Fast and Sensitive Pretargeted Labeling of Cancer Cells via Tetrazine/Trans-Cyclooctene Cycloaddition

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Supporting Information

Materials and Methods

General materials and methods. All chemicals were purchased from Sigma Aldrich unless noted and were used as received. The amine reactive cyanine dye Vivo-Tag 680 (VT680) and cyanine dye Genhance 680 were purchased from VisEn Medical (Bedford, MA), Alexa Fluor 555 (AF555) was purchased from Invitrogen (Carlsbad, CA), and Dylight 488 was purchased from Thermo Fisher Scientific (Rockford, Il). All solvents were of reagent grade or higher and were used without further purification. Analytical HPLC and LC/MS were performed on a Waters 2695 HPLC equipped with a 2996 diode array detector, a Micromass ZQ4000 ESI-MS module, and a Grace-Vydac RPC18 column (model 218TP5210) at a flow rate of 0.3 mL/min. For all HPLC runs, solvent A consists of water with 0.1% TFA and solvent B is composed of acetonitrile with 10% water and 0.1% TFA. All UV/vis spectra and kinetics experiments were recorded on a Thermoscientific Nanodrop 1000 spectrophotometer. All kinetics data were calculated using Prism 4 for Mac.

Synthetic Methods

Tetrazine amine (1) and tetrazine VT680 were synthesized as previously reported.[1]

(*Z*)-9-Oxabicyclonon-4-ene was purchased from Aldrich and used as received. The synthesis of (Z)-cyclooct-4-enol was performed as previously reported.[2]

(*E*)-cyclooct-4-enol (2) was synthesized from (Z)-cyclooct-4-enol using a modification of a previously reported protocol.[3] Briefly, 1 gram of cyclooctenol (2) and 1.1 g methyl benzoate sensitizer was added to 250 mL solvent (9:1 Ether:Hexanes) in a 500 mL quartz reaction vessel (Southern New England Ultraviolet Company). No attempt to degas the solution was made. The vessel was irradiated with 254 nm light in a Rayonet RPR-100 UV reactor (Southern New England Ultraviolet Company) under constant stirring. At 30 minute intervals, the irradiation was stopped and the entire solution was passed through a column packed with silver nitrate (10%) impregnated silica (commercially available from Aldrich). The solution that passes through was then transferred back into the quartz flask and irradiation was continued. After 6 hours the irradiation was stopped and the silica was added to a solution of ammonium hydroxide and stirred for 5 minutes after which ether was added and stirring continued for 5 more minutes. After stirring the ether phase was decanted, washed with water, dried with magnesium sulfate, and evaporated yielding trans-cyclooctenol (40%) as a mixture of isomers as previously reported. The isomers were separated by column chromatography (1:1 Ethyl Acetate Hexanes) and verified by proton NMR using the previously reported proton NMR spectra.[3] The major isomer (more polar isomer) was used for the synthesis of (4)

(*E*)-cyclooct-4-enyl 2,5-dioxopyrrolidin-1-yl carbonate (4). 50 mg of (*E*)-cyclooct-4-enol (major isomer) and 0.2 mL triethylamine were added to 3 mL anhydrous acetonitrile. To this solution was slowly added 250 mg of N,N'-Disuccinimidyl carbonate. The reaction mixture was stirred at room temperature until thin layer chromatography revealed that the reaction was complete

(approximately 48 hours). The acetonitrile was removed by rotary evaporation and the remaining residue was suspended in ether, washed with 0.1M HCl followed by brine, and dried with magnesium sulfate. The ether was evaporated and the resulting oil was purified by column chromatography (1:1 Ethyl Acetate:Hexane) yielding 80 mg (75% yield) of the title compound. ^{1}H NMR (400 MHz $CDCl_3$): δ 5.65-5.54 (m, 1H), 5.5-5.4 (m, 1H), 4.5-4.4 (m, 1H), 2.88-2.78 (s, 4H), 2.45-2.3 (m, 2H), 2.2-1.5 (m, 8H).

HPLC Characterization of reaction between tetrazine amine (1) with trans-cyclooctenol (2)

Tetrazine amine (1) and trans-cycloctenol (2) were combined in 100 µL of PBS at a final concentration of 1 mM for each reagent. The solution was agitated for 10 minutes at room temperature and then analyzed by HPLC and LC-MS (Figures S1 and S2). Multiple peaks were identified with molecular mass corresponding to dihydropyrazine adducts or oxidation products of the adducts. The multiple peaks are expected given the previously demonstrated potential to form several regioisomeric products.[1, 3] The yield of the reaction, based on the remaining signal from the tetrazine amine (1) was greater than 95%.

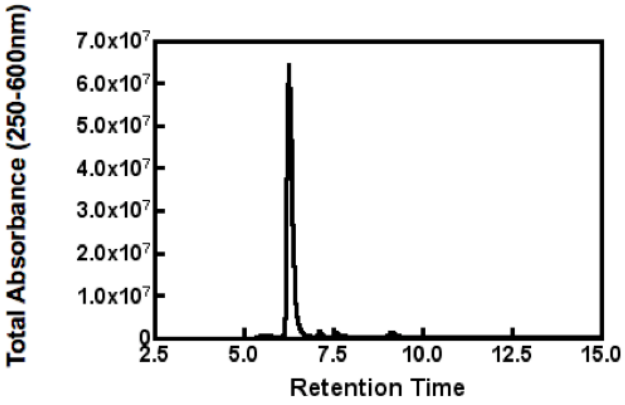

Figure S1:HPLC trace of purified tetrazine amine (1) using a gradient of 0 to 50 % buffer B over 15 min.

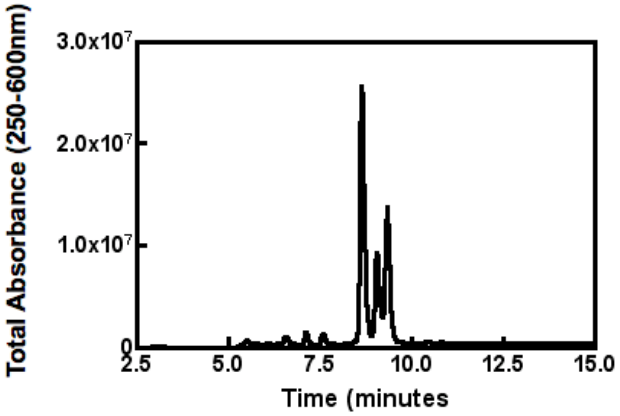

Figure S2: HPLC trace of the reaction products from addition of tetrazine amine (1) to trans-cyclooctene (2). All peaks gave mass identities corresponding to dihydropyrazine adducts.

Labeling Antibody with trans-cyclooctene

Cetuximab (ImClone 2 mg/mL) was purchased and the solvent exchanged for 0.1M $NaHCO_3$ buffered at pH 8.5 with a final concentration of 7 mg/mL. To 200 µL of this stock solution was added 10 µL of DMF. (*E*)-cyclooct-4-enyl 2,5-dioxopyrrolidin-1-yl carbonate was dissolved in anhydrous DMF to make a 40 mM stock solution. For conjugation, the appropriate excess of amine reactive trans-cyclooctene in DMF was aliquoted into the antibody solution, vortexed, and reacted overnight at 4 ^{o}C. In the experiments reported, the final trans-cycloctene loadings of 1, 3, 5, and 6 correspond to using 2, 10, 30, and 100 equivalents of succinimidyl carbonate with respect to antibody. After overnight reaction the antibodies were purified by centrifuge filtration using 5% DMSO PBS, concentrated to 2 mg/mL and stored in PBS at 4 ^{o}C.

Antibody Labeling with Fluorescent Succinimidyl Esters

A solution of antibody (1 mg/mL) in 0.1M NaHCO3 (pH 8.5) was incubated with 2 equivalents of fluorescent succinimidyl ester (VT680, AF555, or Dylight 488) for 2 hours. After incubation, the antibody was purified by centrifuge filtration using 30000 dalton molecular weight cutoff filters (Amicon) and stored in PBS. The number of fluorochromes per antibody was determined by spectrophotometric analysis and determined to be approximately 1 per antibody for all dye succinimidyl esters used.

Kinetic Measurements

trans-cyclooctene modified antibody was physically absorbed onto polystyrene by immersing the surfaces in a 0.1 mg/mL solution of antibody in PBS for 3 hours. After numerous washes with PBS, the surface was exposed to 750 nM tetrazine VT680 in PBS at 37 ^{o}C. After 5 minutes, the tetrazine solution was removed and the surface washed 3 times with PBS. The fluorescence due to the VT680 dye was measured on a fluorescence plate reader (Tecan Safire 2) and corrected for background fluorescence. The surface was again exposed to the tetrazine solution and the entire process repeated at 10, 15, 30, and 60 minutes. The fluorescence measurements were plotted versus time, fitted to a first order exponential growth curve and the pseudo first order rate constant determined (Figure S3 Left). The entire experiment was repeated using two different concentrations of tetrazine (375 nM and 1000 nM) and the pseudo first order rate constants from all three experiments were plotted versus concentration, fitted to a straight line, and the slope taken as the second order rate constant.

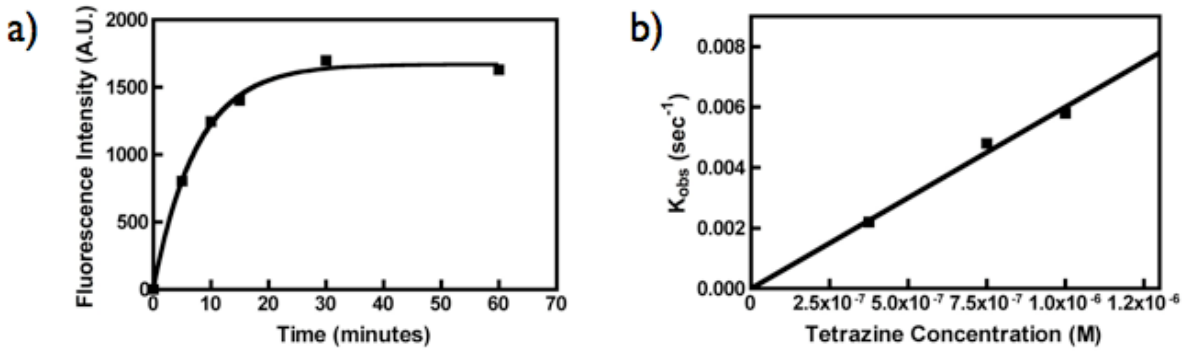

Figure S3: Kinetics of tetrazine VT680 cycloaddition to trans-cyclooctene modified antibody absorbed to a polystyrene plate. (A) Fluorescence monitoring of tetrazine VT680 cycloaddition to a surface array of trans-cyclooctene antibody (1 reactive trans-cyclooctene). The data were fit to a first order exponential (black line). From the fit a pseudo first order rate constant (k_{obs}) is determined. (B) A plot of k_{obs} versus the concentration of tetrazine VT680. The data were fit using linear

regression (black line). The slope of the line was 6000±200 $M^{-1}sec^{-1}$ and was reported at the second order rate constant for the reaction between tetrazine VT680 and trans-cyclooctene bound to antibody.

Cell Culture

The human lung adenocarcinoma epithelial cell line A549 was selected for all experiments due to its mid-level over-expression of EGFR. The cell line was maintained in a standard ATCC formulated F-12K media supplemented with 10% fetal bovine serum and 5% penicillin/streptomycin. In order to facilitate microscopy and visualize intracellular morphology, EGFP labeling of the cell line was done using a third-generation lentiviral vector system. 293T cells were transfected using lipofectamine 2000 in a subconfluent 10-cm dish with the vector pCCLsin.PPT.hPGK (10 µg), into which EGFP had been cloned, as well as pMDLg/p packaging (7 µg) and VSV-G envelope encoding pMD.G (5 µg) plasmids. These plasmids were obtained from Rafaella Sordella at the MGH Center for Cancer Research and Luigi Naldini at the San Raffaele Telethon Institute for Gene Therapy. Viral supernatant was collected after 48 hours, filtered with a 0.45 micron syringe filter, and stored at -80°C. The A549 cell line was infected in subconfluent wells of 24-well plates, using 300 µL of virus in 1 mL of F-12K culture media with 10% fetal calf serum. This protocol yielded an infection rate in excess of 80% (determined by visual assessment using fluorescence microscopy). EGFP-negative cells were removed using a modified 5-laser Becton-Dickinson FACSDiVa with standard techniques.

Confocal Microscopy

Cells were grown on break away glass chamber slides and washed six times after administering either imaging agent. A multichannel upright laser-scanning confocal microscope (FV1000; Olympus) was used to image live cells with a 60X water immersion objective lens. Image collection and fluorophore excitations with lasers at 488nm (EGFP), 543nm (AF555), and 633nm (VT680) were done serially to avoid cross talk between channels. Data were acquired with Fluoview software (version 4.3; Olympus) and image stacks were processed and analyzed with ImageJ software (version 1.41, Bethesda MD).

Flow cytometry

Confluent A549 cells were suspended using 0.05% Tryspin/0.53 mM EDTA, washed by centrifugation with PBS containing 2% FBS (PBS+), and $2.5x10^5$ cells were added to microcentrifuge tubes. Cetuximab antibody with the following modifications was then added at 10 µg/ml concentration in 100 µL PBS+: none (control), 1 trans-cyclooctene per antibody, 3 trans-cyclooctene per antibody, 5 trans-cyclooctene per antibody, 6 trans-cyclooctene per antibody. Following incubation for 15 minutes at room temperature, samples were washed with PBS+. For stability studies, trans-cyclooctene antibodies were labeled with Dylight 488 fluorophore (Pierce, ~1 per antibody) and the cells were resuspended in 100 µL PBS+ and incubated for 15, 30, or 60 minutes at 37ºC before addition of 1 ml PBS+ and 2 washes by centrifugation. For clicking studies, labeled cells were resuspended in 100 µL FBS containing 500 nM tetrazine-VT680 and incubated for 30 minutes at 37ºC before addition of 1 ml PBS+ and 2 washes by centrifugation. VT680 and DyeLight-488 fluorescence was assessed using an LSRII flow cytometer (Becton Dickinson) and analyzed using FlowJo software.

Confocal Microscopy of Control Experiments

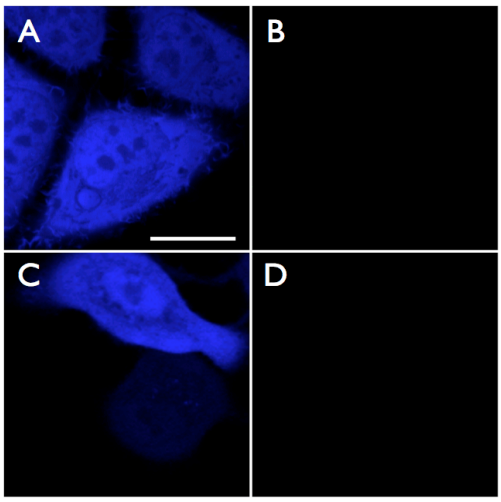

Figure S4: Confocal microscopy of GFP-positive A549 lung cancer cells after control labelings. Cells were exposed to unlabeled Cetuximab antibody, washed, exposed to tetrazine-VT680 probe (500 nM 10 minutes 100% FBS 37°C), washed, and imaged in the GFP channel (Panel A, White scale bar in top left panel denotes 30 microns) and the near-infrared channel (Panel B). In a separate control experiment, cells were exposed to Cetuximab/trans-cyclooctene antibodies, washed, exposed to unlabeled VT680 (Genhance 680, 500 nM 10 minutes 100% FBS 37°C, VisEn Medical), washed, and imaged in the GFP channel (Panel C) and the near-infrared channel (Panel D).

Stability data of trans-Cyclooctene modified Cetuximab

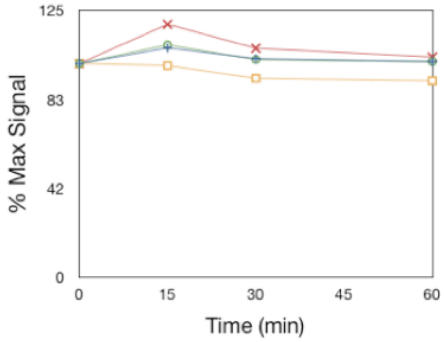

Figure S5: Stability study of Dylight 488 Cetuximab modified with either 1 (blue), 3 (green), 5 (yellow), or 6 (red) reactive trans-cyclooctene. A549 cells were exposed to antibody, washed, and the fluorescence intensity monitored with time using flow cytometry.

References

[1] Devaraj, N. K.; Weissleder, R.; Hilderbrand, S. A. Bioconjug Chem 2008, 19, 2297-2299.
[2] Hillmyer, M. A.; Laredo, W. R.; Grubbs, R. H. Macromolecules 1995, 28, 6311-6316.
[3] Royzen, M.; Yap, G. P.; Fox, J. M. J Am Chem Soc 2008, 130, 3760-3761.