Communication

Fc-small molecule antibody mimetics

Erik D Wold, Jun Y Axup, Brunhilde Felding-Habermann, and Vaughn V Smider

Bioconjugate Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.bioconjchem.5b00530 • Publication Date (Web): 04 Nov 2015

Fc-small molecule antibody mimetics

Erik D. Wold,[†] Jun Y. Axup,[†] Brunhilde H. Felding,*‡ Vaughn V. Smider*[§]

[†] Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037.

‡ Department of Chemical Physiology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037.

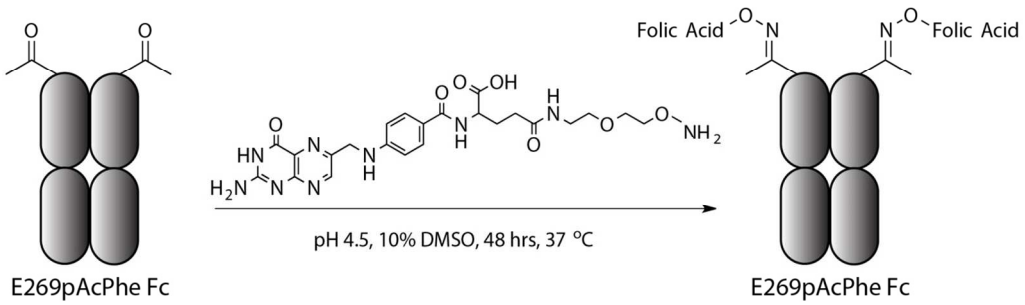
[§] Department of Cell and Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037.

ABSTRACT: Antibody therapeutics are a promising drug class due to their high specificity and favorable pharmacokinetics. While there are many methods for the development of antibodies specific to disease associated antigens, selecting antibodies against functional epitopes with high specificity and affinity can be difficult for certain epitopes. We describe a generalizable method for synthesizing antibody mimetics by site specifically conjugating small molecules (with high affinity and specificity to disease associated antigens) to an Fc fragment to develop drugs with the benefits of an antibody. As a proof of concept, an E269pAcPhe Fc antibody Fc fragment was produced and subsequently site-specifically labeled with a linker-modified folic acid compound to generate an Fc-folic acid antibody-mimetic. This was chosen as the model system because the high-affinity folate receptor FR-α is highly expressed in a number of cancer types including breast and ovarian cancer. The specificity of the Fc-folic acid conjugate was assessed via flowcytometry with the folate-receptor positive breast cancer cell line MDA-MB-231 by measuring Fc-folic acid binding in both the absence and presence of an excess of folic acid. Fc-small molecule conjugates could be developed into a unique class of antibody-like therapeutics.

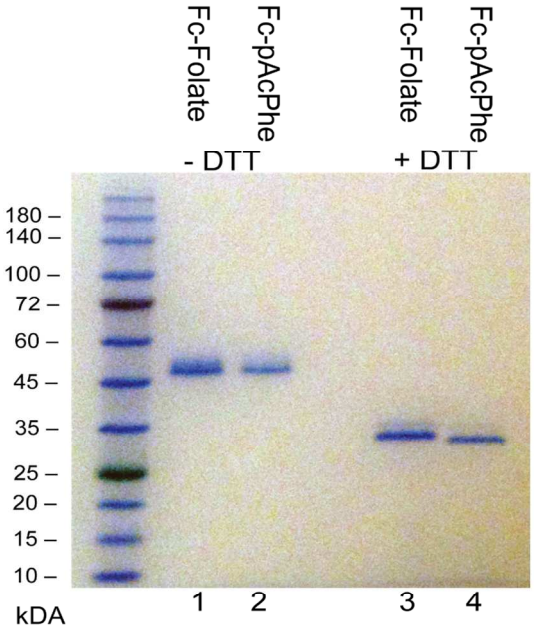
Monoclonal antibody (mAb) therapeutics are a successful and rapidly expanding class of pharmaceuticals due to their high specificity (through the high affinity, bivalent Fab regions of the IgG), activity (either through direct agonist/antagonist activity via antigen binding or via antibody mediated effector function), favorable pharmacokinetics (neonatal Fc receptor (FcRn) binding prevents antibody degradation and increases serum half-life), and standardized manufacturing processes.[1-9] While there are many techniques for the discovery of antibodies against important disease associated antigens, finding antibodies that bind defined functional epitopes with high specificity and affinity can be difficult. Classical hybridoma mAb discovery requires animal immunization, B-cell fusion, clonal screening, and antibody gene cloning before preclinical testing of the mAb as a potential therapeutic can begin.[10-12] Additionally, many hybridoma derived mAbs will have to be humanized before clinical application.[13] Display based approaches circumvent many of the issues associated with animal immunization and hybridoma development. Antibody display allows for the direct selection of fully human antibodies, and the display systems are designed such that much of the cell and molecular biology associated with hybridoma development is unnecessary.[14] However, despite the improvements over classical mAb development approaches, antibody display requires multiple rounds of selection, clonal screening, and can often result in mAbs that bind non-functional epitopes.[14-16]

Recently, in efforts to avoid the procedurally intensive and time consuming process of antibody selection, small molecules that target cell surface receptors conjugated onto antibody scaffolds have been utilized as antibody-like molecules. This enables one to take advantage of the favorable characteristics of small molecules and peptides to achieve target specificity, while still retaining the desired properties of mAb therapeutics.[17]

One technology utilizing small molecules as the



Scheme 1. Synthesis of the Fc-Folate antibody mimetic. E269*p*AcPhe Fc (10 mg/mL) was treated with 30 equivalents of aminoxy-modified folic acid in 100 mM sodium acetate (pH 4.5) with 10% DMSO for 48 hrs at 37 °C. Reaction resulted in quantitative yield verified by SDS-PAGE and ESI-MS.



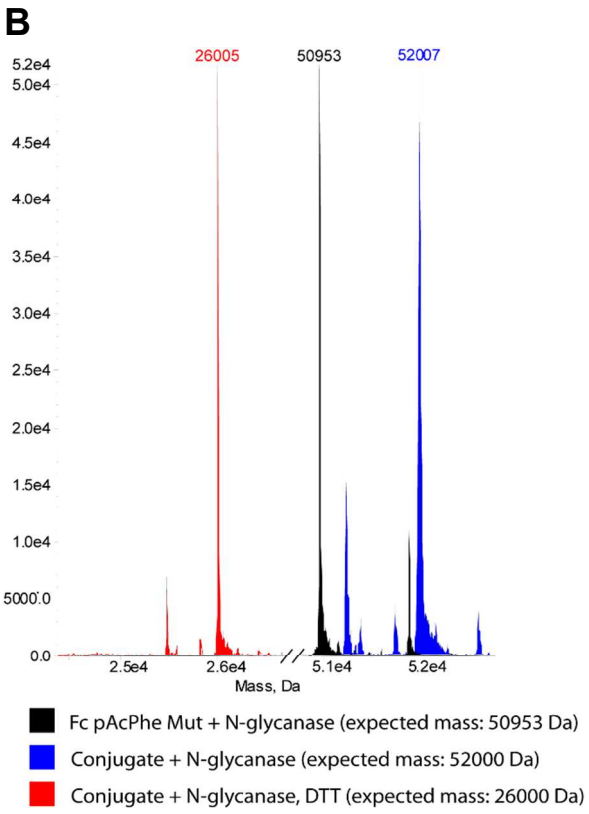


Figure 1. A) SDS-PAGE gel of the Fc-Folate conjugate and the E269pAcPhe Fc starting material. B) ESI-MS data of the Fc-Folate conjugate and the E269pAcPhe Fc starting material.

source of affinity and specificity are Covx-bodies. Covx-bodies, developed by Barbas III et al., utilize a humanized murine aldolase catalytic antibody as the scaffold, and conjugate the small molecule (often peptide ligand mimetics) to the highly nucleophilic lysine in the catalytic active site.[18] Because Covx-bodies are built on the aldolase IgG scaffold, the resulting drugs retain desirable activity and PK.[19] Since every Covx-body is built on the same humanized aldolase mAb, it allows for the rapid development of mAb-like therapeutics against diverse targets.[18-24] The humanized aldolase mAb platform is also a potential drawback of this technology, as humanized antibodies, while they have a substantial reduction in immunogenicity from their murine-derived precursors, still have higher immunogenicity than fully human antibodies.[25-28] Additionally, nearly 2/3 of the Covx-body (the Fab regions) serve as a scaffold for the small molecule. Ideally, an optimized construct would be comprised of a small molecule conjugated directly to the Fc region to eliminate unnecessary and potentially immunogenic protein sequence.

An approach for generating an Fc-small molecule construct was recently described by Chiang et al., wherein expressed protein ligation was utilized to label the C-terminus of an Fc fragment with a high affinity small molecule.[29] This process circumvents some of the drawbacks associated with the Covx-body approach while still retaining the desired effector functions of the Fc domain, however, it is potentially limited by the use of expressed protein ligation. Expressed protein ligation, while a powerful tool in protein engineering, limits labeling of proteins to the C-terminus, limits the chemistries that can be utilized for conjugation, and only allows for a single conjugation site per translated protein (*e.g.* 2 total sites for the Fc homodimer).[30]

Herein, we describe a generalizable method for the synthesis of mAb-like small molecule-antibody mimetics via the site-specific conjugation of high affinity small molecules to a human Fc antibody fragment. The Fc-small molecule conjugate relies on the small molecule for epitope binding, and the Fc portion would provide effector function and half-life of a traditional mAb therapeutic. To enable bio-orthogonal, site-specific small molecule conjugation, we expressed a E269pAcPhe Fc (from human IgG1) antibody fragment using an orthogonal amber suppressor aminoacyl-tRNA synthetase/tRNA pair specific for pAcPhe via previously reported methods.[31,32] Briefly, recombinant Fc containing the pAcPhe UAA was produced by co-expressing, via transient transfection into HEK293-F cells, an orthogonal tRNA/aminoacyl-tRNA synthetase (aaRS) pair specific for pAcPhe and an Fc gene containing a TAG codon at residue E269 (Figure S1) and expressing for 7 days in the presence of 1.3 mM pAcPhe amino acid. The E269pAcPhe Fc provides a platform for which any aminoxy modified molecule can be conjugated to the incorporated ketone moeity in order to produce an antibody mimetic via an oxime linkage at a position that should not interfere with Fc binding or effector function.[33-36] Utilizing a site-specifically incorporated unnatural amino acid for the small molecule conjugation (as opposed to conventional lysine or cysteine conjugation) allows for the generation of a homogenous product, both in terms of conjugate loading and conjugate location. This helps assure that final product has reproducible binding characteristics and that the small molecule conjugation will not disrupt the desired effector functions of the Fc.[37,38] Additionally, this method enables the flexibility to manipulate the construct

at multiple different position; UAA incorporation and biorthogonal conjugation allows for conjugation at any surface exposed site in the protein (including the N or C termini) and even allows incorporation of multiple UAAs (either the same UAA or different UAAs).[39-41]

As a proof of concept, we chose to conjugate folic acid, which binds with very high specificity and affinity to the high affinity folate receptor FR-α.[42] FR-α is overexpressed in a number of cancer types including breast, ovarian, and lung cancer, has been explored as a potential candidate for targeted cancer therapeutics, and

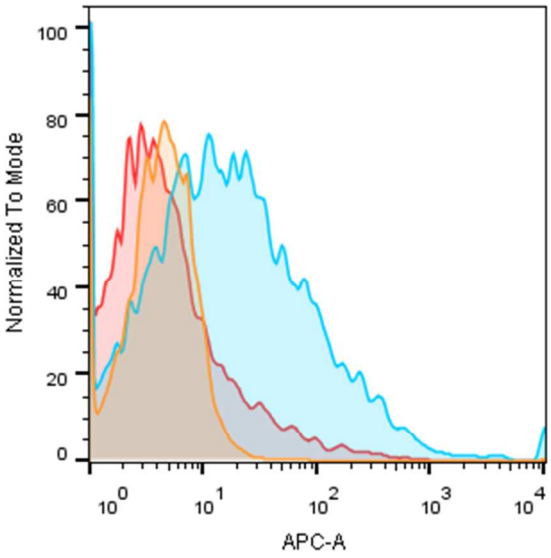


Figure 2. Specificity of Folate-Fc binding to human breast cancer cells. Flow cytometry analysis of Folate-Fc binding to FR-α+ MDA-MB-231 cells detected with an allophycocyanin (APC) conjugated anti-human Fc mAb secondary antibody. Orange is secondary alone, blue is Folate-Fc and secondary and red is Folate-Fc, secondary, and 10eq of free folic acid. Conjugate binding was detected with an allophycocyanin (APC) conjugated anti-human Fc mAb secondary antibody.

higher expression levels is associated with poorer outcome.[43-48] Before folic acid conjugation could be accomplished, first the folic acid had to be aminoxy modified. The folic acid was modified at the carboxylic acid moieties, using previously reported chemistry, adding a short linker and the desired aminoxy group (Scheme S1, Scheme S2).[17,49] The modified folic acid was then used to generate the site specific Fc-folic acid conjugate via the bio-orthogonal oxime ligation (Scheme 1).[31-38] After the 48 hour reaction, the conjugate was purified and buffer exchanged via PD-10 desalting column, before being analyzed for conjugation efficiency and purity via SDS-PAGE and ESI-MS (Figure 1). The reaction proceeded in quantitative yields and as shown in the ESI-MS data, has exactly two conjugates per Fc, with one folic acid per each of the monomers that make up the Fc homodimer.

The specificity of the Fc-folic acid conjugate was assessed via flow cytometry with the folate-receptor positive breast cancer cell line MDA-MB-231(Figure 2). Fc-folic acid binding was assessed in both the absence and presence of an excess of free folic acid. The results revealed that the Fc-folic acid conjugate binds to a cell line which expresses the folate receptor, and showed that the binding is specific to that receptor, as the conjugate can be competed off by an excess of free ligand (10-fold molar excess). Thus, the Fc-folic acid conjugate represents a specific reagent to report FR-positive cells, such as breast cancer cells, and enables Fc-dependent strategies for recognition and targeting.

In summary, we report the development of a generalizable method for the synthesis of site-specifically conjugated Fc-small molecule antibody mimetics. As an example we synthesized an Fc-folic acid conjugate, and showed that it specifically bound to the folate receptor on the folate receptor positive human breast cancer cell line MDA-MB-231. One can envision this technology being applied to other targets with associated high affinity small molecule binders. Such molecules include 2-[3-(1,3-dicarboxy propyl)-ureido] pentanedioic acid (DUPA) which specifically binds and inhibits the protease active site in the prostate specific membrane antigen (PSMA), a specific target in prostate cancer, ligand mimetic peptides against cancer associated integrins (*e.g.* RGD peptides against αvβ1, αvβ3, and αvβ5), and ligands and ligand mimetics of cancer associated peptide receptors (e.g. calcitonin and endothelin receptors).[50-52] In particular, this method would enable the targeting of epitopes that have been historically difficult to select antibodies against (but have available small molecule binders), such as the active sites of enzymes like neuraminidase (*e.g.* Oseltamivir) for the treatment of influenza, or several cancer relevant cell surface enzymes including membrane anchored serine proteases like PSMA.[53-57] Additionally, because the Fc portion of the conjugate should provide both the activity and desirable pharmacokinetics of the conjugate, it enables using molecules with high affinity binding characteristics, but without any innate activity (such as many aptamers), to be used in potential therapeutically relevant applications.[58-62]

Associated Content

Supporting information: Experimental procedures, characterization data, and supplementary figures. This material is available free of charge via the internet at http://pubs.acs.org.

Author Information

Corresponding Author:

*vvsmider@scripps.edu

*brunie@scripps.edu

Acknowledgements

This work was supported by NIH grant 1RC1EBO10745 and American Cancer Society grant RSG-09-1601 (V.V.S.).

References

(1) Akilesh, S.; Huber, T. B.; Wu, H.; Wang, G.; Hartleben, B.; Kopp, J. B.; Miner, J. H.; Roopenian, D. C.; Unanue, E. R.; Shaw, A. S. *Proc Natl Acad Sci U S A* **2008**, *105*, 967.

(2) Ghetie, V.; Popov, S.; Borvak, J.; Radu, C.; Matesoi, D.; Medesan, C.; Ober, R. J.; Ward, E. S. *Nat Biotechnol* **1997**, *15*, 637.

(3) Kacskovics, I.; Kis, Z.; Mayer, B.; West, A. P., Jr.; Tiangco, N. E.; Tilahun, M.; Cervenak, L.; Bjorkman, P. J.; Goldsby, R. A.; Szenci, O.; et.al. *Int Immunol* **2006**, *18*, 525.

(4) Natsume, A.; Niwa, R.; Satoh, M. *Drug Des Devel Ther* **2009**, *3*, 7.

(5) Nelson, A. L.; Reichert, J. M. *Nat Biotechnol* **2009**, *27*, 331.

(6) Ober, R. J.; Radu, C. G.; Ghetie, V.; Ward, E. S. *Int Immunol* **2001**, *13*, 1551.

(7) Sarmay, G.; Lund, J.; Rozsnyay, Z.; Gergely, J.; Jefferis, R. *Mol Immunol* **1992**, *29*, 633.

(8) Scott, A. M.; Wolchok, J. D.; Old, L. J. *Nat Rev Cancer* **2012**, *12*, 278.

(9) Strohl, W. R. *Curr Opin Biotechnol* **2009**, *20*, 685.

(10) Jin, A.; Ozawa, T.; Tajiri, K.; Obata, T.; Kondo, S.; Kinoshita, K.; Kadowaki, S.; Takahashi, K.; Sugiyama, T.; Kishi, H.; Muraguchi, A. *Nat Med* **2009**, *15*, 1088.

(11) Kohler, G.; Milstein, C. *Nature* **1975**, *256*, 495.

(12) Love, J. C.; Ronan, J. L.; Grotenbreg, G. M.; van der Veen, A. G.; Ploegh, H. L. *Nat Biotechnol* **2006**, *24*, 703.

(13) Clark, M. *Immunol Today* **2000**, *21*, 397.

(14) Hoogenboom, H. R. *Nat Biotechnol* **2005**, *23*, 1105.

(15) Levitan, B. *J Mol Biol* **1998**, *277*, 893.

(16) Mao, H.; Graziano, J. J.; Chase, T. M. A.; Bentley, C. A.; Bazirgan, O. A.; Reddy, N. P.; Song, B. D.; Smider, V. V. *Nat Biotechnol* **2010**, *28*, 1195.

(17) Li, H.; Lu, Y.; Piao, L.; Wu, J.; Yang, X.; Kondadasula, S. V.; Carson, W. E.; Lee, R. J. *Bioconjug Chem* **2010**, *21*, 961.

(18) Rader, C.; Turner, J. M.; Heine, A.; Shabat, D.; Sinha, S. C.; Wilson, I. A.; Lerner, R. A.; Barbas, C. F. *J Mol Biol* **2003**, *332*, 889.

(19) Popkov, M.; Rader, C.; Gonzalez, B.; Sinha, S. C.; Barbas, C. F., 3rd *Int J Cancer* **2006**, *119*, 1194.

(20) Bower, K. E.; Lam, S. N.; Oates, B. D.; del Rosario, J. R.; Corner, E.; Osothprarop, T. F.; Kinhikar, A. G.; Hoye, J. A.; Preston, R. R.; Murphy, R. E.; et.al. *J Med Chem* **2011**, *54*, 1256.

(21) Coronella, J.; Li, L. N.; Johnson, K.; Pirie-Shepherd, S.; Roxas, G.; Levin, N. *Anticancer Res* **2009**, *29*, 2243.

(22) Doppalapudi, V. R.; Tryder, N.; Li, L. N.; Aja, T.; Griffith, D.; Liao, F. F.; Roxas, G.; Ramprasad, M. P.; Bradshaw, C.; Barbas, C. F. *Bioorg Med Chem Lett* **2007**, *17*, 501.

(23) Li, L.; Leedom, T. A.; Do, J.; Huang, H.; Lai, J.; Johnson, K.; Osothprarop, T. F.; Rizzo, J. D.; Doppalapudi, V. R.; Bradshaw, C. W.; et.al. *Transl Oncol* **2011**, *4*, 249.

(24) Li, L. S.; Rader, C.; Matsushita, M.; Das, S.; Barbas, C. F., 3rd; Lerner, R. A.; Sinha, S. C. *J Med Chem* **2004**, *47*, 5630.

(25) Pendley, C.; Schantz, A.; Wagner, C. *Curr Opin Mol Ther* **2003**, *5*, 172.

(26) Harding, F. A.; Stickler, M. M.; Razo, J.; DuBridge, R. B. *Mabs* **2010**, *2*, 256.

(27) Hwang, W. Y. K.; Foote, J. *Methods* **2005**, *36*, 3.

(28) Stephens, S.; Emtage, S.; Vetterlein, O.; Chaplin, L.; Bebbington, C.; Nesbitt, A.; Sopwith, M.; Athwal, D.; Novak, C.; Bodmer, M. *Immunology* **1995**, *85*, 668.

(29) Chiang, M. J.; Holbert, M. A.; Kalin, J. H.; Ahn, Y. H.; Giddens, J.; Amin, M. N.; Taylor, M. S.; Collins, S. L.; Chan-Li, Y.; Waickman, A.; et.al. *J Am Chem Soc* **2014**, *136*, 3370.

(30) Muir, T. W.; Sondhi, D.; Cole, P. A. *Proc Natl Acad Sci U S A* **1998**, *95*, 6705.

(31) Kularatne, S. A.; Deshmukh, V.; Ma, J.; Tardif, V.; Lim, R. K.; Pugh, H. M.; Sun, Y.; Manibusan, A.; Sellers, A. J.; Barnett, R. S.; et.al. *Angew Chem Int Ed Engl* **2014**, *53*, 11863.

(32) Lu, H.; Wang, D.; Kazane, S.; Javahishvili, T.; Tian, F.; Song, F.; Sellers, A.; Barnett, B.; Schultz, P. G. *J Am Chem Soc* **2013**, *135*, 13885.

(33) Hutchins, B. M.; Kazane, S. A.; Staflin, K.; Forsyth, J. S.; Felding-Habermann, B.; Schultz, P. G.; Smider, V. V. *J Mol Biol* **2011**, *406*, 595.

(34) Hutchins, B. M.; Kazane, S. A.; Staflin, K.; Forsyth, J. S.; Felding-Habermann, B.; Smider, V. V.; Schultz, P. G. *Chem Biol* **2011**, *18*, 299.

(35) Kazane, S. A.; Sok, D.; Cho, E. H.; Uson, M. L.; Kuhn, P.; Schultz, P. G.; Smider, V. V. *Proc Natl Acad Sci U S A* **2012**, *109*, 3731.

(36) Wang, L.; Zhang, Z.; Brock, A.; Schultz, P. G. *Proc Natl Acad Sci U S A* **2003**, *100*, 56.

(37) Axup, J. Y.; Bajjuri, K. M.; Ritland, M.; Hutchins, B. M.; Kim, C. H.; Kazane, S. A.; Halder, R.; Forsyth, J. S.; Santidrian, A. F.; Stafin, K.; et.al. *Proc Natl Acad Sci U S A* **2012**, *109*, 16101.

(38) Kim, C. H.; Axup, J. Y.; Schultz, P. G. *Curr Opin Chem Biol* **2013**, *17*, 412.

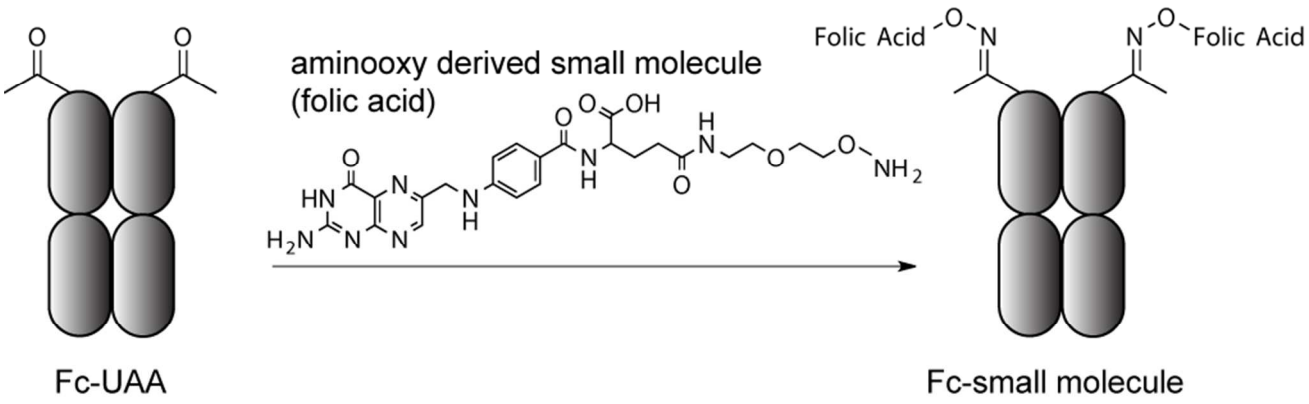
(39) Lammers, C.; Hahn, L. E.; Neumann, H. *Chembiochem* **2014**, *15*, 1800.

(40) Neumann, H.; Wang, K. H.; Davis, L.; Garcia-Alai, M.; Chin, J. W. *Nature* **2010**, *464*, 441.

(41) Schmied, W. H.; Elsasser, S. J.; Uttamapinant, C.; Chin, J. W. *J Am Chem Soc* **2014**, *136*, 15577.

(42) Low, P. S.; Kularatne, S. A. *Curr Opin Chem Biol* **2009**, *13*, 256.
(43) Franklin, W. A.; Waintrub, M.; Edwards, D.; Christensen, K.; Prendegrast, P.; Woods, J.; Bunn, P. A.; Kolhouse, J. F. *Int J Cancer Suppl* **1994**, *8*, 89.
(44) Hartmann, L. C.; Keeney, G. L.; Lingle, W. L.; Christianson, T. J. H.; Varghese, B.; Hillman, D.; Oberg, A. L.; Low, P. S. *Int J Cancer* **2007**, *121*, 938.
(45) Konner, J. A.; Bell-McGuinn, K. M.; Sabbatini, P.; Hensley, M. L.; Tew, W. P.; Pandit-Taskar, N.; Vander Els, N.; Phillips, M. D.; Schweizer, C.; Weil, S. C.;et.al. *Clin Cancer Res* **2010**, *16*, 5288.
(46) Parker, N.; Turk, M. J.; Westrick, E.; Lewis, J. D.; Low, P. S.; Leamon, C. P. *Anal Biochem* **2005**, *338*, 284.
(47) Smith-Jones, P. M.; Pandit-Taskar, N.; Cao, W.; O'Donoghue, J.; Philips, M. D.; Carrasquillo, J.; Konner, J. A.; Old, L. J.; Larson, S. M. *Nucl Med Biol* **2008**, *35*, 343.
(48) Wang, Z. J.; Boddington, S.; Wendland, M.; Meier, R.; Corot, C.; Daldrup-Link, H. *Pediatr Radiol* **2008**, *38*, 529.
(49) Kularatne, S. A.; Deshmukh, V.; Gymnopoulos, M.; Biroc, S. L.; Xia, J.; Srinagesh, S.; Sun, Y.; Zou, N.; Shimazu, M.; Pinkstaff, J.; et.al. *Angew Chem Int Ed Engl* **2013**, *52*, 12101.
(50) Desgrosellier, J. S.; Cheresh, D. A. *Nat Rev Cancer* **2010**, *10*, 9.
(51) Kim, C. H.; Axup, J. Y.; Lawson, B. R.; Yun, H.; Tardif, V.; Choi, S. H.; Zhou, Q.; Dubrovska, A.; Biroc, S. L.; Marsden, R.; et.al. *Proc Natl Acad Sci U S A* **2013**, *110*, 17796.
(52) Reubi, J. C. *Endocrine Reviews* **2003**, *24*, 389.
(53) Bugge, T. H. *J Thromb Haem* **2012**, *10*, E26.
(54) Gubareva, L. V.; Kaiser, L.; Hayden, F. G. *Lancet* **2000**, *355*, 827.
(55) Ivanov, S.; Liao, S. Y.; Ivanova, A.; Danilkovitch-Miagkova, A.; Tarasova, N.; Weirich, G.; Merrill, M. J.; Proescholdt, M. A.; Oldfield, E. H.; Lee, J.; et.al. *Am J Pathol* **2001**, *158*, 905.
(56) Netzel-Arnett, S.; Hooper, J. D.; Szabo, R.; Madison, E. L.; Quigley, J. P.; Bugge, T. H.; Antalis, A. M. *Cancer and Metastasis Reviews* **2003**, *22*, 237.
(57) Nishizuka, Y. *Nature* **1984**, *308*, 693.
(58) Bruno, J. G.; Carrillo, M. P.; Crowell, R. *J Biomed Mater Res Part A* **2009**, *90A*, 1152.
(59) Bunka, D. H. J.; Platonova, O.; Stockley, P. G. *Curr Opin Pharm* **2010**, *10*, 557.
(60) Ireson, C. R.; Kelland, L. R. *Molecular Cancer Therapeutics* **2006**, *5*, 2957.
(61) Lee, J. F.; Stovall, G. M.; Ellington, A. D. *Curr Opin Chem Biol* **2006**, *10*, 282.
(62) Nimjee, S. M.; Rusconi, C. P.; Sullenger, B. A. *Ann Rev Med* **2005**, *56*, 555.

TOC Graphic


aminooxy derived small molecule
(folic acid)
Folic Acid
Folic Acid
Fc-UAA
Fc-small molecule