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RESEARCH PAPER

Nanoformulated Mutant SurR9-C84A: a Possible Key for Alzheimer's and its Associated Inflammation

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ABSTRACT

Purpose Alzheimer's disease (AD) is one of the untreatable neurodegenerative diseases characterised by the pathologic amyloid plaque deposition and inflammation. The aim of this study is to evaluate the neuroprotective effects of nanoformulated SurR9-C84A, a survivin mutant belonging to the inhibitors of the apoptosis (IAP) protein family. The effect of SurR9-C84A was studied against the β-amyloid toxicity and various inflammatory insults in the differentiated SK-N-SH neurons.

Method SurR9-C84A loaded poly(lactic-co-glycolic acid) nanoparticles were prepared following the modified double emulsion technique. The neuroprotective effect of SurR9-C84A was evaluated against the amyloid-β (Aβ) peptide fragment, N-methyl-D-aspartate (NMDA) toxicity and the inflammatory assaults. To mimic the *in vivo* situation, a co-culture of neurons and microglia was also studied to validate these results.

Results SurR9-C84A treatments showed improved neuronal health following Aβ, and NMDA toxicity in addition to inflammatory insults induced in mono and co-cultures. The neuroprotective effect was evident with the reduced neuronal death, accelerated expression of neuronal integrity markers (neurofilaments, beta-tubulin III *etc.*,) and the neuroprotective ERK/MAPK signalling.

Conclusion The current results demonstrated that the SurR9-C84A nanoformulation was very effective in rescuing the neurons and holds a potential future application against AD.

KEY WORDS Alzheimer's disease · inflammation · neurodegeneration · SurR9-C84A and β-amyloid

ABBREVIATIONS

AD	Alzheimer's disease
Aβ	Amyloid β fragment
ICC	Immunocytochemistry
IGF	Insulin like growth factor-1
LDH	Lactate dehydrogenase
MBP	Myelin basic protein
NF	Neurofilament
NGF	Nerve growth factor
NSE	Neuron specific enolase
PLGA	Poly (D,L-lactide-co-glycolide)
PMA	Phorbol-12-myristate-13-acetate

INTRODUCTION

Inflammation is a key prognostic feature for many of the neurological disorders. Being postmitotic cells, neurons are particularly susceptible to the inflammation mediated damage both in acute and chronic disease states. A wide variety of central nervous system disorders like the brain infections, ischemia, trauma and neurodegeneration *etc.* involve the inflammation mediated activation of astrocytes and/or microglial cells (1). Alzheimer's disease (AD) is a typical progressive neurodegenerative disorder characterised by the presence of β-amyloid (Aβ) plaques and neurofibrillary tangles. The senile plaques are formed by the unusual enzymatic cleavage of the amyloid precursor protein (APP) resulting in the generation of Aβ monomers. Following this, the monomers polymerise non-covalently yielding the Aβ-peptides. The other pathological component of AD, neurofibrillary tangles are a result of abnormal phosphorylation of the

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microtubule associated tau protein (1). Several studies have reported that the lesions specific to AD are typically characterised by the presence of mediators similar to those observed in inflammation (1–3). Thus, inflammation can be regarded as a key initiator or a later consequence of AD. In particular, all the neurodegenerative diseases complain the close proximity of reactive glia especially the microglial activation and suffer the inflammatory insult. Astrocytes represent the principal class of neuroglial cells, but they turn active and become phagocytic in the state of neurodegeneration (4). Following this, the disease state is further worsened as they mediate and culminate inflammation secreting cytokines, chemokines and other effector molecules (5). Supportive evidence does exist on the inflammation induced neurotoxicity mediated by the classical microglial activation.

For instance, the serum of AD patients were found to have elevated TNF-α, (1) while some studies observed that the inherent Aβ peptide secreted inflammatory components, TNF-α and interleukin-6 (IL-6) (6). The microglial cells were significantly influenced to secrete these prime inflammatory components including nitric oxide by these Aβ plaques (7,8). Previously, it was also reported that IL-1β a critical pro-inflammatory neurotoxic component is secreted by the activated microglia, which in turn can initiate the stimulation of astrocytes and the inflammatory cascade. Few other studies reported that by any means activation of microglia result in neurodegeneration due to the secreted pro-inflammatory and neurotoxic factors (9,10). In addition, AD is characterised by the deposition of neuritic plaques composing β-amyloid fibrils surrounded by activated microglial cells. *In vitro* studies have evidenced that these microglial cells when exposed to β-amyloid fibrils display an activated phenotype which is characterised by the secretions of cytokines, chemokines and other neurotoxic mediators. THP-1 cell line, a commonly used human microglial model is used for the entire experiments in this study (11,12).

The extended understanding of inflammation and its role in AD provoked us to simulate the inflammatory condition *in vitro* and evaluate the therapeutic potential of SurR9-C84A in protecting the neurons. Thus to conclude, neurotoxicity is inevitable due to the synergistic influence of inflammatory mediators on astrocytes and *vice versa* in the event of inflammation. Several approaches have been tried to study the interactions of neurons and microglial cells *in vitro*. In our study, we tried to demonstrate the neuroprotective activity of SurR9-C84A in AD and for this, differentiated SK-N-SH cells were incubated with β-amyloid fragments and the protective effect of SurR9-C84A was tested.

In addition, we tried to mimic the complete *in vivo* AD inflammatory condition by developing the co-culture model of activated macrophages and the neurons. Also, the supernatants from activated macrophages were incubated with the SK-N-SH cells to study the effect of secreted cytokines and other effector molecules.

MATERIAL AND METHODS

The endotoxin Lipopolysaccharides (LPS) from *Escherichia coli* 055:B5, Phorbol-12-myristate-13-acetate (PMA), ascorbic acid, amyloid-β protein fragment 1-40, retinoic acid, Poly (D,L-lactide-co-glycolide) RESOMER® RG 503 H (PLGA 50:50, inherent viscosity 0.32–0.44 dL/g, Mw 24,000–38,000), poly (vinyl alcohol) (Mw 13,000–23,000, 87–89% hydrolysed) were all obtained from Sigma, Australia. The void and SurR9-C84A loaded nanoparticles used in the study were prepared according to the protocol described in supplementary data (section "Introduction"). Cell lines used in the study, SK-N-SH, and THP-1 were obtained from American type culture collection (ATCC). SK-N-SH cells were grown in Eagle's minimum essential medium (EMEM) and THP-1 cells were maintained in Rosewell park memorial institute (RPMI) 1640 medium supplemented with 10% heat inactivated fetal bovine serum and 2 μM mercaptoethanol.

Stimulation of Microglial and Neuroglial Cells for Cytokine Secretion

Refer supplementary methods section 2.

In vitro Neuroprotective Effect Against Inflammation

Refer supplementary methods section 3.

SurR9-C84A Against the N-methyl D-aspartate (NMDA) Induced Toxicity

Refer supplementary methods section 4.

Neuronal and Microglial Cell Co-Culture

Refer supplementary methods section 5.

Statistical Analysis

The ANOVA with Tukey's post hoc test was employed for calculating the statistical difference and data with $*P<0.05$, $**P<0.01$ and $***P<0.001$ was considered as statistically significant.

RESULTS

In vitro Neuroprotective Effect Against Inflammation

SurR9-C84A Against the LPS and β-amyloid Stimulated THP-1 Secretions

The initial neuroprotector activity of SurR9-C84A was evaluated in differentiated SK-N-SH neurons post inflammatory insult induced by the LPS and β-amyloid stimulated THP secretions. As observed, the LPS stimulated THP secretions induced 16.3% cell death while the inactive/unstimulated THP secretions were negligibly effective in inducing the neuronal death and accounted to only 1% confirming the successful microglial activation. Triton X-100 included in the study served as a negative control and considered inducing 100% cell death and the corresponding cytotoxicity was calculated compared to it. The treatment controls pre-treated with indomethacin, IGF, NGF (positive controls) and the SurR9-C84A were found to be effective in inhibiting the neuronal death

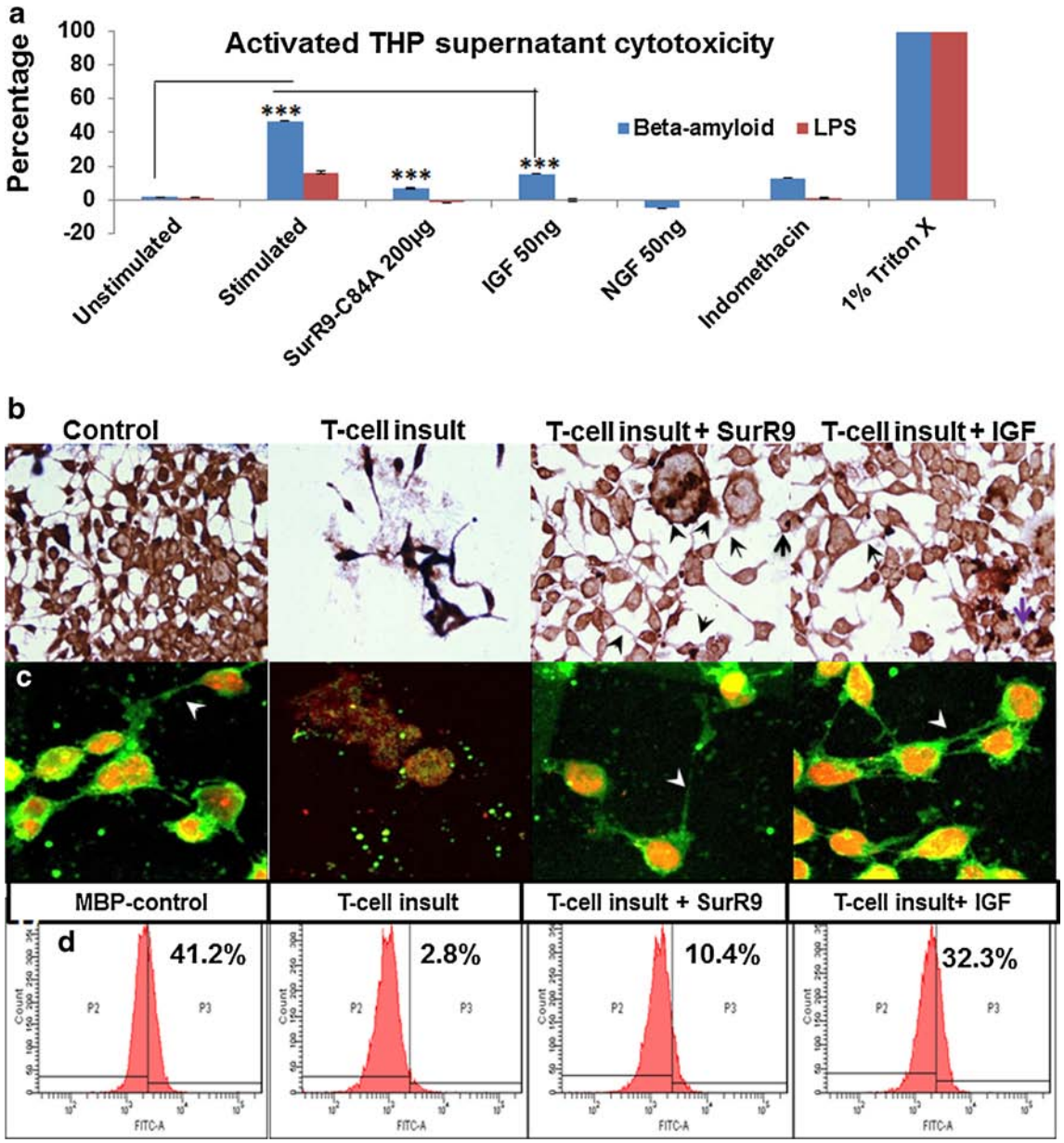


Fig. 1 Neuroprotective activity of SurR9-C84A against the LPS and β-amyloid stimulated THP-1 secretions. (**a**) β-amyloid and LPS stimulated THP-1 cytotoxicity and protective effects of SurR9-C84A were determined from the three independent set of experiments and ***$P < 0.001$ is considered statistically significant. (**b**) Immunocytochemistry performed for myelin basic protein; scale bar = 50 μm. (**c**) Immunofluorescence study showing the SurR9-C84A induced restoration of MBP expression post LPS stimulated T-cell insults. Pre-treatment with SurR9-C84A restored the neuronal integrity compared to the positive control IGF-1. All the images were taken at 40× objective and scale bar = 50 μm. (**d**) Flowcytometry analysis for confirming the expression of neuronal integrity marker, MBP post SurR9-C84A/LPS stimulated insult.

with a percentage reduction to 1.1, −0.52, −0.5 and −1.8% respectively (Fig. 1a). Interestingly, the percentage neuronal toxicity increased to 46.8% ($P<0.001$) with the β-amyloid stimulated THP secretions compared to 16.3% with LPS stimulation while the inactive/unstimulated THP secretions were able to induce 12.7% cell death. However, following pre-treatments with indomethacin, IGF, NGF and the SurR9-C84A cytotoxicity was reduced to 10.3, 15.1, −4.9 and 8.3% respectively (Fig. 1a, $P<0.001$). Interestingly, pre-treatment with SurR9-C84A 200 μg prior to activation with LPS reduced the NO levels to 15.4% (Fig. S1G). In addition, the neuronal integrity marker, myelin basic protein (MBP) expression was studied using the qualitative immunocyto-chemistry (Fig. 1b) and immunofluorescence (Fig. 1c) studies. Further, flowcytometry results showed a quantitative 3.6 folds increment in the expression of (MBP) following SurR9-C84A treatment compared to the insult control (Fig. 1d). Thus, pre-liminary results gave an initial clue to proceed and evaluate the neuroprotective potential of SurR9-C84A.

SurR9-C84A Against the β-amyloid Toxicity

In order to determine the neuroprotector potential of SurR9-C84A, the neurons were directly subjected to the β-amyloid insult with or without SurR9-C84A, IGF, NGF and indo-methacin pre-treatments. 1% Triton-X-100 was calculated to be 100% cytotoxic and corresponding to it β-amyloid only treated control showed 79% cell death while the pre-treatments with SurR9-C84A, IGF, NGF and indomethacin reduced the neuronal death to 13.2, −2.4, −4.5 and 18.6% respectively. Thus, SurR9-C84A treatment showed a 6 fold increased protection towards the neurons compared to the β-amyloid only treated group (Fig. 2a). Next the gene expression studies were performed to elucidate the characteristic neuroprotector mechanism of SurR9-C84A and its ability to surmount the β-amyloid induced cytotoxicity. Quantitative RT-PCR was performed sequentially for the inflammatory, apoptotic, neuronal integrity markers, Alzheimer's related genes and ERK/MAPK genes (Table S1). In the case of

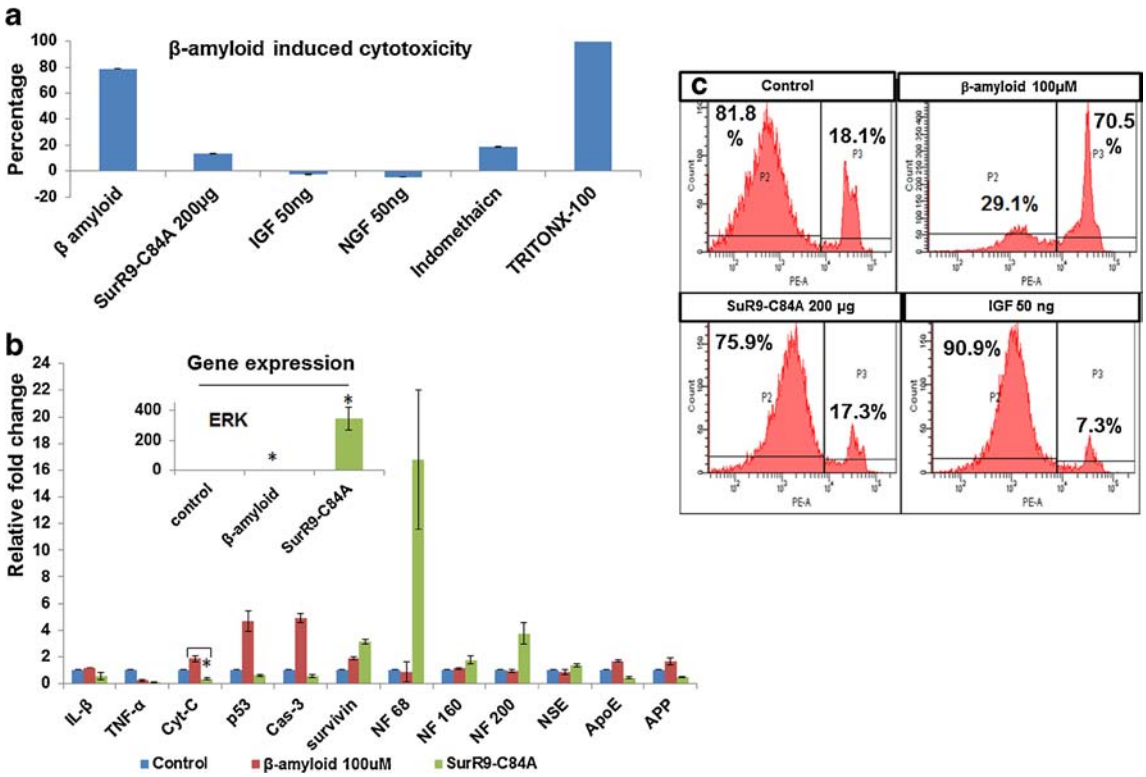


Fig. 2 Neuroprotective actions of SurR9-C84A against β-amyloid induced toxicity. (**a**) SK-N-SH cells were pre-treated with 200 μg/ml of SurR9-C84A along with positive controls IGF, NGF and indomethacin for 24 h followed by treatment with β-amyloid for 24 h. Pre-treatment with SurR9-C84A proved to be effective in lowering the cytotoxicity. The % cytotoxicity was determined using LDH assay and data are representative of at least three independent experiments and expressed as avg ± SD. (**b**) Gene expression study post SurR9-C84A/Aβ insult in the neurons. SurR9-C84A showed reduced expression of inflammatory (TNF-α and IL-β), apoptotic (Cyt-c, p53 and Cas-3) genes with an increase in the survival and integrity markers (survivin, NF 68, NF 160, NF 200 and NSE). Alzheimer related genes ApoE and APP were considerably reduced and the gene responsible for neuroprotective signalling ERK was significantly increased, $P<0.05$. The relative expression of all the genes was calculated relative to β-actin that served as an internal control. Lanes 1–3 are β-amyloid only treated control, void NPs and SurR9-C84A 200 μg respectively. (**c**) PI staining analysis post SurR9-C84A/Aβ insult. SurR9-C84A showed a considerable decrease in the percentage PI positive cells (17.3%) compared to the untreated Aβ only treated control (70.5%) confirming its potential in restoring the neuron health. Data shown are the result of three independent set of experiments and represented as avg ± SD. *$P<0.05$ is considered statistically significant.

inflammatory genes, pro-inflammatory IL- β and TNF-α showed 1.9 and 7.8 fold reduced expression respectively.

Apoptotic markers Cyt-C, p53 and Cas-3 were reduced by 2.8 ($P<0.05$), 1.7 and 1.9 folds while the neuronal integrity markers endogenous survivin, NF 68, 160, 200 and β tubulin III showed a corresponding increment of 3.1, 16.7, 1.7, 3.7 and 1.3 folds respectively. The Alzheimer's related genes ApoE and APP were respectively reduced by 2.4 and 2 folds while the ERK showed a massive increase of 345 folds ($P<0.05$) suggesting its role in the neuroprotection mechanism exhibited by SurR9-C84A (Fig. 2b). Added to this β-amyloid induced toxicity and the counteracting effect of SurR9-C84A was further confirmed with the PI staining using flow cytometry analysis.

Apoptotic cells tend to loose significant amount of the DNA content on account of the fragmentation and PI staining being specific to the DNA gives a clear picture of the percentage dead cells by quantifying the PI positive cells (13). As observed from the (Fig. 2c), a significant amount of dead cells (70.5% PI positive cells) were observed upon incubation with β-amyloid while the SurR9-C84A pre-treatment ameliorated the cytotoxic effects displaying an enhanced neuroprotector activity. The % PI positive cells were reduced to 17.3 while the PI negative cells were increased to 76% confirming the neuron protector ability. This positive effect of SurR9-C84A was compared against the positive control IGF which showed 91% of PI negative cells (Fig. 2c).

Protein Expression Through Flow Cytometry

In order to confirm the neuroprotector activity of SurR9-C84A against the β-amyloid insult, flow cytometry was performed for the expression of Cyt-C, Cas-3, Bcl-2 and thereby determining the anti-apoptotic activity. The results showed a significant reduction of Cyt-C and Cas-3 to 1.3 and 57.6% respectively, while the Bcl-2 expression increased to 95.2%.

The analysis for neuronal integrity marker expression (NF 68, 160, 200 and neuron specific enolase (NSE)) confirmed the ability of SurR9-C84A to maintain the neuronal integrity with a drastic increase to 63, 55.4, 99.4 and 99% respectively. Further to this, ERK/MAPK analysis showed a 96.3% increase compared to 43% of β-amyloid only treatment comprising of 2.2 fold increase. Thus, this significant increment in ERK expression revealed the strategic mechanism of SurR9-C84A involving the ERK pathway in protecting the neurons from the β-amyloid toxicity (Fig. 3a, b). Table S2, gives a clear picture of SurR9-C84A effect post β-amyloid insult.

Cytokine and Apoptotic Array Analysis

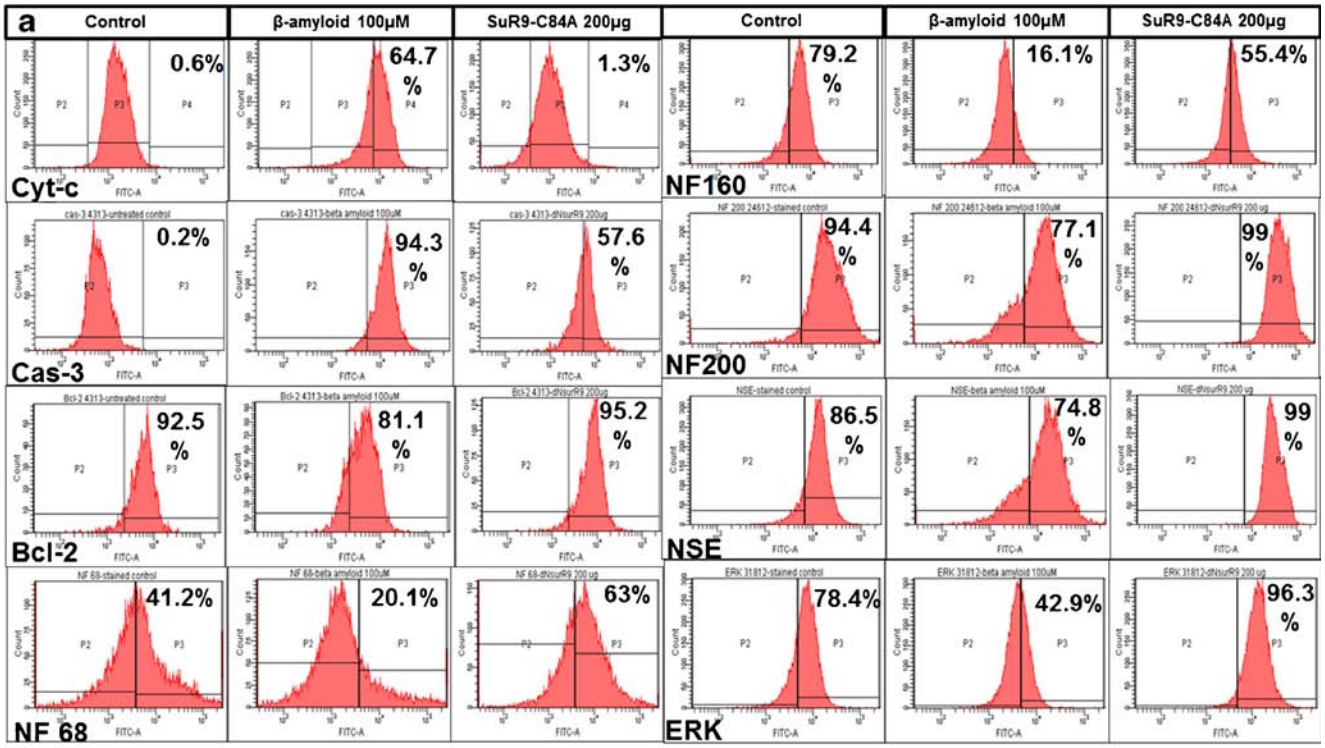
In addition, to confirm the enhanced neuroprotective effect of SurR9-C84A, cytokine and apoptotic array analysis was carried where in SurR9-C84A treatment exhibited reduced level of all the cytokine secretions and apoptotic markers post β-amyloid insults in mono and co-cultures. As observed, it is evident that pre-treatment with SurR9-C84A not only reduced the inflammatory cytokines (cytokine array Fig. 4a–d) but also rescued the neurons from the toxic effects of the cytokines (apoptotic array Fig. 4f–h). Western blot was also performed to confirm this and analysed for the expression of wild type survivin, β tubulin III, proliferating cell nuclear antigen (PCNA) and ERK. All of them showed an enhanced expression in the SurR9-C84A/β-amyloid treatment group compared to the β-amyloid only treated control (Fig. 4e). Thus, it confirms the neuroprotective actions of SurR9-C84A against the β-amyloid induced insult with a possible involvement of ERK signalling pathway as described in Fig. 2b. All the above protein expression was compared against β-actin as the internal standards.

Finally, in support of the neuroprotective effect exhibited by SurR9-C84A, the DNA fragmentation a classical hallmark of apoptosis was analysed. Substantiating the results obtained earlier, the β-amyloid only treated group showed complete DNA fragmentation indicating the severe toxicity and even the void NP treated group showed the same result. Interestingly, SurR9-C84A devoid of the nanoformulation also didn't show any protective effect on the DNA and this is explained because of its shorter half-life and reduced bioavailability in the native form. Supporting this hypothesis the nanoformulated SurR9-C84A showed an enhanced protective effect on account of its improved availability and half-life (Fig. 4i).

Neuronal and Microglial Cell Co-Culture

Percentage Cytotoxicity and Nitrite Measurement

A co-culture model of activated THP-1 and differentiated SK-N-SH neurons was established to closely mimic the *in vivo* neuro-inflammation condition prevailing in AD (Fig. 5a). As observed from the Fig. 5c, post co-culturing for 5 days β-amyloid stimulated THP cells showed high degree cytotoxicity accounting to 89% and while the unstimulated THP cells showed only 20% cell death. A β-amyloid only treated group was also included in the study showing 49% cell death confirming the fact that THP cells turned aggressive after activating with β-amyloid in inducing the neuronal death. Considering the neuroprotective activity of SurR9-C84A, the percentage cell death was significantly reduced to 21% while the other treatments, IGF and NGF showed only 8 and 11% cytotoxicity. On the whole, the neuroprotective actions of SurR9-C84A were in agreement with the previous results and were effective both in mono and co-cultures (Fig. 5a–c).



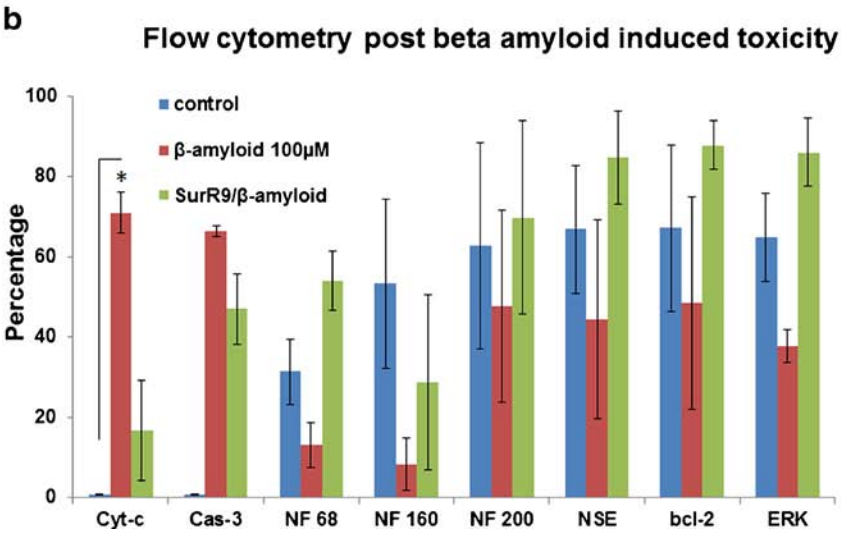


Fig. 3 Flowcytometry analysis for protein expression in the neurons following SurR9-C84A/Aβ treatments. (**a**) SurR9-C84A pre-treatment confirmed the reduced expression of apoptotic markers (Cyt-C and Cas-3) along with an increased expression of neuro-integrity markers (NF 68, 160, 200, NSE). The neuroprotective signalling molecule ERK was also elevated confirming the neuroprotective potential of SurR9-C84A. (**b**) Histogram showing the flowcytometry analysis, $^*P < 0.05$ is considered statistically significant.

Flowcytometry and Confocal Microscopy for NF 200 Expression Post Co-Culture

Finally, to authenticate the neuroprotective actions of SurR9-C84A the co-cultured cells were studied for the neuronal marker NF 200 expression with the confocal microscopy. SurR9-C84A pre-treatment retained 61% of it ($P<0.001$), while the untreated activated THP and SK-N-SH showed only 7% expression (Fig. 5d, e). Finally, flowcytometry was performed to validate the expression of NF 200 and CD 163 (a macrophage specific marker) in activated THP-1 cells.

As observed (Fig. 5f), untreated SK-N-SH only cells expressed 58.5% of NF 200 while the co-cultured inactive THP and SK-N-SH showed negligible reduction to 46%. A substantial reduction to 6% was noticed in activated THP and SK-N-SH group while the pre-treatment with SurR9-C84A and IGF showed a concrete increase in the expression corresponding to 64 and 79% respectively.

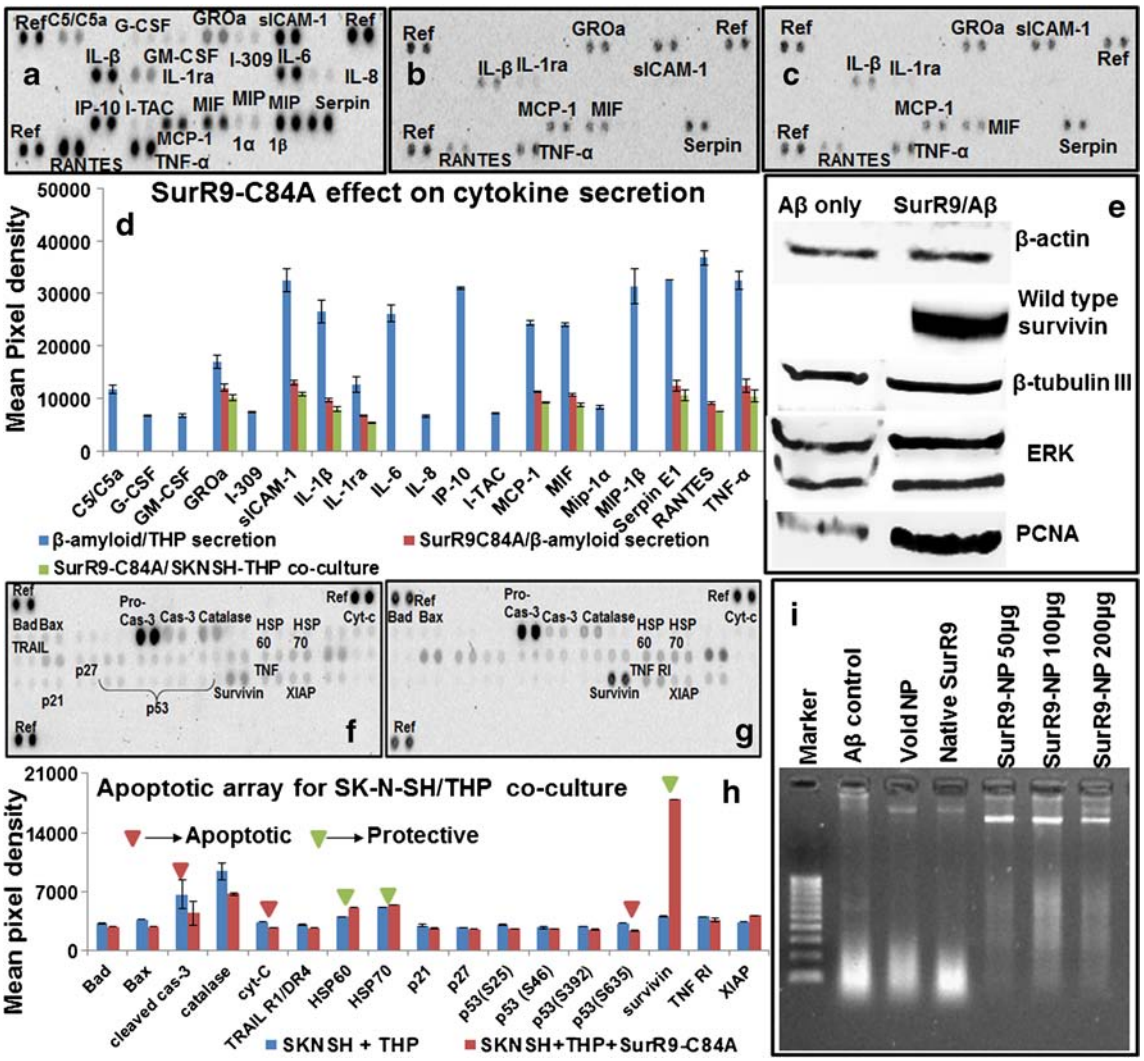
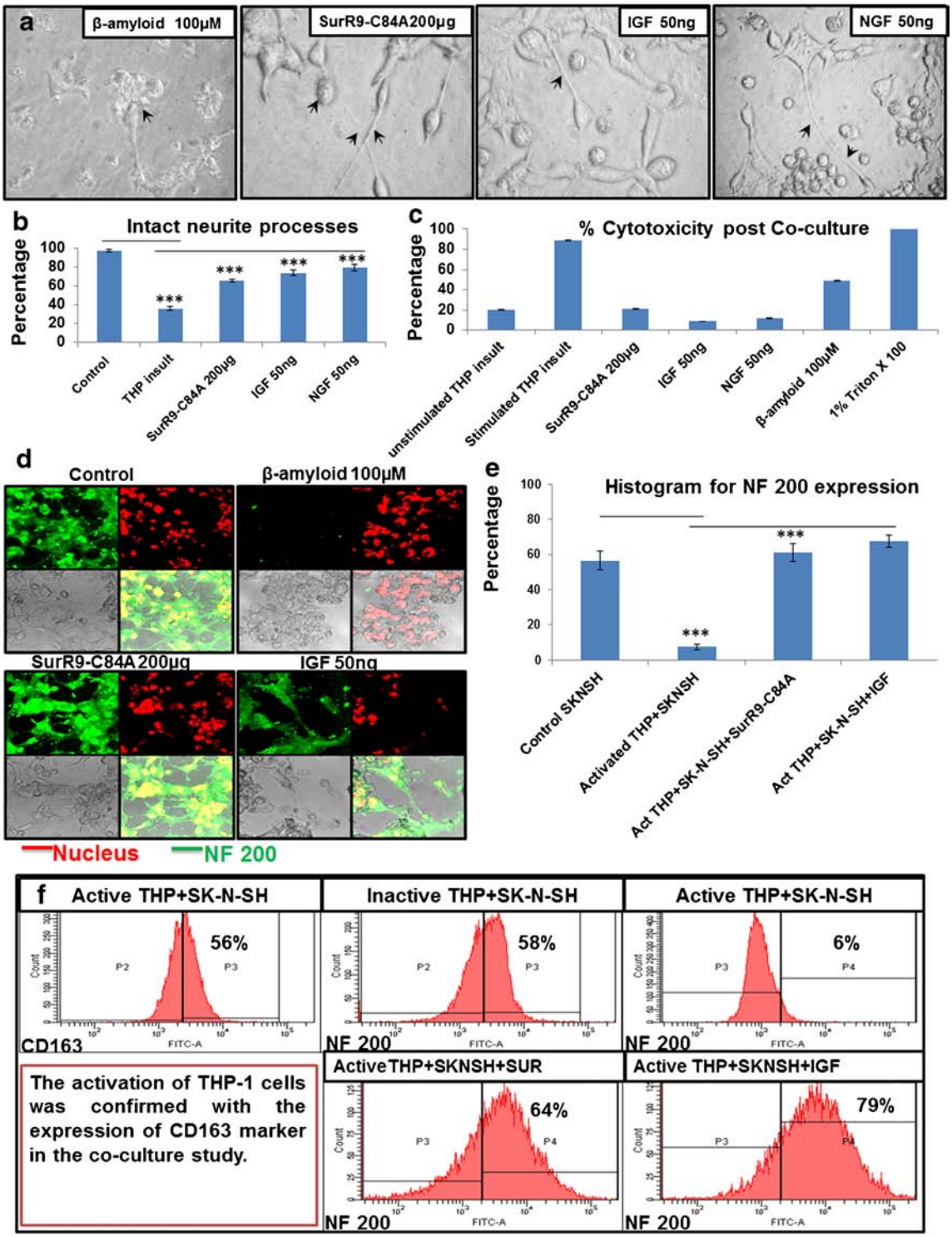


Fig. 4 Protein expression study following SurR9-C84A/Aβ treatments. (**a**) Cytokine array for Aβ stimulated THP-1 secretion. (**b**) SK-N-SH cells were pre-treated with SurR9-C84A followed by Aβ stimulated THP secretion and then the supernatants were evaluated for cytokine expression. (**c**) SK-N-SH cells were pre-treated with SurR9-C84A followed by co-culture with the Aβ stimulated THP-1 cells for 5 days. Then the supernatants were evaluated for cytokine expression. (**d**) Histogram showing the reduced cytokine expression following SurR9-C84A treatments in SK-N-SH and Aβ activated THP co-culture. (**e**) Western blot analysis confirming the expression of survival (survivin), proliferative (PCNA) and neuronal integrity markers post SurR9-C84A treatment compared to the Aβ only treated controls. Lane 1 is the Aβ only treatment control and lane 2 represents the SurR9-C84A/Aβ treatment. Apoptotic array for: (**f**) SK-N-SH treated with Aβ stimulated THP secretion. (**g**) SK-N-SH treated with SurR9-C84A/Aβ stimulated THP secretion. (**h**) Histogram confirming the reduced expression of apoptotic markers with SurR9-C84A compared to Aβ THP treatment. *Arrow heads* showing reduced apoptotic and increased survival protein expression post SurR9-C84A treatments. (**i**) DNA fragmentation was evident with the β amyloid induced insult while treatments with SurR9-C84A (starting from 50–200 μg only in nanoformulated form) served to maintain the integrity of the DNA post insult. Data shown are the representative of three independent set of experiments

DISCUSSION

Neuroglial cells comprising of micro and macroglia play a diverse role in the CNS and are implicated in the inflammation and developing brain circuits respectively. Microglia represents the resident macrophages while the macroglia encompasses the oligodendrocytes and astrocytes. Astrocytes in majority participate in maintaining and modulating the intrinsic brain physiological processes but turn aberrant during neuronal insult and secrete inflammatory cytokines and chemokines. The pro-inflammatory TNF-α and IL-β are predominantly secreted by them explaining the importance of astrocytes in mediating the neuronal inflammation and degeneration (14–16). It is fascinating to observe that neuropathological damage is often brain region specific and characterised by the pathological events and neuronal loss. This condition is better explained considering the presence of pro-inflammatory stimuli such as LPS and the region specific damage is attributed to the presence and function of microglia (17). The activation and execution of innate immune responses by the microglial cells is often accompanied by the astrocytes. This condition aptly describes AD pathological process where



the neuronal and neuroglial cell crosstalk leads to the malicious autocrine-paracrine interactions amplifying the neuronal inflammation and its loss (18–20). Considering the prime role of neuroglial cells in mediating the neuro-inflammation in AD we tried to mimic this pathologic condition by activating the microglial cells with the bacterial endotoxin LPS and β-amyloid fragment respectively. In the present work, we tried to evaluate the neuroprotective potential of SurR9-C84A NPs against the microglia induced inflammatory insults, β-amyloid toxicity along with the co-culture of neuronal and microglial cells.

With respect to the microglial induced insults, efforts were made closely to mimic the *in vivo* insults where THP-1 cells were stimulated with bacterial LPS and β-amyloid fragment

◀ **Fig. 5** Neuroprotective activity of SurR9-C84A post activated THP/neuronal co-culture. (**a**) Phage contrast images post SK-N-SH and Aβ stimulated THP-1 cells. *Arrow heads* indicating the neurite processes. (**b**) Histogram showing the neurons with intact neurite processes post co-culture for 5 days. SurR9-C84A treatment retained 60% of neurite processes as compared to the positive controls IGF-1 and NGF, $P < 0.001$. (**c**) Co-cultured neurons and active THPs were pre-treated with 200 μg/ml of SurR9-C84A along with positive control IGF-1 for 24 h followed by activation with β-amyloid for 24 h. Pre-treatment with SurR9-C84A proved to be effective in lowering the cytotoxicity throughout the co-culture period and was determined using LDH assay. ***$P < 0.001$ is considered statistically significant. (**d**) Immunofluorescence study for the expression of NF 200 in the neurons post co-culture period to evaluate the SurR9-C84A neuroprotector activity. Surprisingly, SurR9-C84A retained 64% of NF 200 expression compared to only 6% expression in untreated THP + SK-N-SH neurons. NF 200 expression is shown in FITC channel (*green*), nucleus is stained red with PI; scale bar = 50 μm. (**e**) Histogram for NF 200 expression post co-culture. All the images were taken at 40× objective and the histogram is representative of avg ± SD with ***$P < 0.001$ is considered statistically significant. (**f**) Flowcytometry analysis for the co-culture. CD163 expression was significantly observed in the active THPs confirming their activated stage. The inactive/unstimulated THP/SK-N-SH control showed negligible lowering of NF 200 expression while the stimulated THP/SK-N-SH co-culture showed massive reduction in the integrity marker NF 200 which corresponded to only 6%. However, NF 200 expression was restored in the co-culture post SurR9-C84A treatments which increased to 64% substantiating the previous results. Thus, SurR9-C84A was able to restore the NF 200 expression in the activated microglia-neuronal co-culture. Data shown are the result of three independent set of experiments and represented as avg ± SD.

separately and cytokine expressions were evaluated using the cytokine array kits (Fig. S1A-F). Though β-amyloid activation of THP-1 cells showed enhanced expression of cytokines, SurR9-C84A, however was effective in rescuing the neurons from cytokines as observed from Fig. 1a–d. SurR9-C84A also restored the expression of neuronal integrity marker MBP confirming its ability to protect the neurons against active T-cells (Fig. 1b–d). To mimic the *in vitro* AD model, the neurons were incubated with the β-amyloid fragment (the pathological hallmark of AD) and the neuroprotective effect of SurR9-C84A was evaluated against it. SurR9-C84A pre-treatment reduced the percentage cytotoxicity compared to β-amyloid only treated control and this accounted to a significant 6 fold increased protection inferring the neuroprotective potential.

Further to this, qRT-PCR studies revealed the down regulation of inflammatory, apoptotic and Alzheimer's related genes (Fig. 2b) along with a substantial increase in neuronal integrity markers post SurR9-C84A/β-amyloid treated groups compared to β-amyloid only treated controls. These results gave an initial clue about the protective capacity of the protein employed and supportive evidences were drawn from the results of flowcytometry and western blot analysis. Considering the PI staining analysed by flowcytometry, 70.5% of cells were dead upon treatment with β-amyloid while SurR9-C84A was able to reduce the number drastically to 17.3% only (though less than positive control, IGF employed in this study). Further analysis also supported the PCR data where apoptosis inducing Cyt-C and Cas-3 were significantly reduced while the expression of survival and neuronal integrity markers Bcl-2 and NF 68, NF 160, NF 200, NSE were increased noticeably. Western blot analysis also confirmed the above results showing a noticeably increased expression of ERK/MAPK in the SurR9-C84A/β-amyloid treated group (Fig. 4e). To conclude, ERK/MAPK signalling pathway is predicted to be involved in the neuroprotective mechanism exhibited by SurR9-C84A against the β-amyloid insult. Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinase signal transducers that have a pivotal role in modulating both the cellular events of death and survival (21,22). Of all the three MAPKs, the extracellular regulated kinase (ERK/MAPK) signalling is commonly implied in the cell survival (23). Although, few reports revealed the involvement of ERK/MAPK signalling in enhanced proliferation and apoptosis inhibition in cancer cells (24), most of the recent studies also conferred the neuroprotective effects of this pathway (25–28). As observed from the results, SurR9-C84A was able to upregulate the expression of ERK/MAPK both at the mRNA level and protein levels with no noticeable changes in untreated controls (Figs. 2b, 3a, and 4e) ($P < 0.05$).

Furthermore to ERK, the downstream effector molecule Bcl-2, a well identified anti-apoptotic factor (29–31) was also increased following the SurR9-C84A pre-treatment to 1.2 folds. Increased Bcl-2 levels further blocked the release of cytochrome-C (32) and reduced it by 50 folds that in turn affected the activation of Cas-3 that was reduced to 58% following pre-treatment with SurR9-C84A. In addition to this pathway, SurR9-C84A was also found to increase the expression of wild type survivin, a critical anti-apoptotic factor post β-amyloid insult that is in compliance with earlier results (33).

Finally, concrete evidences for the neuroprotective activity exhibited by SurR9-C84A were obtained with the expression of neuronal integrity markers NF68, 160, 200 and NSE post amyloid insult (34–36). Thus, these results confirm the neuroprotective potential of SurR9-C84A along with its ability to maintain the integrity and to regain the proliferative potential in the neurons post β-amyloid insult. Intriguing results were obtained post treatments with SurR9-C84A where activated microglia showed lowered the secretion of pro-inflammatory TNF-α and IL-β along with IL-6 and IL-8 at mRNA level. This could also be the reason behind the extended neuroprotective actions of SurR9-C84A against the cytokine rich secretions of the microglia. Pre-treatment with SurR9-C84A potentially limited the neuronal death from the cytotoxic effects of LPS, Aβ stimulated microglial toxicity (Fig. 1f). In addition, substantial results also proved the extended neuroprotective effects of SurR9-C84A where it significantly aborted the direct β-amyloid induced neuronal toxicity (Fig. 2a). The neuro-inflammatory condition prevailing in the AD brain is closely mimicked by the co-culture model developed between the

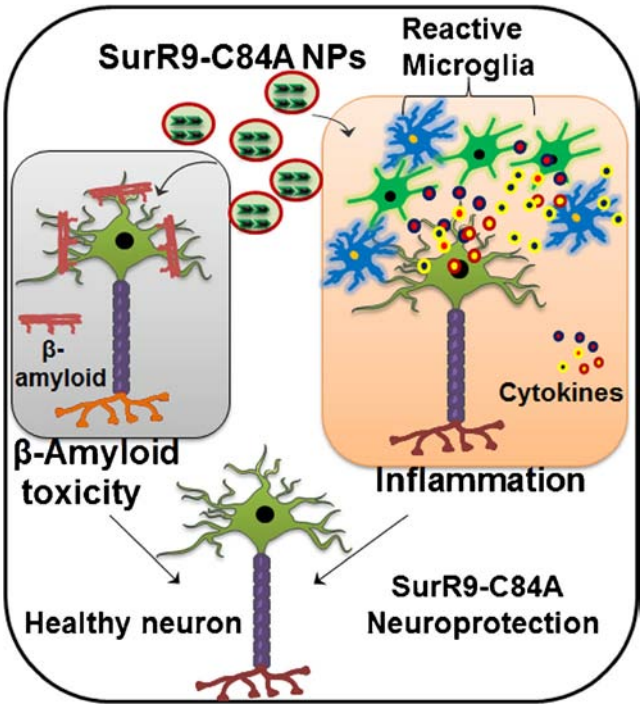


Fig. 6 Neuroprotective mechanism of SurR9-C84A NPs against the AD insults. AD is typically characterized by the presence of β-amyloid plaques and neuro-inflammation. The latter phenomenon is particularly caused by the reactive neuroglia by secreting a plethora of cytokines and chemokines. However, mimicking this situation, the nanoformulated SurR9-C84A mutant rescued the neurons against the β-amyloid toxicity and activated neuroglia mediated inflammation. It executed this function, by enhancing the neuronal integrity marker expression and simultaneously inhibiting the inflammatory secretions. Further, the nanoformulation also added to the increased bioavailability and half-life of the mutant protein and therefore it showed an enhanced efficacy.

neurons and activated microglial cells. SurR9-C84A was found to surmount the toxic effects in this instant too and also marked increased NF 200 expression (Fig. 5) $P<0.001$.

In addition to the above, excitotoxicity insult mediated by NMDA associated with AD (37) was also included and surprisingly, SurR9-C84A pre-treatment protected the neurons (Fig. S2A-S2C). This was further confirmed with the mitochondrial membrane staining where SurR9-C84A reduced the % depolarised cells corresponding to 6.6 folds (Fig. S2D). To our knowledge, this is the first report of its kind where nanoformulated SurR9-C84A has shown significant neuroprotective potential against a variety of AD related insults (Fig. 6).

CONCLUSION

To conclude, SurR9-C84A NPs exhibited significant neuroprotector activity against the β-amyloid toxicity and inflammatory secretions from neuroglial cells. In addition, it also lowered the secretion of inflammatory cytokines in the activated microglia. Therefore, the nanoformulated SurR9-C84A holds potential future application against AD both in terms of delivery and efficacy.

FUTURE PERSPECTIVE

Neuroglial cells have a vital role to play in regulating the host defence in various neurological disorders involving inflammation and other insults. In response to these insults neuroglia turn reactive secreting a plethora of inflammatory cytokines and chemokines as observed with LPS and β-amyloid in this case. Considering their potential in progressing and worsening the AD an ideal therapeutic has to be developed to alleviate the toxicity. In this instance, SurR9-C84A NPs displayed remarkable neuroprotector activity against the Aβ toxicity and the associated inflammatory component in addition to lowering the cytokine secretions in the activated microglia.

Therefore, future studies warrant the fabrication and preclinical evaluation of surface functionalized brain specific SurR9-C84A NPs against AD and its associated inflammation.

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