Communication

Bioorthogonal Removal of 3-Isocyanopropyl Groups Enables the Controlled Release of Fluorophores and Drugs in Vivo

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J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/jacs.8b05093 • Publication Date (Web): 21 Jun 2018

Bioorthogonal Removal of 3-Isocyanopropyl Groups Enables the Controlled Release of Fluorophores and Drugs In Vivo

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Supporting Information Placeholder

ABSTRACT: Dissociative bioorthogonal reactions allow for chemically controlling the release of bioactive agents and reporter probes. Here we describe 3-isocyanopropyl substituents as masking groups that can be effectively removed in biological systems. 3-isocyanopropyl derivatives react with tetrazines to afford 3-oxopropyl groups that eliminate diverse functionalities. The study shows that the reaction is rapid and can liberate phenols and amines near-quantitatively under physiological conditions. The reaction is compatible with living organisms as demonstrated by the release of a resorufin fluorophore and a mexiletine drug in zebrafish embryos implanted with tetrazine-modified beads. The combined benefits of synthetic ease, rapid kinetics, diversity of leaving groups, high release yields, and structural compactness, make 3-isocyanopropyl derivatives attractive chemical caging moieties for uses in chemical biology and drug delivery.

The ability to chemically control the release of reporter probes, bioactive compounds, and biomacromolecules using bioorthogonal reactions is opening opportunities for the development of innovative research tools, diagnostics, and therapeutics.[1] Applications in diverse fields such as biosensing,[2] cell imaging,[3] gasoemission,[4] and activity control of nucleic acids[5] and proteins[6] exemplify the potential of such transformations. Moreover, there is an interest in the clinical translation of dissociative bioorthogonal reactions as chemically-responsive antibody-drug conjugates[7] and prodrugs.[8]

The growing demand for click-reactions linked to a payload release has motivated the invention of several such transformations. Examples make use of the Staudinger reaction,[2b,8a,9] inverse-electron demand Diels-Alder cycloaddition,[10] borane-induced deoxygenation,[11] strain-promoted [3+2] azide-alkene cycloaddition,[12] and metal-catalyzed uncaging.[13] The utility of several of these reactions to control the release of drugs and fluorophores has been demonstrated in vitro and in living vertebrates.[6c,7,8c,13d,13e] To further advance the scope of bond-cleavage reactions compatible with biological systems, we were interested to develop structurally compact bioorthogonal reagents that are facile to synthesize while meeting the key requirements of rapid reaction kinetics, high release yields, broad range of leaving groups, lack of toxicity, and extended serum stability.

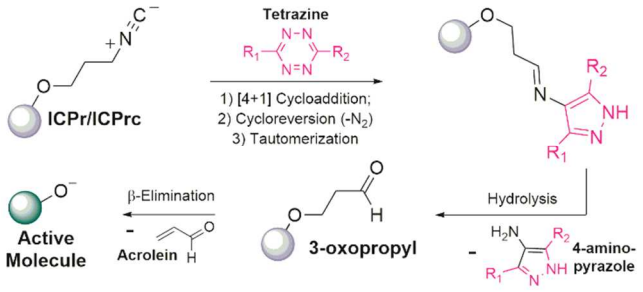


Figure 1. Tetrazine-mediated release of molecules caged with 3-isocyanopropyl (ICPr) and 3-isocyanopropyl-1-carbamoyl (ICPrc) groups.

Here we describe 3-isocyanopropyl (ICPr) and 3-isocyanopropyl-1-carbamonyl (ICPrc) modifications as masking groups that can be effectively removed from diverse molecules by bioorthogonal reactions with 1,2,4,5-tetrazines (Tz; Fig. 1). The design of ICPr/ICPrc groups is based on the precedence that Tz at room temperature converts isonitriles into aldehydes.[14] We rationalized that the acidity of the aldehyde's α-proton would make it possible to release drugs and reporter molecules via β-elimination. Several reports have previously shown that 3-oxopropyl groups spontaneously eliminate diverse functionalities.[15] We anticipated that ICPr/ICPrc groups would undergo a [4+1] cycloaddition reaction with Tz followed by rapid expulsion of N_2 and formation of a pyrazole-imine intermediate. Hydrolysis to the aldehyde will induce the spontaneous elimination of leaving groups at the C-1 position of the resulting 3-oxopropyl moiety (Fig. 1).

Scheme 1. Synthesis of ICPr-tos, ICPr-OH, and ICPr-nc.

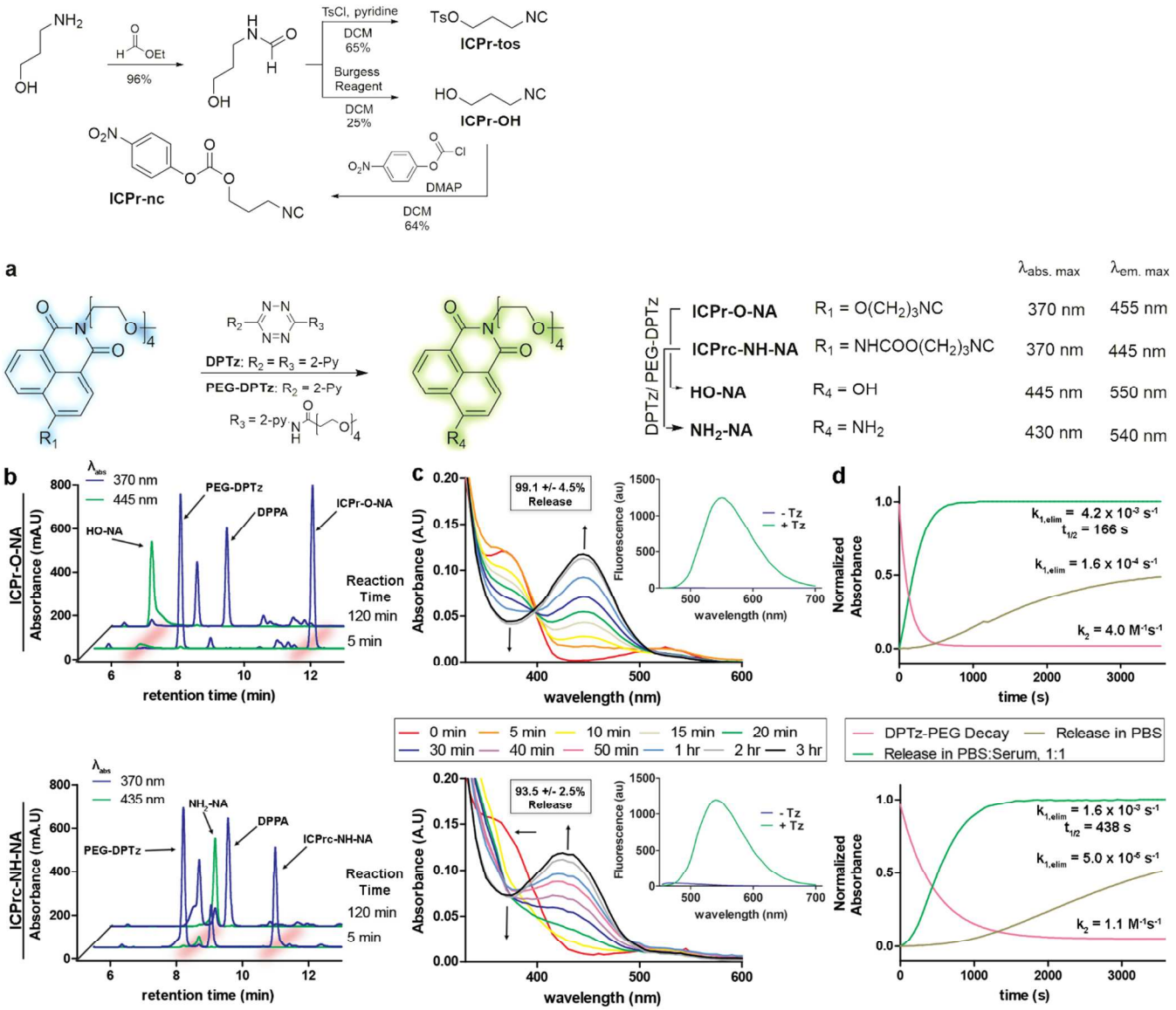


Figure 2. Tz-mediated uncaging of phenols and amines from 3-isocyanopropyl and 3-isocyanopropyl-1-carbamoyl derivatives. (a) Structures of caged 1,8-naphthalimide reporter probes and products of their reactions with tetrazine. b) HPLC analysis of tetrazine-mediated uncaging of ICPr-O-NA and ICPr-NH-NA by PEG-DPTz (c(probe) = 1 mM, c(Tz) = 2 mM, PBS:DMSO (9:1), T = 37 °C; DPPA: 3,5-di(pyrid-2-yl)-1H-pyrazol-4-amine). (c) Time-dependent absorbance changes associated with the reaction of ICPr-O-NA and ICPr-NH-NA with DPTz (c(probe) = 1 mM, c(Tz) = 3 mM, PBS:DMSO (1:1), T = 37 °C). (d) Kinetics of tetrazine consumption and accumulation of products HO-NA and NH_2-NA (c(probe) = 2 mM, c(Tz) = 0.2 mM, PBS:DMSO (9:1), T = 37 °C).

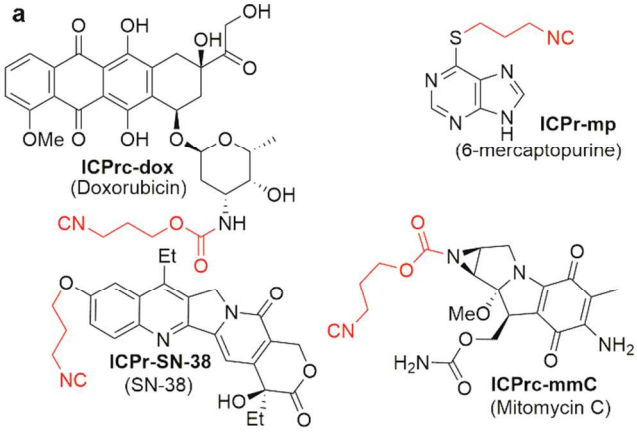
Reagents for the preparation of ICPr/ICPrc-derivatives were straightforwardly accessible (Scheme 1). 3-isocyano-1-tosylpropane (ICPr-tos) for the alkylation of phenols and other nucleophiles could be prepared on a multi-gram scale from inexpensive 3-amino-1-propanol in two steps and 62 % yield. ICPr-tos is stable for months at -20 °C. To mask amines with ICPrc groups, we prepared 3-isocyano-4-nitrophenyl carbonate (ICPr-nc) along a similar synthetic route. The ease of preparation of these molecules favorably contrasts the tedious synthesis of some bioorthogonally-removable groups.

To investigate the Tz-mediated removal of ICPr/ICPrc-groups, we synthesized probes that report unmasking by ratiometric changes in absorbance and fluorescence spectra (Fig. 2). 1,8-naphthalimides were modified on 4-OH/4-NH_2 functionalities with ICPr/ICPrc groups (ICPr-O-NA, ICPrc-NH-NA; see Supporting Information for synthesis). A PEG4-group at the imine nitrogen endowed the probes with excellent water solubility. With these reporter molecules, we confirmed that Tz elicits the traceless removal of ICPr/ICPrc groups from phenols and amines. Incubation of ICPr-O-NA and ICPrc-NH-NA (c = 1 mM) with the water-soluble tetrazine PEG-DPTz (Fig. 2a; c = 2 mM; T = 37 °C, PBS:DMSO (9:1, v/v)) led to the complete consumption of the masked dyes and the formation of the parental fluorophores as indicated by HPLC analysis (Fig. 2b)

We further monitored ICPr/ICPrc unmasking by UV-Vis spectrophotometric analysis (Fig. 2c). The introduced modifications caused a hypsochromic shift of the absorbance and emission bands of these fluorophores (Fig. 2a,c). In case of ICPr-O-NA (c = 1 mM), the absorbance band with a maximum at 370 nM disappeared rapidly in the presence of excess 3,6-di-2-pyridyl-1,2,4,5-tetrazine (DPTz; c = 3 mM, T = 37 °C, PBS:DMSO (1:1)) concomitant with the appearance of the absorbance peak characteristic for HO-NA ($\lambda_{abs,max}$ = 445 nm) and an isobestic point at 395 nm. The Tz-mediated conversion of

ICPrc-NH-NA to H_2N-NA, accompanied by an absorbance shift from 370 to 430 nm, provided a comparable result with the exception of a hypsochromic shift at early time points (Fig. 2c), which indicated the formation of an intermediate with decreased electron-density on the amine. The formation of a cyclic 4-hydroxy-1,3-oxazinan-2-one at equilibrium with the 3-oxopropyl carbamate is a possible explanation for this observation as reported for related molecules.[16] Measurement of unmasking yields based on the absorbance intensity of the product indicated near-quantitative release for the reaction of DPTz with ICPr-O-NA (99.1 ± 4.5%) and ICPrc-NH-NA (93.5 ± 2.5%). Liberation of the 1,8-naphthalimides further led to a strong fluorescence turn-on signal (ICPr-O-NA: 1210-fold; ICPrc-NH-NA: 76-fold; (Fig. 2c, inset). Removal of ICPr-groups also occurred with 7-hydroxycoumarin and resorufin (ICPr-res) fluorophores (Fig. S1-4 in Supporting Information). Based on previous studies of β-eliminations from 3-oxopropyl substituents,[15a,17] it appears likely that ICPr/ICPrc chemistry can be used to mask diverse functional groups.

We further studied the kinetics of the Tz-induced release of phenols and amines from ICPr/ICPrc groups (Fig. 2d). Fitting the disappearance of PEG-DPTz (c = 0.2 mM) absorbance in the presence of excess ICPr-O-NA or ICPrc-NH-NA (c = 2 mM; T = 37 °C, PBS:DMSO (9:1)) to a pseudo-first order rate equation provided the bimolecular rate constants k_2(ICPr-O-NA) = 4.0 ± 0.2 $M^{-1}s^{-1}$ and k_2(ICPrc-NH-NA) = 1.1 ± 0.2 $M^{-1}s^{-1}$, respectively. Release of the fluorophores in PBS from the postulated 3-oxopropyl intermediate was delayed relative to the reaction of the ICPr/ICPrc-groups with PEG-DPTz ($k_{1,elim}$(HO-NA) = 1.6 × 10^{-4} s^{-1}; $k_{1,elim}$(H_2N-NA) = 5.00 × 10^{-5} s^{-1}; Fig. 2d) in agreement with studies of cargo release from such groups.[15,18] The aldehyde intermediate was detectable by NMR in DMSO-d_6:D_2O (9:1) (Fig. S1 in the Supporting Information). To our delight, fluorophore release in diluted human serum (T = 37 °C, PBS:serum (1:1)) reached completion in few minutes because serum albumin catalyzes the β-elimination reaction.[15b,16,18a] The apparent first-order rate constants for this step in serum were calculated as $k_{1,elim}$(ICPr-O-NA) = 4.2 × 10^{-3} s^{-1} and $k_{1,elim}$(ICPrc-NH-NA) = 1.6 × 10^{-3} s^{-1}. In the absence of Tz, ICPr-O-NA and ICPr-NH-NA were completely inert to serum for at least three days (Fig. S5 in Supporting Information). These outcomes demonstrate that stable ICPr/ICPrc-modifications can be rapidly and near-quantitatively removed from phenols and amines under physiological conditions.



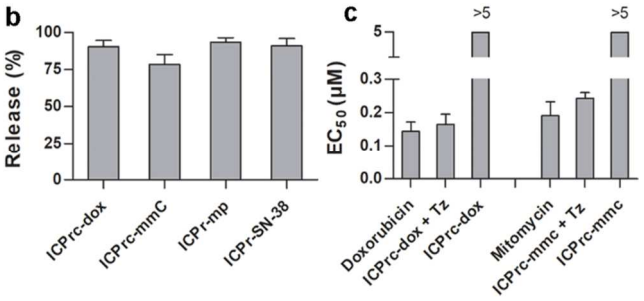


Figure 3. Tetrazine-mediated activation of isocyanopropyl-derivatized prodrugs. (a) Structures of drugs caged with ICPr/ICPrc groups. (b) Release percentages for prodrugs (c(prodrug) = 1 mM, c(DPTz) = 2 mM, PBS:DMSO (1:1), T = 37 °C). (c) EC_{50} values in cytotoxicity studies (A549 cells) of prodrug alone, prodrug + Tz (c = 80 μM) and parent drug (for dose-response curves see Fig. S6 in Supporting Information).

Considering the appeal of bioorthogonal chemistry for prodrug[8c] and therapeutic pretargeting approaches,[7] we evaluated the reaction in the context of anticancer agents (Fig. 3). We synthesized ICPr/ICPrc-prodrugs of doxorubicin (ICPrc-dox), mitomycin C (ICPr-mmc), mercaptopurine (ICPr-mp), and SN-38 (ICPr-SN-38) by reacting the drugs with ICPr-tos or ICPr-nc (see Supporting Information for synthesis). The prodrugs (c = 1 mM) were incubated with DPTz (c = 2 mM) in PBS:DMSO (1:1; T = 37 °C; 2 mM GSH was added with exception of ICPr-SN-38 to suppress Michael addition of acrolein to 6-mercaptopurine) and the release was assessed by HPLC (t = 4 h; Fig. S7 in Supporting Information). Each of the drugs was released in high yields (Fig. 3b; ICPr-SN-38, 91 ± 5 %; ICPrc-dox, 91 ± 4 %; ICPr-mp, 94 ± 3 %; ICPrc-mmC = 79 ± 6 %). To confirm that the released molecules were active, we performed cytotoxicity experiments with ICPr-dox and ICPrc-mmc (Fig. 3c). Combinations of the prodrugs with excess PEG-DPTz (c = 80 μM) elicited dose-dependent cytotoxicity in A549 adenocarcinoma cells. The potencies of the prodrugs combined with PEG-DPTz (EC_{50}(ICPrc-dox) = 0.165 ± 0.035 μM; EC_{50}(ICPrc-mmC) = 0.244 ± 0.017 μM) rivaled those of the free drugs (EC_{50}(dox) = 0.144 ± 0.028 μM; EC_{50}(mmC) = 0.191 ± 0.042 μM). In contrast, the prodrugs alone showed little toxicity in the tested concentration range (EC_{50} > 5 μM; Fig. S6) and PEG-DPTz was non-toxic at concentrations as high as 100 μM. In conclusion, ICPr/ICPrc modifications can be used to generate tetrazine-responsive prodrugs for diverse bioactive compounds.

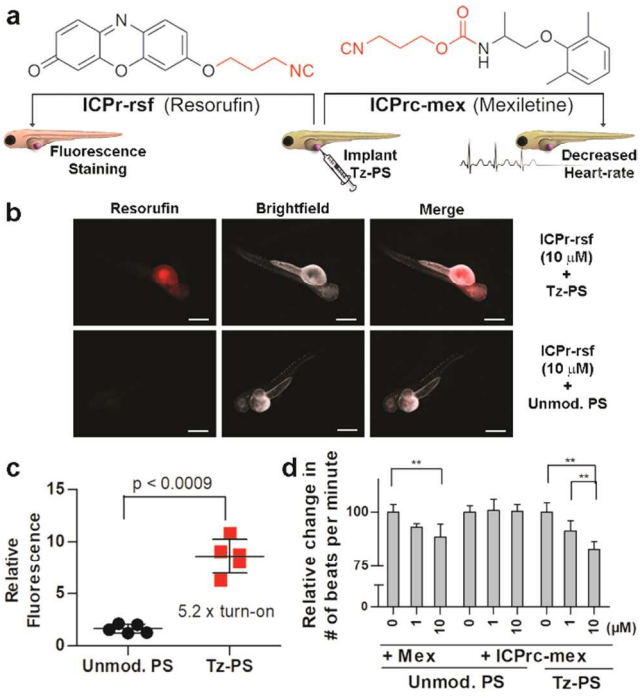


Figure 4. Release of resorufin and mexiletine inside zebrafish embryos upon the reaction of ICPr/ICPrc groups with tetrazine-modified beads (Tz-PS). (a) Cartoon representation of experiments involving the implantation of zebrafish with Tz-PS followed by incubation with either ICPr-rsf for fluorescence imaging or ICPrc-mex leading to a decreased heart rate. (b) Detection of resorufin fluorescence upon tetrazine-mediated uncaging in zebrafish (scale bar = 200 μm). (c) Fluorescence increase in zebrafish with either Tz-PS or unmodified beads in when incubated with ICPr-rsf (c = 10 μM; t = 8 h). (d) Decrease in heart rate in zebrafish implanted with either Tz-PS or unmodified beads treated with ICPrc-mex (t = 8 h; normalized to heart rate at t = 0 h; ** = p-value < 0.005).

Having established that it is possible to remove ICPr/ICPrc groups in aqueous solution at room temperature, we aimed to demonstrate that this chemistry allows for activation of molecules in vivo using zebrafish embryos as model organisms (Fig. 4a). A Tz-modified polystyrene bead (Tz-PS) was implanted into the yolk sac of zebrafish embryos followed by incubation with ICPr-rsf (c = 10 μM). Fish implanted with Tz-PS exhibited a significantly higher fluorescence staining when exposed to ICPr-rsf than controls with unmodified beads (5.2-fold, p-value = <0.0009, t = 8 h; Fig. 4b,c). To corroborate that ICPr chemistry is compatible with living systems, we analyzed the phenomenological effects of releasing an active drug inside zebrafish (Fig. 4a). We synthesized an ICPr-prodrug of mexiletine (ICPrc-mex, Fig. 4a), a voltage-gated sodium channel blocker, which induces cardiac arrhythmia and decreases heart rate.[19] Incubation in ICPrc-mex containing medium (c = 0, 1, 10 μM) caused a dose-dependent decrease in heart rate in fish with implanted Tz-PS similar to the effect observed for the free drug, whereas no changes were observed in control fish bearing unmodified beads (Fig. 4c). These experiments demonstrate that ICPr/ICPrc-derivatized molecules can be effectively unmasked in living organisms and liberate active compounds.

In conclusion, the study established the usefulness of ICPr/ICPrc moieties as masking groups that can be removed by reaction with Tz. In a series of steps, we demonstrated that the bimolecular reaction occurred rapidly, that release yields were near-quantitative, and that the chemistry was compatible with diverse molecules including reporter fluorophores and cytotoxic agents. Experiments in zebrafish models exemplified the utility of the chemistry for in vivo drug and probe release, and efforts to test the reaction in mammalian cells and other animals are ongoing. A limitation of the ICPr/ICPrc groups is the delayed elimination of molecules from the 3-oxopropyl intermediate. In addition to the possibility of using albumin to accelerate the release,[18a] we will explore simple structural modifications to the design to afford near-instantaneous release. The release of acrolein is a possible limitation of this reaction in drug delivery; however, acrolein-releasing prodrugs are in clinical use and addition of Mesna can effectively minimize associated adverse effects. An intriguing aspect of ICPr/ICPRc groups is their structural compactness. Such moieties might be engineered into proteins for chemical control of activity while minimally disrupting their secondary structure or alternatively be used for designing prodrugs with little impact on their pharmacokinetics. The ease of synthesis will further make the outlined chemistry attractive for diverse applications in chemical biology and smart therapeutics.

ASSOCIATED CONTENT

Supporting Information

Experimental details, additional data, and characterization of synthetic intermediates (PDF).

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Author Contributions

‡ These authors contributed equally.

ACKNOWLEDGMENT

R.M.F. gratefully acknowledges financial support from the University of Utah, the Huntsman Cancer Institute, and the USTAR initiative. This work was supported by the L. S. Skaggs Presidential Endowed Chair (R.T.P.).

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