Bioorthogonal Prodrug Activation Driven by a Strain-Promoted 1,3-Dipolar Cycloaddition

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A. Manos-Turvey,
Wipf Group Current Literature
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Prodrugs for Cancer Therapies

- Non-selectivity in cancer treatments leads to off-target side-effects
- Prodrug activation is seen as a viable method allowing for direct drug delivery
  - Cleavage of a deactivating linker, leading to activation
  - Can react with off-target sources due to hydrolysis

- Antibody-Drug Conjugates (ADCs)
  - ADCs can elicit an immune system response
  - The linkers need to be fine tuned between stability and “cleavability”
  - Drugs become diluted as this is dependant on cell surface receptors, leading to a potent...


Prodrugs for Cancer Therapies

- **Antibody-Directed Enzyme Prodrug Therapy (ADEPT)**
  - Targets an antibody-enzyme conjugate to a cancer cell
  - Limited to human enzymes, to avoid anti-enzyme immune responses

Prodrugs for Cancer Therapies

- Bioorthogonal Chemistry
  - Not many examples for *in vitro* prodrug activation
  - Staudinger and tetrazine-TCO (Inverse-Electron-Demand Diels-Alder Cycloadditions) reactions have been used.

Prodrugs for Cancer Therapies

- Bioorthogonal Chemistry

- TCO/tetrazine prodrug activation difficulties:
  - Lower TCO-conjugate activity
  - Low tumour-to-background ADC ratio, leading to off target effects
  - TCO-prodrug variant may isomerise to cis form if administered separately

Bioorthogonal Chemistry: TCO and Azide

- First reported in 1992
- Triazoline product is unstable

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\[ \text{Product} \] = \text{TCO} + \text{Azide} \]

Prodrugs for Cancer Therapies: 1,3-Dipolar Click Reaction with TCO and Azide

- TCO and Azide for Prodrug Activation!
  - Attach electron-deficient linker to inactive drug
  - TCO identifies target
  - Aqueous environment is key

![Diagram of 1,3-Dipolar Click Reaction](image_url)

- **Step 1**: 1,3-Dipolar Cycloaddition
- **Step 2**: Acid-catalyzed breakdown of the imine bond
- **Step 3**: Formation of unstable 1,2,3-triazoline
- **Step 4**: PABC incorporation
- **Step 5**: Formation of active cytotoxic drug
Proof of Concept

- Coumarin probes were synthesised to investigate rate and mechanism of reaction
- A doxorubicin azido-PABC prodrug was synthesized to investigate in vitro biorthogonal activation
- Two TCOs were made

![Chemical structures and reaction equations]

- **8a**
  - Reaction 1: 4-NO₂PhCO₂Cl, pyridine, DCM, rt, Et₃N, DMF, rt
  - Product: 38%
  - TCO-OH

- **8b**
  - Reaction 2: Doxorubicin.HCl, Et₃N, 4A sieves, DMF, rt
  - Product: 69%
  - TCO-ε-lysine

- **9**
  - Reaction 3: Doxorubicin.HCl, Et₃N, 4A sieves, DMF, rt
  - Product: 69%
  - TCO-ε-lysine
Proof of Concept

- Tested by spectrofluorometry, measuring fluorescence (ex 360 nm, em 455 nm)
  - in PBS:MeCN (1:1) n=3

![Chemical Structures](image)

![Graph](image)
A series of 1H NMR experiments were then carried out
- 8a carbonate was used at 6.7 mM, TCO at 18.7 mM
# Mechanism

![Chemical structures and spectra](image)

**a)** C<sub>6</sub>D<sub>6</sub>

<table>
<thead>
<tr>
<th>Time</th>
<th>Spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td><img src="image" alt="0 h spectrum" /></td>
</tr>
<tr>
<td>3 h</td>
<td><img src="image" alt="3 h spectrum" /></td>
</tr>
<tr>
<td>24 h</td>
<td><img src="image" alt="24 h spectrum" /></td>
</tr>
<tr>
<td>5 days</td>
<td><img src="image" alt="5 days spectrum" /></td>
</tr>
</tbody>
</table>

- **8a** - SM carbonate
- **13a** - Coumarin
- **11a** - Triazoline
- **12a** - Aldimine
Mechanism

8a - SM carbonate

13a - coumarin

14a

b) CD₃CN / D₂O

47 h

23 h

270 min

0 h

11a - triazoline

12a - aldimine
Rate of Reaction of 1,3-Dipolar Cycloaddition

- TCO/TCO-OH activation results in coumarin release
- RP-HPLC was used to determine the second order rate of the initial 1,3-dipolar cycloaddition
  - MeCN:PBS (1:1, 37 °C), measuring disappearance of SM at 254 nm
  - Comparable rates to those seen with first generation SPAAC (10⁰-10⁻³ M⁻¹ s⁻¹)

![Chemical Structures]

\[ k_2 = 0.017 \text{ M}^{-1} \text{ s}^{-1} \]
\[ k_2 = 0.027 \text{ M}^{-1} \text{ s}^{-1} \]

8b and TCO were reacted in CD$_3$CN and DMSO-$d_6$ for 19 h to give 11b.

An aliquot of the NMR was then diluted into PBS and the rate of triazoline and imine degradation was monitored spectroscopically by the appearance of 13b.
Rate of Reaction for Coumarin Release

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Rate of Reaction for Coumarin Release

- Degradation and release (3 steps) follows pseudo first-order kinetics in polar protic solvents
  - Assumption that either triazoline degradation or imine hydrolysis is the rate limiting step
  - *In vivo* this rate is less significant as both intermediates will be fixed to a cancer cell surface

![Release of 7-amino-4-methylcoumarin](image)

11b - triazoline

ii) dilute 1000-fold into PBS

13b

t_{1/2} = 19 min
Bioorthogonal Potential

Using a model murine melanoma cell line (B16-OVA), the reaction strategy was evaluated in vitro following 72 h incubation with 9 and 10 at 37 °C

- 9 cytotoxicity alone is low

The authors propose that 15 is released outside of targeted tumour cells and then diffuses into the closely located cancer cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>doxorubicin (15)</td>
<td>0.71</td>
</tr>
<tr>
<td>9 pro-drug</td>
<td>49.9</td>
</tr>
<tr>
<td>9 + CCO-OH (100 µM)</td>
<td>55.0</td>
</tr>
<tr>
<td>9 + TCO-OH (mix) (100 µM)</td>
<td>0.96</td>
</tr>
<tr>
<td>9 + TCO-OH (eq) (100 µM)</td>
<td>1.47</td>
</tr>
<tr>
<td>9 + TCO-OH (ax) (100 µM)</td>
<td>1.34</td>
</tr>
<tr>
<td>9 + TCO-OH (eq) (10 µM)</td>
<td>4.98</td>
</tr>
</tbody>
</table>
Bioorthogonal Potential

- Attachment of 1-6 TCOs to each monoclonal antibody with binding with $10^5$ cell surface receptors, [TCO] = 0.4-2.5 µM on tumour cell surfaces
  - 9 rate of reaction still needs improvement
  - Need to overcome rapid clearance rates from mice ($t_{1/2} = \text{mins}$) for similar compounds
- *In vivo* suitability was evaluated by monitoring stability and activation of 9 in 50% mouse serum:PBS and PBS only (HPLC analysis)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Degradation of 9 (15 release) PBS only</th>
<th>Degradation of 9 (15 release) PBS + MS</th>
<th>Degradation of 9 (15 release) + TCO-OH eq PBS only</th>
<th>Degradation of 9 (15 release) + TCO-OH eq PBS + MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100% (0%)</td>
<td>100% (0%)</td>
<td>100% (0%)</td>
<td>100% (0%)</td>
</tr>
<tr>
<td>4</td>
<td>106% (0%)</td>
<td>95% (0%)</td>
<td>84% (34%)</td>
<td>47% (51%)</td>
</tr>
<tr>
<td>24</td>
<td>121% (0%)</td>
<td>68% (0%)</td>
<td>39% (77%)</td>
<td>12% (59%)</td>
</tr>
<tr>
<td>48</td>
<td>112% (0%)</td>
<td>56% (6%)</td>
<td>12% (79%)</td>
<td>2% (5%)</td>
</tr>
</tbody>
</table>
Bioorthogonal Potential

- No reaction deactivation from serum derived byproducts
  - a problem seen with Staudinger prodrug activations
- Investigated if serum protein interactions may reduce effective [9] and therefore activity
  - SPAAC cyclooctyne reactants (added after tumour targeting) interact with serum and show reduced in vivo reaction rates
- The reaction proceeds faster in the presence of serum than the model systems of 8a and 8b in MeCN:PBS (k₂ = 0.017-0.027 M⁻¹ s⁻¹)
  
\[
\begin{align*}
&\text{9} \\
&\xrightarrow{\text{TCO-OH (10)}} \\
&50\% \text{ serum:PBS} \\
&k₂ = 0.137 \text{ M}⁻¹ \text{ s}⁻¹
\end{align*}
\]
Conclusions and Scope

- Successfully use a 1,3-dipolar cycloaddition between TCO-OH and an azide, to facilitate prodrug activation
  - Activation is 1-2 orders of magnitude faster than the Staudinger reaction variants (and faster still in serum:PBS mixtures)
  - TCO-OH could isomerise, but will be modified with antibody linkers known to stabilise isomerization
  - Still room to expand on reactivity through modification of the azido-prodrug
  - Good stability in mouse serum (min to h)
- Next step: need to move from the hypothetical to the actual prodrug antibody system