinase inhibitors is an elegant way to confirm changes in kinase activity of a particular kinase between AD patients and non-demented controls. We hence propose protein kinase activity profiling of post mortem human brain tissue as an alternative approach to evaluate the effectiveness of protein kinase inhibitors directly in the tissue.