Inhibiting autophagosomal turnover targets therapy resistant leukemia cells through metabolic disruption

Kaitlyn Dykstra, PhD
Department of Medicine – Leukemia
Lab of Eunice Wang, MD
April 3, 2018
Hematopoiesis

Hematopoietic Stem cell

Lymphoid Progenitor

Myeloid Progenitor

B cell precursor

T cell /NK precursor

RBC precursor

Megakaryocytic precursor

Granulocytic precursor

Plasma Cell

T cell

NK cell

RBC

Platelets

Neutrophils

Macrophage

Slide courtesy Monica Guzman, Weill Cornell
Acute Myeloid Leukemia (AML)

- Pluripotent Stem cell
- Myeloid Progenitor
- Granulocytic precursor
- Megakaryocytic precursor
- B cell precursor
- T cell/NK precursor
- RBC precursor
- Platelets
- Neutrophils
- Plasma Cell
- T cell
- NK cell
- RBC
- Macrophage

Slide courtesy Monica Guzman, Weill Cornell
AML Survival Rates Remain Low

**Estimated New Cases (2018)**

- **CML**: 14%
- **ALL**: 10%
- **CLL**: 35%
- **Other**: 9%
- **AML**: 32%

**Leukemia Subtype** | **5-year survival rate**
--- | ---
Acute lymphocytic (ALL) | 79.0%
Chronic lymphocytic (CLL) | 68.2%
**Acute myeloid (AML)** | 26.9%
Chronic myeloid (CML) | 66.9%

SEER Cancer Statistics Review (CSR), 1975-2014

American Cancer Society
Why is AML difficult to cure?

- Heterogeneous disease
  - 23 different mutated genes
- Compare to CML
  - Almost all cases are caused by “Philadelphia chromosome” translocation
  - Can be treated with tyrosine kinase inhibitors

Why is AML difficult to cure?

- AML has an older patient population
  - Less tolerant to chemotherapy
  - Less tolerant to bone marrow transplant
- Compared to ALL
  - Most patients are pediatric

[Sallan, SE. Hematology Am Soc Hematol Educ Program, 2006.](#)
Outcomes are particularly poor in older adults

“Young” < 60 years old  “Old” ≥ 60 years old

Juliusson et al., Blood 119:3890-3899, 2012
Juliusson et al., Blood 113(18): 4179-4187, 2009
Standard of Care for AML

Standard 7+3 induction chemotherapy

- Treatment has not changed since the 1970s
- Despite most patients achieving remission, relapse inevitably occurs

7 days
Cytarabine (AraC)
+ 3 days
Daunorubicin or Idarubicin
Relapse occurs due to minimal residual disease including leukemia stem cells in the bone marrow.

Minimal residual disease (MRD)
- No signs or symptoms of disease (remission)
- Disease below the threshold of diagnostic detection by microscopy or flow cytometry

How can we eradicate MRD?
Hypoxia in the bone marrow microenvironment contributes to chemoresistance in AML

Oxygen Level
Cellularity

O₂ levels
- 6% in normal tissues
- 1-3% in bone marrow

Portwood et al., Clinical Cancer Research, 2013
Hypoxia contributes to chemoresistance in AML cell lines

Live Cells: Annexin V negative/7-AAD negative

* p<0.05 ** p<0.005 *** p<0.0005 n=3
How does hypoxia lead to chemoresistance?

- AraC targets proliferating cells
- Hypoxia reduces cell proliferation
- Is reduced proliferation in hypoxia sufficient to explain AraC resistance?
Hypoxia does not reduce the anti-proliferative or DNA damaging effects of AraC

**Cells/ml (Trypan Blue)**

**DNA Damage (p-H2.AX)**
How does hypoxia lead to chemoresistance?

- Hypoxia has previously been shown to upregulate anti-apoptotic proteins (e.g. Bcl-2) and downregulated pro-apoptotic proteins (e.g. Bax).
- Is reduced chemosensitivity due to changes in expression of these proteins?
Expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax are not affected by hypoxia
How does hypoxia lead to chemoresistance?

- Other survival pathways altered under hypoxia
  - Autophagy?
Autophagy is a prosurvival process that is upregulated under hypoxic conditions.
Autophagic flux is upregulated in AML cell lines after prolonged exposure to hypoxia

**Graphs and Images:**
- **Hypoxia Exposure:**
  - **Hel-Luc:** Hypoxia: +, Normoxia: -
  - **HL60:** Hypoxia: +, Normoxia: -
  - **MOLM13:** Hypoxia: +, Normoxia: -

- **Western Blot:**
  - LC3-I, LC3-II, p62, Actin
  - **DAPI/CytoID Images:**
    - **Normoxia**
    - **Hypoxia**
  - % CytoID Positive Cells: 21% O2: 0, 1% O2: *p<0.05 n=3
Does autophagy drive chemoresistance?
Autophagosome formation does not drive chemoresistance under hypoxia

Cyto-ID

MRT68921
(ULK1 inhibitor)

Spautin-1
(Class II PI3K inhibitor)

* p<0.05  n=3
Autophagosome formation does not drive chemoresistance under hypoxia

**Diagram:**
- **Early Autophagy**
- **Graph:**
  - x-axis: % Live Cells
  - y-axis: 0-100
  - Bars represent different treatments:
    - Unt
    - AraC
    - Non-silencing shRNA
    - Atg7 shRNA
    - LC3B shRNA
  - Conditions: 21% O2 and 1% O2
  - ns (not significant)

**Images:**
- N.S. Atg7-:
  - Atg7
  - LC3-I
  - LC3-II
  - Actin
- N.S. LC3B-:
  - Atg7
  - LC3-I
  - LC3-II
  - Actin
BafA1 and Chloroquine inhibit autophagosome turnover through distinct but related mechanisms

BafA1 and Chloroquine

Bafilomycin A1

Vacuolar-type H+-ATPase inhibitor
- Increases lysosomal pH

Chloroquine

Lysosomotropic agent
- Trapped in lysosomes
- Increases lysosomal pH

Increased lysosomal pH reduces fusion competence.
Inhibiting autophagosome turnover overcomes hypoxia induced chemoresistance

**Bafilomycin A1**

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Live Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unt</td>
<td>100</td>
</tr>
<tr>
<td>AraC</td>
<td>80</td>
</tr>
<tr>
<td>BafA1 AraC</td>
<td>70</td>
</tr>
<tr>
<td>Unt AraC BafA1</td>
<td>60</td>
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</tbody>
</table>

**Chloroquine**

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Live Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unt</td>
<td>100</td>
</tr>
<tr>
<td>AraC</td>
<td>85</td>
</tr>
<tr>
<td>CQ AraC</td>
<td>75</td>
</tr>
<tr>
<td>Unt AraC CQ</td>
<td>65</td>
</tr>
</tbody>
</table>

*MOLM13* 21% O₂ 1% O₂

* p<0.05 n=3
Relapse occurs due to minimal residual disease (MRD) and leukemia stem cells (LSCs) in the bone marrow.
Can late stage autophagy inhibitors target leukemia stem cells?

LSC Characteristics

- CD34+/CD38-/Lin-  
  - not exclusively
- Preferentially localized in hypoxic bone marrow
- Quiescent
- Increased autophagy

Neng Yang, Guzman Lab
Functional definition for LSC

Leukemia stem cell → Leukemia progenitor → Colony forming assays (CFU)
Functional definition for LSC

Leukemia stem cell → Leukemia progenitor → Xenotransplantation in sub-lethally irradiated immunodeficient mice

Guzman Lab
Late stage autophagy inhibitors inhibit primary AML colony formation

Neng Yang, Guzman Lab
Bafilomycin A1 selectively eradicates leukemia stem cells

Neng Yang, Guzman Lab
Why do late stage autophagy inhibitors target LSCs?

- Inhibiting OXPHOS has been demonstrated to target leukemia stem cells and therapy resistant cells

- Do late stage autophagy inhibitors modulate OXPHOS?

Viale et al., Cancer Research, 2015
Late stage autophagy inhibitors block OXPHOS under hypoxia in AML cells

**Basal respiration**

**Maximal Respiration**

**Average Basal Respiration**

* p<0.05 ** p<0.005 *** p<0.0005 n=3
Late stage autophagy inhibitors block OXPHOS under hypoxia in AML cells

Average Basal Respiration

Maximal Respiration

Basal respiration

0 20 40 60 80
Time (minutes)

OCR (pmol/min)

Normoxia Untreated
Hypoxia Untreated
Normoxia 50µM CQ
Hypoxia 50µM CQ

0 100

basal respiration

0 100

100

50

25µM 50µM 100µM Unt Unt CQ

* p<0.05  ** p<0.005  *** p<0.0005 n=3
Late stage autophagy inhibitors block OXPHOS in BafA1 sensitive primary AML samples

Patient Sample 1

Patient Sample 2

Patient Sample 3

- Normoxia Untreated
- Hypoxia Untreated
- Normoxia 25nM BafA1
- Hypoxia 25nM BafA1
Late stage autophagy inhibitors block OXPHOS in BafA1 sensitive primary AML samples

Patient Sample 1

- Normoxia Untreated
- Hypoxia Untreated
- Normoxia 25nM BafA1
- Hypoxia 25nM BafA1

n=1
Late stage autophagy inhibitors block OXPHOS in BafA1 sensitive primary AML samples

Patient Sample 3

OCR (pmol/min) vs Time (minutes)
- Normoxia Untreated
- Hypoxia Untreated
- Normoxia 25nM BafA1
- Hypoxia 25nM BafA1

Patient Sample 3

%Apoptotic
- Unt
- 25nM Baf

Unt treated
Normoxia
Hypoxia

n=1
Early stage autophagy inhibitors do not modulate OXPHOS

* p<0.05 ** p<0.005 *** p<0.0005 n=3
OXPHOS inhibition by late stage autophagy inhibitors is not due to decreases in mitochondrial mass
Late stage autophagy inhibitors induce mitochondrial ROS production

![Graph showing relative MitoSOX MFI under normoxia and hypoxia conditions with different treatments.]

- Untreated
- 25nM BafA1
- 50nM BafA1
- 50uM CQ
- 100uM CQ

**Normoxia**

- 4h
- 8h
- 24h

**Hypoxia**

- 4h
- 8h
- 24h

*n=2*
BafA1 reduces glycolytic function in hypoxia

Glycolytic Capacity

Average Glycolysis

- * p<0.05
- ** p<0.005
- *** p<0.0005

n=3
Chloroquine does not upregulate glycolytic function in hypoxia

Glycolytic Capacity

Glycolysis

Average Glycolysis

* $p<0.05$  ** $p<0.005$  *** $p<0.0005$  n=3

- Normoxia Untreated
- Hypoxia Untreated
- Normoxia 50uM CQ
- Hypoxia 50uM CQ
Conclusions

• Hypoxia reduces chemosensitivity in AML cell lines and increases autophagic flux.

• Inhibiting the late stages of autophagy overcomes both hypoxia induced chemoresistance and eradicates LSCs.

• Late stage autophagy inhibitors disrupt both mitochondrial respiration and glycolysis under hypoxia.
Future directions

- Test BafA1 and chloroquine in combination with AraC and in an MRD model of AML
- Elucidate the connection between blocking autophagosome turnover/lysosomal disruption and metabolism
  - Mitochondrial ROS
  - Ca^{2+}
- Identify new inhibitors with better specificity/distribution
  - Bone marrow targeted nanoparticles
Acknowledgements

Wang Lab
- Eunice Wang, MD
- Matthew Johnson
- Scott Portwood, MS, MBA
- Amanda Przespolewski, DO
- Dirkje Hanekamp, MS
- Megan Johnson

Weill Cornell
- Monica Guzman, PhD
- Neng Yang, PhD

NCI Cancer Center Support Grant 5P30 CA001656
  - Flow and Image Cytometry Shared Resource
  - Immune Analysis Facility

Roswell Park Alliance Foundation
Jacquie Hirsch Leukemia Research Fund