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The essentiality of Bcl-2, PKC and proteasome–ubiquitin complex activations in the neuroprotective-antiapoptotic action of the anti-Parkinson drug, rasagiline

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Abstract

The anti-Parkinson drug, rasagiline, a irreversible propargyl possessing monoamine oxidase B inhibitor can protect neurons *in vitro* and *in vivo* from a variety of neurotoxic insults including SIN-1, glutamate, the parkinsonism inducing neurotoxin, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, *N*-methyl-(*R*)-salsolinol and including beta amyloid protein. Recent studies have shown that rasagiline rapidly modulates intracellular signaling pathways involved in cell survival and death. Specifically rasagiline activates Bcl-2, Bcl-xl, protein kinase C (PKC) and reduces Bax in a variety of cells including PC-12 and neuroblastoma human dopamine derived SH-SY5Y cells. These enzymes play key roles in cellular events including modulation of apoptotic processes, neuronal plasticity and amyloid precursor protein processing. This pharmacological action of rasagiline is also associated with the prevention of the neurotoxin induced fall in mitochondrial membrane potential, opening of mitochondria permeability transition pore, activation of proteasome–ubiquitin complex, inhibition of cytochrome *c* release and prevention of caspase 3 activation, similar to the actions of cyclosporin A or Bcl-2 over expression in SH-SY5Y cells. Rasagiline and its various derivatives induces PKC dependent release of soluble amyloid precursor protein alpha and which is blocked by inhibitors of α-secretase, PKC and MAPK-dependent signaling. Structure–activity relationship with various propargyl containing derivatives of rasagiline including propargylamine itself has shown that the above described pharmacological action of these compounds resides in the propargylamine moiety. These results have provided a new understanding into the mechanism of neuroprotective actions of rasagiline and its anti-Alzheimer drug derivatives TV3326 and TV3279, which are relevant for therapy of Parkinson's disease, Alzheimer's disease and other neurodegenerative diseases.

Keywords: Proteasome; Ubiquitin; Rasagiline

1. Parkinson's disease and neuroprotective properties of rasagiline

Parkinson's disease (PD) is a progressive disorder of basal ganglia where nigrostriatal dopamine neurons degenerate and specific etiology for it has not been established. However, numerous studies on human brain autopsy and its animal models have indicated mechanisms that may involve oxidative stress and that inflammatory process resulting in apoptotic or necrotic death of the neurons. Many attempts are being made to identify and determine whether certain anti-Parkinson, antioxidant, iron chelators

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Abbreviations: MAO, monoamine oxidase; MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, dihydropyridinium ion; L-DOPA, L-dihydroxyphenylalanine; NGF, nerve growth factor; PKC, protein kinase C; SOD, superoxide dismutase; iNOS, inducible nitric oxide synthase; 6-OHDA, 6-hydroxydopamine; *N*-M-(*R*)-Sal, *N*-methyl-(*R*)-salsolinol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VDAC, voltage-dependent anion channel; MPT, mitochondrial permeability transition; AD, Alzheimer's disease; PD, Parkinson's disease; APP, amyloid precursor protein; sAPPα, soluble amyloid precursor protein alpha; Aβ, beta amyloid protein.

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and bioenergetics can be neuroprotective in animal and cellular model, which in turn can be studies in such patients. Here, we discuss the mechanism of neuroprotective action of anti-Parkinson drug, rasagiline [1].

Rasagiline (*N*-propargyl-1-(*R*)-aminoindan) is a potent selective irreversible inhibitor of monoamine oxidase (MAO)-B, while its *S*-isomer, TVP1022, is more than 1000 times less active [2]. It is structurally similar to selegiline, has a 10- to 20-fold higher potency in humans and experimental animals as MAO-B inhibitor, but does not have the sympathomimetic activity side effect of selegiline [3,4]. Unlike the latter, it is not metabolized to sympathomimetic amphetamine, but rather to aminoindan [2]. At a dose that selectively inhibits brain MAO-B it does not potentiate the sympathomimetic action of tyramine [3,4], but increases the behavioral response to phenylethylamine, a substrate of MAO-B [5]. Similar to selegiline and other MAO-B inhibitors, it protects against the Parkinson inducing neurotoxin, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice and monkeys by preventing its conversion to MPP^+ (dihydropyridinium ion) [6]. Recent multi-center double-blind monotherapy in phase 3 studies by the Parkinson Study Group [7] and as adjunct therapy to L-dihydroxyphenylalanine (L-DOPA) [8] have shown that rasagiline confers significant symptomatic improvement and long-term studies suggests possible alterations in disease progression at doses of 1 and 2 mg. These dosages are 5–10 times lower than those for selegiline. Rasagiline has been shown to have potent antiapoptotic activity in partially differentiated PC-12 cells deprived of serum and nerve growth factor (NGF) [9] and against endogenous neurotoxin *N*-methyl-(*R*)-salsolinol (*N*-M-(*R*)-Sal) [10–12], 6-hydroxydopamine (6-OHDA) [13] and SIN-1, a peroxynitrite donor in rat phaeochromocytoma (PC-12) and human dopamine-derived neuroblastoma (SH-SY5Y) cells [14,15], glutamate toxicity [16] and *in vivo* studies with several models including MPTP, global ischemia and closed head injury [17,18]. These studies prompted us to investigate more closely the molecular mechanism of neuroprotective activity of rasagiline and its non-MAO inhibitor *S*-isomer (TVP1022) in neuronal cell cultures and *in vivo*.

2. Antiapoptotic properties of rasagiline and mitochondrial permeability transition (PT) pore

Rasagiline, which has structural resemblance to selegiline, has antiapoptotic and neuroprotective activities [19] in partially-neuronally differentiated rat PC-12 cells after serum and NGF deprivation [9]. However, since both these compounds are inhibitors of MAO-B, it was not known whether MAO-B inhibition contributes to the antiapoptotic and neuroprotective activities. We investigated the antiapoptotic actions of rasagiline and TVP1022 in partially-NGF-differentiated PC-12 cells during serum and NGF deprivation. Both drugs showed a dose (10^{-3} to 10^{-10} M)-dependent inhibition of apoptosis, while the aminoindan metabolite of rasagiline, TVP136, which lacks the propargyl moiety, was inactive [9]. The antiapoptotic activity of rasagiline and TVP1022 correlated with the prevention of the fall in mitochondrial potential observed during serum and NGF withdrawal [12–14].

Synthesis of new protein is required for the antiapoptotic action of rasagiline in partially neuronally differentiated PC-12 cells deprived of serum and NGF. This effect can be observed by the use of transcription and translation inhibitors actinomycin and cycloheximide without causing the death of PC-12 cells. The antiapoptotic actions of rasagiline are blocked by cycloheximide [10] and actinomycin suggesting that these compounds act by altering gene transcription of antiapoptotic proteins induced by rasagiline [9,20]. This is supported by our recent studies with rasagiline with gene expression employing cDNA microarray in rats and mice striatum and hippocampus. The most notable increases in gene expression were those of PKC, Bcl-2, superoxide dismutase (SOD) and catalase together with a down-regulation of genes suspected to play a role in neurodegeneration such as oxidative stress proteins, glutamate and AMPA receptors, inducible nitric oxide synthase (iNOS) and inflammatory transcription factor NFκB, Bad and Bax [21]. Up regulation of NFκB has been reported in nigrostriatal dopamine neurons of PD as compared to controls and suggested to be a consequence of inflammatory reaction resulting from proliferation of reactive microglia. One consequence of which is increased release of cytotoxic cytokines IL1, IL2 and TNFα, which is thought to contribute to the process of neurodegeneration. Both parkinsonism neurotoxins MPTP and 6-hydroxydopamine (6-OHDA) activate NFκB resulting in its translocation from cytoplasm to the nucleus in PC-12 and SH-SY5Y cells in culture. Indeed neuroprotective antioxidants and iron chelators (EGCG, *R*-apomorphine) down regulate NFκB and prevent its translocation to the nucleus. Similar findings have also been seen in striatum of 6-OHDA treated rats *in vivo* [22]. Whether rasagiline has a similar action has not been investigated.

Indeed, the consequences of serum and NGF withdrawal from partially neuronal-differentiated PC-12 cells are the fall in the antiapoptotic proteins Bcl-2 and SOD and their mRNAs and mitochondrial membrane potential and which are prevented by rasagiline and TVP1022, but not by their aminoindan metabolite. The results of these studies have indicated that MAO-B inhibition is not a pre-requirement, but rather that the propargylamine moiety in these molecules is directly involved, as supported by the studies of Maruyama *et al.* [23] with other propargylamines. Carrillo *et al.* [24] examining the chronic effect of various dosage (0.1–1 mg/kg per day × 24) of rasagiline *in vivo* on various tissues of rats reported highly significant dose dependent increases in SOD and catalase activities in substantia nigra, striatum, heart and kidney.

Using cultured human neuroblastoma (SH-SY5Y) and rat PC-12 cells, with neurotoxins SIN-1 (peroxynitrite donor), *N*-M-(*R*)-Sal, and 6-hydroxydopamine (6-OHDA), we found that rasagiline, TVP1022, were relatively potent, antiapoptotic, neuroprotective agents. However, aminoindan, the metabolite of rasagiline, which does not possess the propargylamine moiety, had no antiapoptotic activity [15]. The antiapoptotic activity of rasagiline and TVP1022 were attributed to the ability of these drugs to prevent neurotoxin induced fall in mitochondrial membrane potential ($\Delta\psi_m$) [14,15] and the opening of the mitochondrial permeability transition (MPT) pore, which has the voltage-dependent anion channel (VDAC) associated with it. Overexpression of Bcl-2 in SH-SY5Y cells had similar antiapoptotic effect as that of rasagiline and prevented the opening of the MPT pore in response to *N*-M-(*R*)-Sal [11,12] (Fig. 1). Furthermore these cells were resistant to apoptosis induced by neurotoxins SIN-1 and an endogenous neurotoxin *N*-M-(*R*)-Sal derived from dopamine. Bcl-2 is known to regulate mitochondrial membrane potential and be tightly associated with the MPT pore regulation, the release of cytochrome *c* and other apoptosis-inducing factors [25,26]. Pharmacologically rasagiline, similar to over expression of Bcl-2 in SH-SY5Y cells, prevented *N*-M-(*R*)-Sal induced fall in mitochondrial membrane potential release of cytochrome *c*, the translocation of pro-apoptotic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and blocked the opening of VDAC in such cells [12,15]. However, rasagiline does not prevent Ca^{2+} induced opening of MPT suggesting it might act at some other ion site on the mitochondria [12]. The similarity of the action of rasagiline to over expression of Bcl-2 in SH-SY5Y cells can be explained by our recent findings that rasagiline on one hand causes activation of Bcl-2 and Bcl-xl and on the other reduction of Bax gene expression and proteins in a time and concentration dependent manner [20] (Fig. 1). Similarly it activates ubiquitin–proteasome system with the resultant inhibition of mitochondrial cytochrome *c* release [27,28]. The neuroprotective action of rasagiline is not limited to neuronal cell culture studies, the limitation of which is well recognized.

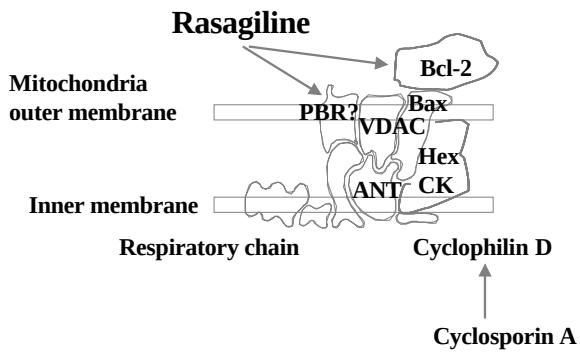

Fig. 1. Possible site of action of rasagiline and its derivatives at the mitochondrial VDAC, which is part of MPT. The exact protein constituents of MPT is not known but several of the proteins, such as anti and proapoptotic proteins Bcl-2 and Bax, respectively; porin; PBR, peripheral benzodiazepine receptor; ANT, adenosine nucleotide translocator; Hex, hexokinase and CK, creatine kinase have been identified. In a number of respects mechanism of neuroprotective action of rasagiline and its interaction with MTP resembles that of cyclosporin A, a drug with neuroprotective activity.

3. Neuroprotective effects of rasagiline—*in vivo* models

The potential neuroprotective effects of rasagiline and (*S*)-enantiomer, TVP1022, were examined against the effects of the dopaminergic neurotoxin, MPTP (*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) in mice [21] and against the sequelae of closed head injury in rats [29] and postnatal anoxia [30]. When injected at a dose that selectively inhibited MAO-B in mice, rasagiline, but not TVP1022, prevented MPTP induced degeneration of nigrostriatal dopamine neurons[1] similar to what we had observed with its racemic form [6]. However both rasagiline and TVP1022 when injected once, subcutaneously (s.c.), 5 min after closed head injury in mice, accelerated the recovery of motor function and spatial memory and reduced the cerebral edema. Scopolamine prevented the neuroprotective effects of these drugs on motor function and spatial memory, but not on cerebral edema. When rasagiline was injected from 3 to 8 days after injury, recovery of spatial memory but not motor function was accelerated [29]. Furthermore when injected 1 and 3 hr after global ischemia in gerbil significant reduction in occlusion was observed [31]. These studies demonstrated that early administration of rasagiline or TVP1022 could reduce the immediate sequelae of brain injury. They can also hasten the recovery even if given after the sequelae of brain trauma have fully developed. Since rasagiline and TVP1022 were protective in this model it suggests that MAO-B inhibition is not involved confirming the results obtained with neuronal cell cultures described above [18].

Impairment of motor function and memory produced by brain injury has been shown to be associated with a deficit in cholinergic transmission. Since the actions of rasagiline and TVP1022 were antagonized by scopolamine, it suggests the drugs preserved cholinergic transmission in relevant brain neurons. However, neither drug inhibits cholinesterase (ChE) or has any direct effect on cholinergic receptors. It is possible that they may increase the activity of choline acetyl-transferase by an unknown mechanism similar to what has been reported for selegiline. The neuroprotective activity of rasagiline and TVP1022 may also involve other mechanisms such as activation of antioxidant enzymes such as SOD, catalase glutathione peroxidase and Bcl-2, since oxidative stress and inflammatory processes, which have been demonstrated in closed head

[1] Unpublished data.

injury. Certainly chronic treatment of rats with various dosages of rasagiline (0.1–1.0 mg/kg for 24 days) significantly increases the activities of Cu–Mn SOD, Zn–SOD and catalase by more than 200% in the substantia nigra, striatum, cerebral cortex, heart and kidney [24].

4. Amyloid precursor protein processing activities of rasagiline and its cholinesterase inhibitor derivatives

Significant percentage of Parkinsonian subjects may also develop Alzheimer's disease (AD) dementia. One of the main characteristics of AD is the deposition of the supposedly neurotoxic beta amyloid protein (Aβ), which is derived from a larger amyloid precursor protein (APP). APP can be processed proteolytically *via* alternative pathways: cleavages at the N and C terminal of Aβ domain by β- and γ-secretases, respectively. This leads to the formation of the Aβ peptide, and cleavage in the α-secretase pathway, also occurs within the sequence of Aβ. In addition, the α-secretase pathway cleavage occurs within the sequence of Aβ peptide and generates a large, secreted form of soluble amyloid precursor protein alpha (sAPPα) which has been shown to have neuroprotective and neurotrophic activities. Thus precluding the formation of the toxic amyloidogenic Aβ [31–34]. Proteolytic processing of APP can be regulated by various neurotransmitters, growth factors, cytokines and hormones. Indeed, the potential importance of regulated cleavage of APP was previously indicated by the ability of acetylcholine, a critical neurotransmitter altered in AD and working through muscarinic receptors, to stimulate APP cleavage [35,36]. The loss of the basal forebrain cholinergic system is one of the most significant aspects of neurodegeneration in the brains of AD patients, and it is thought to play a central role in producing cognitive impairments. Therefore, AD drug development strategies have focused on the augmentation of cholinergic transmission as a means of restoring cognitive function in these patients. Indeed, two classes of agents, cholinergic agonists and acetylcholinesterase inhibitors (AChEIs), have shown efficacy in treating the symptoms of AD. In addition to the inhibition of ChE, some, but not all AChEI drugs were found to alter the metabolism of APP to sAPPα, which might in turn affect the process of toxic Aβ deposition (Cf [34]). Recently, we have examined the APP processing activities of rasagiline and its ChEI derivatives, TV3326 and TV3279. These drugs were developed from pharmacophore of rasagiline to combine the neuroprotective activity of rasagiline [20,37] with carbamate ChE inhibitory moiety of rivastigmine, an established anti-Alzheimer drug [38,39]. TV3326 is both ChE and brain selective MAO-A and -B inhibitor, while its *S*-isomer, TV3279, is only ChE inhibitor [39]. The two AChEI derivatives of rasagiline [40] were shown to processes APP to sPPα *via* MAP kinase and PKC dependent activation of α-secretase [35] (Fig. 3). Rasagiline, which has no cholinergic activity, also significantly stimulates the release of the non-amyloidogenic α-secretase form of soluble APP (sAPP) from both SH-SY5Y and PC-12 cells, and reduces full length APP in rat and mice hippocampus [41,42]. Rasagiline is significantly more potent than other propargylamines and this effect was mediated *via* α-secretase activity. Structure–activity have clearly shown that α-secretase dependent processing of APP is associated with the propargylamine moiety of these drugs, since propargylamine itself has similar action, but not aminoindan, the metabolite of rasagiline. Using several signal transduction inhibitors, we have shown that PKC-, mitogen-activated protein (MAP) kinase- and tyrosine kinase-dependent pathways are involved in the effect of rasagiline and its ChEI derivatives TV3326 and TV3279 on the enhancement of sAPP release. In addition all three drugs induced the phosphorylation of p44 and p42 MAP kinase and their effect was abolished by PD98059 and U0126, specific inhibitors of MAP kinase activation (Fig. 3). Furthermore they activate and initiate the translocation of PKCα and ε both in SH-SY5Y and *in vivo* in rat and mice hippocampus [42]. The generation of sAPP precludes the formation of amyloidogenic derivatives. The fact that these novel neuroprotective drugs can stimulate the non-amyloidogenic α-secretase pathway, though the expression of Bcl-2 family of proteins and PKC activation both *in vitro* and *in vivo* suggests that they may influence the basic pathogenic mechanisms underlying AD and could be of clinical importance for the treatment of the PD dementia and AD. Furthermore since rasagiline does not possess any known cholinergic activity, the APP processing attributed to some but not all cholinergic drugs by other investigator so far may not be correct. It may however depend on the ability of such drugs to activate the PKC-MAP kinase pathway. Thus, non-cholinergic neuroprotective-antiapoptotic drugs, such as rasagiline, which can activate PKC-MAP kinase may also have therapeutic value in the treatment of AD.

5. Discussion

The synthesis of rasagiline and related aminoindan propargylamine derivatives has made it possible to assess the relationship between MAO-B inhibition and their neuroprotective activities in several models. Rasagiline has been shown to possess neuroprotective and antiapoptotic activities in neuronal cell cultures and *in vivo* and to be superior to selegiline in its activity and also because of the lack of L-amphetamine (the major metabolite of selegiline) metabolites, which has been shown by Tatton *et al.* [43] and Abu-Raya *et al.* [44] to interfere with neuroprotective activity of selegiline and rasagiline *in vitro*. Indeed we and others have clearly shown that L-amphetamine metabolite of selegiline interferes with neuroprotective activity

of rasagiline. But the aminoindan metabolite of rasagiline is not only devoid of this property, but itself may have some neuroprotective activity [17,29]. This may be one reason why in contrast to selegiline, the side effects of rasagiline in anti-Parkinson trials were no worse than placebo [7].

By comparing the neuroprotective property of rasagiline, with that of its *S*-isomer, TVP1022, which is at least 1000-fold weaker as an inhibitor of MAO, we have been able to demonstrate that MAO-B inhibition is *not* a prerequisite for their neuroprotective activity. However, all the aminoindan derivatives of rasagiline [20], which contain the propargyl moiety, whether they are MAO inhibitors or not, but not aminoindan itself, exhibit neuroprotective and antiapoptotic activities. Furthermore they release PKC–MAP kinase dependent release of neuroprotective-neurotrophic sAPPα in PC-12 and SH-SY5Y cells (Fig. 3). These pharmacological actions have been demonstrated to be associated with the propargylamine moiety on these drugs, since propargylamine itself has similar action [38]. It is apparent that rasagiline's antiapoptotic activity is dependent on several factor including induction of Bcl-2 family proteins, inhibition of the translocation of proapoptotic GAPDH and activation of proteasome–ubiquitin system in dopamine-derived neuroblastoma SH-SY5Y cells [27], which results in the modulation of mitochondrial permeability pore and prevention of VDAC opening [45]. In this regard the pharmacological actions of rasagiline and its derivatives resembles that of Bcl-2 over expression in such cells [11,12,28]. However, other factors may also be involved. Consideration should also be given to the ability of rasagiline to increase brain (striatum, cortex and hippocampus) SOD, and catalase. Over-expression of Bcl-2 and SOD in mice and PC-12 and SH-SY5Y cells has been shown to make them resistant to neurotoxin-induced neurodegeneration. Thus the mechanism of neuroprotection induced by propargylamines, such as rasagiline, involves a complex set of neurochemical events and may involve other factors besides those so far discussed. Indeed, our recent studies with rasagiline employing cDNA microarray gene expression have identified several other previously unknown gene pathways including over expression of AKT1 and 2, hexokinase and synaptophysin and down regulation of NFκB, glutamate and AMPA receptors, and Huntington.

The observation that rasagiline and its derivatives upregulate the expression of antiapoptotic Bcl-2 family proteins (Fig. 2) and plasticity dependent PKC and MAP-kinase (Fig. 3) is indicative that these drugs either possess two separate pharmacological actions or that there is a close interaction between them. Our present efforts are directed at determining the influence of each on the other, even though it has previously been demonstrated that PKC activation has antiapoptotic activity. Future neuroprotective drug development should consider compounds similar to rasagiline, which interact with the proteins of MPT pore. These include adenosine nucleotide translocator, PBR and creatine kinase, which have been shown to play crucial role in MTP function. Whether rasagiline interacts directly with

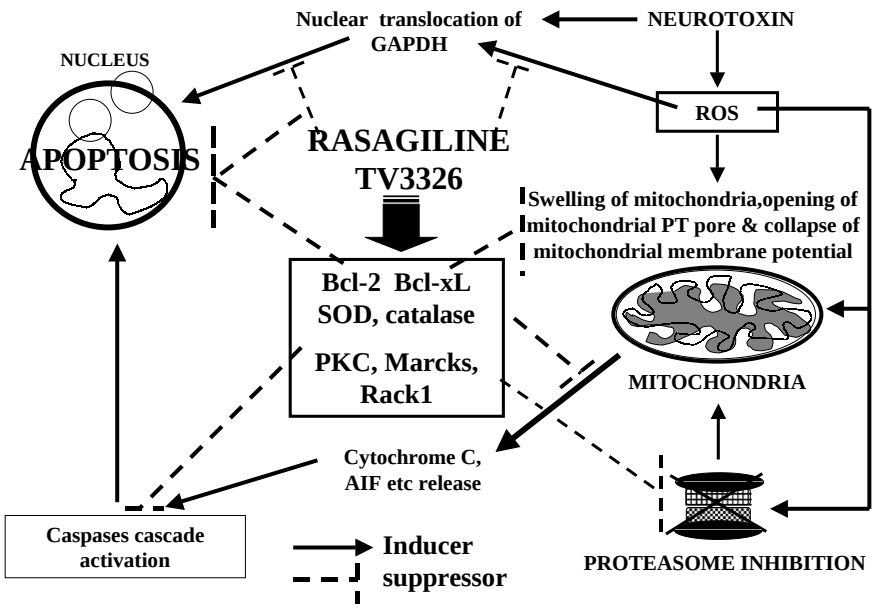

Fig. 2. Mechanism of neuroprotective-antiapoptotic action of rasagiline and its anti-Alzheimer derivative ChE-MAO inhibitor, TV3326. Both drugs are *N*-propargyl-1-(*R*)-aminoindan derivatives with TV3326 possessing a carbamate ChEI moiety. It is the propargyl moiety in these drugs that confers the neuroprotective-antiapoptotic, Bcl-2 inducing activities and PKC activating properties. Rasagiline inhibits neurotoxin (SIN-1, *N*-M-(*R*)-Sal) initiated apoptosis in SH-SY5Y and PC-12 cells by preventing the collapse of mitochondrial membrane potential, opening of the MPT, release of ubiquitin–proteasome dependent cytochrome *c* and caspase 3 activation resulting in its antiapoptotic activity. It also prevents the translocation of pro-apoptotic GAPDH in these cells. Its neuroprotective activity may also depend on its activation of SOD and catalase as has been observed *in vivo* in various tissues including brain and heart [10–13,24,41].

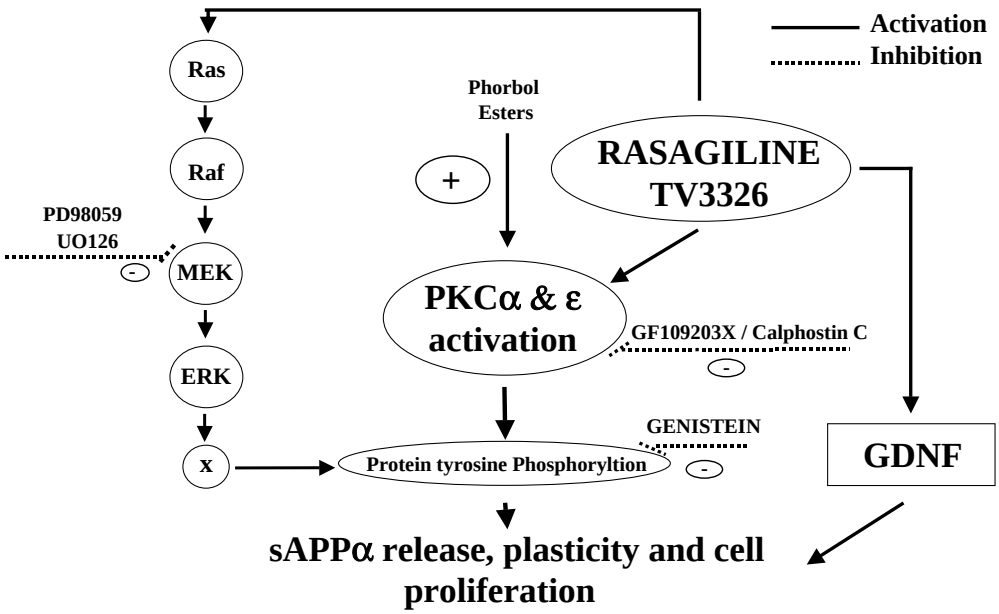

Fig. 3. Signal transduction pathways mediating the activation of PKC dependent neuroprotection and plasticity by rasagiline and TV3326. Both drugs activate PKC and MAPK pathways in a time and concentration (0.1–10 μM) dependent manner in PC-12 and SH-SY5Y cells in culture, resulting in activation of α-secretase dependent release of sAPPα. Phorbol esters have similar action and inhibitors of PKC and MAPK pathways, as indicated, prevent rasagiline and TV3326 induced release of sAPPα. *In vivo* both drugs activate mice and rat hippocampal PKCα and ε promote their translocation from cytoplasm to the mitochondrial membrane [40–42,46].

these proteins or at some site further upstream with sinal transduction is now being investigated.

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