Melatonin and N-acetyl-serotonin offer neuroprotection in experimental models of ischemic injury

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Stroke is one of the leading causes of death worldwide. According to The World Stroke Organization, every six seconds someone somewhere will die from a stroke and one in six people will at some time have a stroke.

Central Nervous System Agent In Medicinal Chemistry. 2011. 11, 80
Current Molecular Medicine 2012. 12, 1282-1296
The long-term aim

No effective treatment has been found to prevent stroke except TPA (tissue plasminogen activator) with narrow therapeutic window. Developing novel drugs for neuroprotection from stroke is urgently needed.

The long-term aim of our Neuroapoptosis Drug Discovery Laboratory is to find new neuroprotective drugs for cure adult ischemic stroke and newborn hypoxic-ischemic (H-I) brain injury (H-I encephalopathy).
1) Ischemic Stroke
The most common type of stroke, accounting for almost 80 - 85% of all strokes, is caused by a clot or other blockage within an artery leading to the brain.

2) Intracerebral Hemorrhage (ICH)
A type of stroke caused by the sudden rupture of an artery within the brain. Blood is then released into the brain compressing brain structures.

3) Subarachnoid Hemorrhage (SAH)
Also a type of stroke caused by the sudden rupture of an artery. A SAH differs from an ICH in that the location of the rupture leads to blood filling the space surrounding the brain rather than inside of it.
CNS agents for ischemic stroke with neuroprotective mechanisms

ISCHEMIC STROKE

ANTI-OXIDANTS

ANTI-INFLAMMATORY

ANTI-EXCITOTOXIC

MULTI-ACTION AGENTS

ANTI-APOPTOSIS

HORMONAL THERAPY

COMBINED AGENTS

Humanin
Nicotinamide
EPO
Acetaminophen
Baicalin
Neural stem cells
Aminoguanidine

Estrogen
progesterone

Ethyl pyruvate & aspirin
Velcade & tPA
Urokinase & topiramate
Caspase inhibitors & MK 801
Eliprodil & rt-PA

Melanocortins
IFN-β
6-Mercaptopurine
BM derived MNC

Suramin
YM-202074
Mefenamic acid
Nortryptiline
Memantine
Valdecoxib
Felbamate
Diazepam
Resveratrol
NROX

AM 36
Tempol
Ebselen
4-HBA
GSRD
Carvedilol
IAC
PPBP
Bromocriptine

Central Nervous System Agent In Medicinal Chemistry. 2011. 11, 81-97
Melatonin

- A natural hormone secreted by the pineal gland; FDA approved drug with human safety and easily crosses Blood Brain Barrier (BBB);
- Beneficial in circadian rhythm/sleep wake cycle, cancer, aging.

Neuroprotection in neurological disorders

- Stroke
  - adult ischemic stroke
    (X Wang et al., Stroke 2009)
  - newborn hypoxic-ischemic (H-I) brain injury
- Amyotrophic lateral sclerosis (ALS)
  (Y Zhang et al., Neurobiol Dis 2013)
- Huntington’s disease (HD)
  (X Wang et al., J Neurosci 2011)
- Alzheimer’s disease (AD)
- Parkinson’s disease (PD)
  (X Wang, review, CNS Neurosci & Ther. 2009 and Taylor & Francis-CRC Press 2014)
Melatonin decreases damage in Middle Cerebral Artery Occlusion (MCAO) mice

Stroke. 2009. 40, 1877-1885
Central Nervous System Agent In Medicinal Chemistry. 2011. 11, 81-97
Melatonin inhibits NMDA-mediated cell death in PCNs while MT1 antagonist luzindole blocks this neuroprotection.
Melatonin inhibits Oxygen–Glucose Deprivation (OGD)-mediated cell death in PCNs, while MT1 antagonist luzindole blocks this neuroprotection.
Melatonin inhibits H$_2$O$_2$-mediated cell death in PHNs while MT1 antagonist luzindole blocks this neuroprotection.
Melatonin inhibits temperature shift with SDM-mediated cell death in ST14 striatal cell lines, while MT1 antagonist luzindole blocks this neuroprotection.
Melatonin inhibits H2O2-mediated PCN cell death, while MT1 antagonist luzindole blocks its role -- TUNEL staining

Control

H2O2

H2O2 + melatonin

Luzindole + H2O2 + melatonin

Stroke. 2009. 40, 1877-1885
CNS neuroscience & therapeutics 2009. 15, 345-357
Luzindole is an MT1 antagonist.

Molecular docking analysis indicates a strong π-π stacking interaction and the bending of luzindole causes a shift of the hydrogen bond contribute to luzindole’s effective antagonistic properties on the MT1 receptor.
Molecular mechanisms of MT1 agonists

Is melatonin receptors involved in the neuroprotective effect of MT1 agonists in experimental models of stroke?

We focus on melatonin receptor 1A (MT1)
MT1 is lost/reduced in apoptotic cultured neurons and MCAO mice, while melatonin restores its deficiency.

Cellular model of stroke

MCAO mouse model of ischemic stroke

Journal of Neuroscience. 2011. 31, 14496-14507
Knockdown of MT1 in cultured neurons by siRNA assay

Cellular model of stroke

MT1siRNA(1) and MT1siRNA (2) targeting MT1 expression Cells -- transiently transfected with the siRNAs using HiPerFect Transfection Reagent (Qiagen):

MT1 siRNA (1) targets sequence AACGCAATCATATACGGACTA and consists of 5’-CGCAAUCAUAUACGGACUAtt-3’ and 3’-ttGCGUUAGUAUAUGCCUGAU-5’

MT1 siRNA (2) targets sequence CGGGATCGCTATGAACCGCTA and consists of 5’-GGAUCGCUAUGAACGCUAtt-3’, and 3’-GCCCUAGC GAUACUUGGCGAU-5’
Neuroprotection by melatonin is eliminated/deleted by MT1siRNAs

Cellular model of stroke

Journal of Neuroscience. 2011. 31, 14496-14507
MT1 receptor itself is neuroprotective
Melatonin decreases damage from neonatal H-I brain injury.
Knockdown of MT1 sensitizes pups to death – Significantly increased mortality in MT1 KO mice

MT1 KO mice were subjected to unilateral carotid ligation followed by 45 mins of hypoxia.

One day, Bharati MT1 KO mice pups all died
MT1 did good thing, without MT1 Pups easily to die

Number of pups death (%)

MT1-/- mice  MT1+/- mice (wild type mice)

B. Sinha, et al., 2015. In the preparation
Molecular Mechanisms of melatonin

Melatonin offers neuroprotection through

1) inhibiting mitochondrial cell death pathway
2) activating anti-apoptotic survival signal pathway
Melatonin inhibits apoptotic death pathway and activates survival signal pathway.

CNS neuroscience & therapeutics 2009. 15, 345-357
Inhibition of apoptotic cell death pathway by melatonin

<table>
<thead>
<tr>
<th>Inhibits death pathway event</th>
<th>Diseases/Models</th>
<th>Effects of melatonin</th>
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<td>Cyto. c</td>
<td>Neurodegeneration</td>
<td>Inhibits cyto. c release from purified mitochondria</td>
<td>Mouse</td>
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<td>Stroke/MCAO</td>
<td>Decreases cyto. c release</td>
<td>Rat; Mouse, PCN</td>
<td>[18,71]</td>
<td></td>
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<tr>
<td>PD</td>
<td>Prevents cyto. c release</td>
<td>Astrocyte</td>
<td>[117]</td>
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<td>Smac/Diablo</td>
<td>HD Neurodegeneration</td>
<td>Neuroprotective in HD models</td>
<td>Mu-htt ST14A [unpublished data]</td>
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<tr>
<td>AIF</td>
<td>Stroke</td>
<td>Neuroprotective in PCN</td>
<td>PCN</td>
<td>[18]</td>
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<td>ΔΨm</td>
<td>Stroke</td>
<td>Neuroprotective in PSN and PCN</td>
<td>PSN; PCN</td>
<td>[18,71]</td>
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<tr>
<td>PD</td>
<td>Prevents ΔΨm depolarization</td>
<td>Astrocyte</td>
<td>[117]</td>
<td></td>
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<td>mtPTP</td>
<td>Stroke</td>
<td>Inhibits mtPTP in brain ischemia</td>
<td>PSN</td>
<td>[71]</td>
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<tr>
<td>PD</td>
<td>Prevents mtPTP opening</td>
<td>Astrocyte</td>
<td>[117]</td>
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<td>Bax</td>
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<td>Attenuates Aβ25-35-induced apoptosis</td>
<td>Microglial cell</td>
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<td>Bad</td>
<td>Stroke/MCAO</td>
<td>Attenuates cerebral ischemic injury</td>
<td>Rat</td>
<td>[65,78]</td>
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<td>ROS</td>
<td>PD</td>
<td>Prevents ROS formation</td>
<td>Astrocyte</td>
<td>[117]</td>
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<td>ALS</td>
<td>Reduces ROS in ALS model</td>
<td>NSC34 motoneuron</td>
<td>[1]</td>
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<td>PARP</td>
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<td>Attenuates cerebral ischemic injury</td>
<td>Rat</td>
<td>[65]</td>
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<td>Caspase-3</td>
<td>Stroke/MCAO</td>
<td>Prevents caspase-3 activation</td>
<td>Rat; Mouse, PCN</td>
<td>[18,71,77]</td>
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<td>AD</td>
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<td>PD</td>
<td>Blocks caspase-3 activation</td>
<td>Astrocyte; Dopaminergic neuron; CGN</td>
<td>[116–118]</td>
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<td>Caspase-9</td>
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<td>Caspase-1</td>
<td>Stroke</td>
<td>Neuroprotective in PCN</td>
<td>PCN</td>
<td>[18]</td>
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<td>IL-1β</td>
<td>Stroke</td>
<td>Neuroprotective in PCN</td>
<td>PCN</td>
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<td>Rip2</td>
<td>HD</td>
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<td>Mu-htt ST14A [unpublished data]</td>
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Inhibition of the anti-apoptotic cell death pathway by melatonin

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<tr>
<td>DNA Fragmentation</td>
<td>Stroke/MCAO</td>
<td>Displays decreased DNA fragmentation; Neuroprotective in PCN</td>
<td>Rat; PCN</td>
<td>[18,71]</td>
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<tr>
<td>AD</td>
<td></td>
<td>Attenuates Aβ25-35- or Aβ1-42-induced apoptosis</td>
<td>Astroglia C6 cell</td>
<td>[102]</td>
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<tr>
<td>PD</td>
<td></td>
<td>Prevents DNA fragmentation</td>
<td>SK-N-SH cell; PC12 cells; Astrocyte; mesencephalic cell; striatal neuron; mouse;</td>
<td>[54,117,121,122]</td>
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<tr>
<td>TUNEL-positive</td>
<td>Stroke/MCAO</td>
<td>Reduces number of DNA breaks</td>
<td>Rat</td>
<td>[80]</td>
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<tr>
<td>Stroke/OGD</td>
<td></td>
<td>Decreases TUNEL-positive cells</td>
<td>Rat</td>
<td>[65,78,79]</td>
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<td>AD/OVX</td>
<td></td>
<td>Neuroprotective in PCN</td>
<td>PCN</td>
<td>[18]</td>
</tr>
<tr>
<td>AD</td>
<td></td>
<td>Improves spatial memory performance; Reduces apoptosis</td>
<td>Rat</td>
<td>[89]</td>
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<tr>
<td></td>
<td></td>
<td>Protects the wortmannin-induced tau hyperphosphorylation</td>
<td>N2a cells</td>
<td>[94]</td>
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<tr>
<td>JNK</td>
<td>PD</td>
<td>Inhibits cell death</td>
<td>SK-N-SH cell</td>
<td>[54,55]</td>
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<td>Par-4</td>
<td>AD</td>
<td>Reducts Par-4 upregulation</td>
<td>Mouse</td>
<td>[92]</td>
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<tr>
<td>NF-κB</td>
<td>AD</td>
<td>Blocks Aβ25-35-induced apoptosis</td>
<td>Microglial cell; Mouse</td>
<td>[5,25]</td>
</tr>
<tr>
<td>AD</td>
<td></td>
<td>Anti-inflammatory effect on Aβ vaccination in mice</td>
<td>Mouse</td>
<td>[26]</td>
</tr>
</tbody>
</table>

Abbreviations: OVX, ovariectomized.
<table>
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<th>Activates element of survival pathway</th>
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<tr>
<td>PI3-K/Akt</td>
<td>Stroke/MCAO</td>
<td>Restores phosphorylated Akt, Protects against brain injury</td>
<td>Mouse; Rat</td>
<td>[56,77,78]</td>
</tr>
<tr>
<td></td>
<td>AD</td>
<td>Impairs NADPH oxidase via PI3K/Akt signaling pathway</td>
<td>Microglia</td>
<td>[93]</td>
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<tr>
<td>Bcl-2</td>
<td>Stroke/MCAO</td>
<td>Enhances Bcl-2 upregulation</td>
<td>Rat</td>
<td>[79,82]</td>
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<td>AD/Aβ25-35</td>
<td>Attenuates Aβ25-35-induced apoptosis</td>
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<td>Bcl-xL</td>
<td>Stroke/MCAO</td>
<td>Elevates Bcl-xL in brain injury</td>
<td>Mouse</td>
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<td>JNK1/2</td>
<td>Stroke/MCAO</td>
<td>Increases JNK1/2 phosphorylation</td>
<td>Mouse</td>
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<td>ERK1/2</td>
<td>Stroke/MCAO</td>
<td>Increases ERK1/2 phosphorylation</td>
<td>Mouse; Rat</td>
<td>[56,65]</td>
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<td>Raf-1</td>
<td>Stroke/MCAO</td>
<td>Attenuates cerebral ischemic injury</td>
<td>Rat</td>
<td>[65]</td>
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<td>MEK1/2</td>
<td>Stroke/MCAO</td>
<td>Attenuates cerebral ischemic injury</td>
<td>Rat</td>
<td>[65]</td>
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<tr>
<td>NF-κB</td>
<td>Stroke</td>
<td>Relates with NF-kB-mediated protective signaling</td>
<td>Primary neuron</td>
<td>[27]</td>
</tr>
</tbody>
</table>
Melatonin, is an inhibitor of cytochrome c release (like minocycline does) – Luzindole blocks the role of melatonin.
Melatonin, reduces the activation of caspase-3, while luzindole blocks the role of melatonin.

<table>
<thead>
<tr>
<th></th>
<th>pro-caspase-3</th>
<th>active caspase-3</th>
<th>β-actin</th>
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<tbody>
<tr>
<td>H2O2</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Melatonin, 5 μM</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Luzindole, 25 μM</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

*PNAS 2003. 100, 16012-16017*
Conclusion

MT1 agonist melatonin-mediated neuroprotection through inhibiting of cell death pathway and activating survival signaling pathway in experimental models of stroke requires the MT1 receptor.
The other agonists of MT1 may offer protection in stroke as well -- novel neuroprotective agents for stroke.

146 MT1 agonists have been documented from the G-protein coupled receptor GPCR-Ligand Database. They are generally characterized by an indole core skeleton.
Eighteen relatively common MT1 agonists

There are 3 G-protein-coupled melatonin receptors: MT1, MT2, and MT3. They are generally characterized by an indole core skeleton. We aim to identify novel drugs from MT1 agonists for stroke.

- Melatonin
  - Neuroprotective; antioxidant; cell proliferation stimulator; reproductive function; treatment of sleep disorders; antidepressant; anti-inflammatory; antigonadotropic effect

- 2-Iodomelatonin
  - Reproductive function; contraceptive and menstrual cycle controlling

- 2-Iodo-N-Butanoyl-melatonin
  - Induction of calcium signaling; negative regulation of cytosolic phospholipase A2; induction of duodenal bicarbonate secretion

- N-Butanoyl-melatonin
  - Antagonadal activity; induction of pigment aggregation

- N-Propionyl-melatonin
  - Induction of pigment aggregation

- 6-Chloromelatonin
  - Reproductive effect

- 2-Methyl-7-melatonin
  - Reduction of tumor size; antigonadotropic effect

- Agomelatine (S 20098)
  - Antidepressant agent; Treatment of sleep disorder

- S26131 (Agomelatine dimer derivative)

- 8-M-PDOT
  - Reproductive function

- (-)-AMMTC
  - Acceleration of development

- LKK7
  - Treatment of insomnia

- Ramelton (TAK 375)
  - Treatment of insomnia

- GR196429
  - Modulation of circadian rhythm

- 5-Methoxytryptamine
  - Reproductive function

- GR128107
  - Induction of pigment aggregation

- N-Acetyltryptamine
  - Antioxidant

- N-Acetylsertotonin
  - Neuroprotective; antioxidant; antidepressant; anti-aging;
NAS (N-acetyl-serotonin, Normelatonin)

- NAS is the immediate precursor of melatonin. NAS is a chemical intermediate, which produced from serotonin and is converted to melatonin.

Serotonin $\rightarrow$ NAS $\leftrightarrow$ Melatonin

indole core skeleton
Common:
Both NAS and melatonin are 1) MT1 agonists; 2) NINDS 1040 compounds; 3) anti-oxidant; 4) anti-aging; 5) dietary supplements.

Different:
NAS is a better antioxidant.
NAS has wider distribution (brainstem, cerebellum, hippocampus, motor nuclei). Melatonin is secreted mainly from the pineal gland, it distributes in brain tissue (neurons and glia), brain ventricles, and hippocampus.
NAS decreases damage in Middle Cerebral Artery Occlusion (MCAO) mice

Journal of Neuroscience. 2014. 34:2967-78
Increased Mortality in MT1KO mice

Figure 3: MT1 KO mice were subjected to unilateral carotid ligation followed by 45 minutes of hypoxia. There was significantly increased mortality in MT1 KO group compared to WT C57BL6 mice. **p<0.005

NAS inhibits cell death (PI staining) in organotypic hippocampal slice cultures

Journal of Neuroscience. 2014. 34:2967-78
Molecular Mechanisms of NAS

NAS offers neuroprotection through inhibiting

1) mitochondrial cell death pathways
2) autophagic cell death pathways
NAS determination in brains of mice with ischemic injury

Liquid chromatography/mass spectrometry (LC/MS)

Endogenous NAS secretion in the brains of MCAO mice

NAS brain level in the brain of MCAO mice treated with NAS (~100 fold than non-NAS treated one)

Journal of Neuroscience. 2014. 34:2967-78
NAS reduces the release of cytochrome c and activation of caspase-3 in MCAO mice
NAS offers neuroprotection through inhibiting mitochondrial fragmentation in PCNs.
NAS offers neuroprotection through inhibiting autophagic cell death in cell model of ischemic stroke

Primary cerebrocortical neurons (PCNs)

2 hours

- LC3-I
- LC3-II
- Beclin1
- p62

MW: 19 kDa - 17 kDa
MW: 60 kDa
MW: 65 kDa

Ratio of LC3-I/β-actin
Ratio of LC3-II/β-actin
Ratio of Beclin1/β-actin
Ratio of p62/β-actin

Control, H2O2, NAS

18 hours

- LC3-I
- LC3-II
- Beclin1
- p62

MW: 19 kDa - 17 kDa
MW: 60 kDa
MW: 65 kDa

Ratio of LC3-I/β-actin
Ratio of LC3-II/β-actin
Ratio of Beclin1/β-actin
Ratio of p62/β-actin

Control, H2O2, NAS

* Significant difference
** Highly significant difference

Journal of Neuroscience. 2014. 34:2967-78
Monodansylcadaverine (MDC) is a specific marker for autophagic vacuoles/autophagosomes. H$_2$O$_2$ caused accumulation of autophagic vacuoles in PCNs.
NAS offers neuroprotection through inhibiting autophagic activation in MCAO mouse model of ischemic injury.

**Figure:**

- **LC3-I** and **LC3-II** proteins are shown with molecular weights of 18 kDa and 16 kDa.
- **Bclin 1** protein is shown with a molecular weight of 60 kDa.
- **p62** protein is shown with a molecular weight of 62 kDa.

**Graphs:**

- Ratios of LC3-II to β-actin, Bclin 1 to β-actin, and p62 to β-actin are presented for different conditions.

**Legend:**

- Cerebral ischemia: - (no ischemia), + (ischemia)
- NAS: - (no NAS), + (NAS treatment)
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Yi Zhang (FDA)
Jiang Zeng (Univ. of Toronto, Canada)
Mingchang Li (Wuhan Univ., China)
Happy Global Summit on Stroke-2015!