Suppression of AKT/mTOR pathway and activation of mitophagy by melatonin via mitochondrial regulation in head and neck cancer

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It is necessary to explore molecular mechanisms to find more effective therapeutic strategies.

The mTOR pathway plays an important role in HNSCC progression, metastasis and resistance to therapy.
mTOR pathway

Chiang, GG, Trends in Molecular Medicine, 2007
NEGATIVE FEEDBACK REGULATION OF mTOR SIGNALING

Growth Factors → PI3K → PDK1 → Akt

Akt → mTORC2 → S6K1

S6K1-dependent negative feedback loop

Akt → mTORC1 → Cell survival

mTORC1 → S6K1, 4E-BP1, ATG13, HIF-1α

Growth Factors
mTORC1: an anti-tumor target

Many cancer types show resistance to rapamycin treatment
It is necessary to find a therapeutic strategy to block Akt activation.

Melatonin induces downregulation of the mTOR pathway in various cancers.
Experiment design

**In vitro studies**

- *SCC-9*  
  - *Cal-27*  
  - Cell culture conditions:  
    - Seed cells
    - 60-70% confluence
    - 24 h
    - 48 h
    - 48 h

**In vivo studies**

- *Cal-27 xenograft*
  - Tumor size: 100–200 mm$^3$
  - Days: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11

Control:
- Rap 20 nM
- Rap 20 nM+aMT 0.1 mM
- Rap 20 nM+aMT 0.5 mM
- aMT 1 mM

Rap + aMT 300 mg/kg Intraperitoneally
Rap + aMT 300 mg/kg Subcutaneously
mTOR pathway

<table>
<thead>
<tr>
<th>aMT (mM)</th>
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<th>0.1</th>
<th>0.5</th>
<th>1</th>
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<td>Rap (nM)</td>
<td>20</td>
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- **pS6/GAPDH**
- **pT308AKT/GAPDH**
- **pS473AKT/GAPDH**
- **AKT/GAPDH**

**mTORC1**

- **mTORC2**
Melatonin inhibits the Akt activation

PI3K → PDK1 → Akt (T308, S473) → mTORC1 (S6K1) → Cell survival

- Rapamycin inhibits mTORC1
- Melatonin inhibits Akt activation
Melatonin enhances the effects of rapamycin

Cell viability

**Cal-27**

**SCC-9**
Apoptosis

- **aMT (mM)**: — — 0.1 0.5 1 1
- **Rap (nM)**: — 20 20 20 20 —

**Bcl-2**

**GAPDH**

**Bax**

**GAPDH**

**Bax/Bcl-2 (Relative to control)**

**Bcl-2/GAPDH (Relative to control)**

**Bax/GAPDH (Relative to control)**

**Bax/Bcl-2 (Relative to control)**
Mitochondria are the main target of melatonin

We explored the possibility that melatonin enhances the effects of rapamycin through mitochondrial pathway
Rapamycin-treated cells exhibited reduced capacity for oxidative phosphorylation

The combined treatment decreased metabolic rate in HNSCC
Melatonin enhances the effects of rapamycin, in terms of decreasing the number of mitochondria or the number of functional mitochondria.

To confirm this hypothesis, we next examined OXPHOS, mitochondrial mass, and mtDNA.
Mitochondrial OXPHOS expression in HNSCC

- Complex V (CVa/CII)
  - aMT (mM): — — 0.1 0.5 1 1
  - Rap (nM): — 20 20 20 1 1

- Complex IV (Cox I/CII)
  - aMT (mM): — — 0.1 0.5 1 1
  - Rap (nM): — 20 20 20 1 1

- Complex III (Core 2/CII)
  - aMT (mM): — — — — 0.5 1
  - Rap (nM): — 20 20 20 20 —

- Complex I (ND6)
  - aMT (mM): — — 0.1 0.5 1 1
  - Rap (nM): — 20 20 20 20 —

- Mitochondrial OXPHOS expression in HNSCC

- Cox I (Complex IV)
- Core 2 (Complex III)
- CVa (Complex V)
- Complex II
- ND6 (Complex I)
Mitochondrial mass and mtDNA

![Mitochondrial mass and mtDNA graph]

- **NAO (arbitrary units)**
  - Relative to control
  - 0.0, 0.5, 1.0, 1.5, 2.0

- **mtDNA/nDNA**
  - Relative to control
  - 0, 1, 2, 3, 4

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- **Statistical significance**
  - *: p < 0.05
  - **: p < 0.01
  - ***: p < 0.001
  - #: p < 0.05 (right graph)
We hypothesized that melatonin enhances the cytotoxic effects of rapamycin by augmenting the number of dysfunctional mitochondria.
ROS and LPO levels

DCF fluorescence (relative to control)

LPO (nmol/mg prot) (relative to control)

DCFA fluorescence (relative to control)

LPO (nmol/mg prot) (relative to control)
GSSG/GSH ratio

(aMT (mM) — — 0.1 0.5 1 1
Rap (nM) — 20 20 20 20 —)
High concentration melatonin (1mM) → Oxidative stress

We supposed that at high concentration of melatonin, ROS accumulation under respiratory conditions may have resulted in mitochondrial protein degradation and even mitophagy.
These results indicated that high concentrations of melatonin contribute to induce mitophagy and to eliminate dysfunctional mitochondria.
Synergistic effect of melatonin and rapamycin
These results were in contrast to the expectation that the combined treatment would be more effective based on our *in vitro* results.
We thought that melatonin didn’t reach the tumor at a sufficient concentration to inhibit tumor growth.
Combination of melatonin and rapamycin for head and neck cancer therapy: Suppression of AKT/mTOR pathway activation, and activation of mitophagy and apoptosis via mitochondrial function regulation

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Abstract
Head and neck squamous cell carcinoma (HNSCC) clearly involves activation of the Akt mammalian target of rapamycin (mTOR) signalling pathway. However, the effectiveness of treatment with the mTOR inhibitor rapamycin is often limited by chemoresistance. Melatonin suppresses neoplastic growth via different mechanisms in a variety of tumours. In this study, we aimed to elucidate the effects of melatonin on rapamycin-induced HNSCC cell death and to identify potential cross-talk pathways. We analysed the dose-dependent effects of melatonin in rapamycin-treated HNSCC cell lines (Cal-27 and SCC-9). These cells were treated with 0.1, 0.5 or 1 mmol/L melatonin combined with 20 nM rapamycin. We further examined the potential synergistic effects of melatonin with rapamycin in Cal-27 xenograft mice. Relationships between inhibition of the mTOR pathway, reactive oxygen species (ROS), and apoptosis and mitophagy reportedly increased the cytotoxic effects of rapamycin in HNSCC. Our results demonstrated that combined treatment with rapamycin and melatonin blocked the negative feedback loop from the specific downstream effector of mTOR activation S6K1 to Akt signalling, which decreased cell viability, proliferation and clonogenic capacity. Interestingly, combined treatment with rapamycin and melatonin-induced changes in mitochondrial function, which were associated with increased ROS production, increasing apoptosis and mitophagy.
Thank you very much