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PRODUCTION OF RADIOLABELED MONOCLONAL ANTIBODY CONJUGATES BY PHOTOAFFINITY LABELING

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Introouction

Since initiation of this project on September 1, 1989, we have:

- 1) Synthesized ethyl N,N'-bis(benzoylmercaptoacetyl)-2,3-diaminopropanoate (Figure 1), a ligand to be used as a bifunctional chelating agent (BFCA), to form ^{186}Re or ^{188}Re ($^{186}\text{Re}/^{188}\text{Re}$) complexes. $^{186}\text{Re}/^{188}\text{Re}$, in reducing media, reacts with this ligand to form $^{186}\text{Re}/^{188}\text{Re}$ -CO₂DADS chelates that will be used to formulate new radiolabeled photoaffinity labels (RPALs).
- 2) Initial steps have been taken to synthesize R-As-dithiol compounds. This approach will be used to produce ^{77}As -RPALs or covalently link ^{77}As directly to monoclonal antibodies (MAbs). The R group will contain a group that can be used for conjugation reactions.
- 3) Spectral and photochemical properties of various types of photoaffinity labels (PALs) have been studied. Most of the work has concentrated on PALs containing azido groups. Acrylo-azido compounds and 9-azido acridine have been studied as well as several other photoprobes.
- 4) The binding characteristics of the azido-based PALs to HSA have been studied and progress has been made on developing techniques for efficiently separating of non-covalently bound PALs.
- 5) The Nd-YAG laser was purchased and arrived in 1990. It has been assembled and tested and is now operational.

1. Synthesis and characterization of ethyl N,N'-bis(benzoylmercaptoacetyl)-2,3-diaminopropanoate (Bz=CO₂DADS)

¹⁸⁶Re/¹⁸⁸Re-CO₂DADS (Figure 1) should be an ideal chelate to be used as part of a BFCA for conjugation of ¹⁸⁶Re/¹⁸⁸Re to MABs since ¹⁸⁶Re-CO₂DADS has been shown to have exceptional in vitro and in vivo stability and is rapidly cleared from circulating blood via the kidney, into the urine. For these reasons, we chose to synthesize BzCO₂DADS so it would be available when a PAL with efficient photo-induced conjugation properties is identified. The long-term objective is to link ¹⁸⁶Re/¹⁸⁸Re-CO₂DADS to a PAL which, in turn, is photochemically linked to a MAb. Figure 1 outlines the synthetic scheme used for synthesis of BzCO₂DADS.

Synthesis of ethyl N,N'-bis(benzoylmercaptoacetyl)-2,3-diaminopropanoate

STEP I:

STEP II:

Figure 1

STEP III:

Figure 1 (Cont'd)

The NMR, m.pt. and IR of the intermediates have been obtained. These analysis of the respective intermediates are consistent with published values and confirm its chemical identity.

BzCO₂DADS reacts with Re(V)O-citrate and reductant to form ReO-CO₂DADS⁻². This chelate is stable in vivo and is rapidly cleared into the urine with minimal uptake in the liver or other organs. The -CO₂H group appended to the DADS backbone can be used to link the ReO-CO₂DADS chelate (via an activated ester or CDI reagent) to PALs or to MAbs.

2. Synthesis of RAs-dithiols

R-As-dithiols form very stable As compounds where R is an aryl or alkyl group. we are synthesizing compounds where the R group contains a group that can be readily converted to a chemically reactive site for linking these ^{77}As -compounds to a PAL or directly to a protein. We plan to use either benzyl thiocyanate or an activated ester for conjugation. Figure 2 is an outline of the reaction scheme that will lead to an ^{77}As reagent to be used to form a benzylthiocyanate ^{77}As -BFCA.

Figure 2

In the reaction scheme shown in Figure 2, ^{77}As is added to RHgR where $\text{R}=\text{H}_2\text{N}-\text{C}_6\text{H}_4$. The reactions to form RAsO as the subsequent reaction with 3,4-dimercaptotoluene form Cmpd I were found to be efficient. Cmpd I can be activated to couple to PALs containing a primary amine group or to directly conjugate a terminal lysine-amine group on MABs by first converting the $-\text{NH}_2$ group on Cmpd I to thiocyanate ($-\text{NCS}$) using thiophosgene. This activation reaction and subsequent coupling conjugation via benzyl-thiocyanate has been shown to work well for conjugating with amine groups on MABs.

^{77}As is obtained at no-carrier added (NCA) levels as ^{77}As iodide from a $^{77}\text{Ge}/^{77}\text{As}$ radionuclide generator we developed. The ^{77}Ge parent is produced by the (n,γ) reaction with ^{76}Ge -oxide in the University of Missouri Research Reactor (MURR). ^{77}As has a $t_{1/2}=1.6\text{d}$ and emits a medium energy beta particle ($E_{\text{max}}=680\text{ KeV}$). This radionuclide will have value as a therapeutic radionuclide agent when attached to MABs or MAB fragments. ^{77}As is generated as the iodide when separated from ^{77}Ge (and ^{76}Ge) and is directly used in the reaction sequence shown in Figure 2.

3. Spectral and photochemical properties of PALs evaluated

We have investigated a number of commercially available labels for potential use as photolabels. The desired characteristics are: light absorption at or above 350 nm with high extinction coefficient and high quantum yield for attachment to antibody. In addition, the probe must have side chains which can be easily attached to the radiolabel and/or are stable enough for this derivatization to be carried out.

Thus far several probes have been tried and some excluded from further study: These are summarized below:

PALs with poor properties:

- a. 4-fluoro-3-nitrophenyl azide. This probe has a λ_{max} at 340 nm and $\epsilon=1200\text{-}1500\text{ M}^{-1}\text{cm}^{-1}$. This wavelength and low extinction coefficient did not permit excitation of the probe in the presence of antibody.
- b. 2-diazo-3,3,3-trifluoropropanoic acid. This probe has λ_{max} at 410 nm and $\epsilon=450\text{-}500\text{ M}^{-1}\text{cm}^{-1}$ was found to be too weak of an absorbance to be useful.
- c. N-succinimidyl-6-(4'-azido-2'-nitrophenylamine) hexanoate (SANPAH). This compound has λ_{max} at 260 and 480 nm with $\epsilon=1250\text{ M}^{-1}\text{cm}^{-1}$ at the longer wavelength. Unfortunately the quantum yield (ϕ) was found to be approximately 0 at the longer wavelength and a high photolysis rate in the 260 nm region which is in the region where MABs have strong absorbance.
- d. sulfo-SANPAH: This analog of SANPAH has greater water solubility and a maximum absorption at 480 nm. Unfortunately, like SANPAH, the ϕ was found to be about 0. It is likely that the long wavelength chromophore for this compound is not adjacent to the azido active group.

- e. 5-azidonaphthalene sulfonic acid, sodium salt. $\lambda_{\max}$ at 310 nm and $\epsilon = 8000 \text{ M}^{-1}\text{cm}^{-1}$. The quantum yield for this compound was low and the absorption maximum overlaps absorption by Abs.
- d. N-(((p-azido benzoyl)amino)ethyl)-4-amino-3,6-disulfo-1,8-naphthalimide, dipotassium salt (Lucifer Yellow) was investigated. This compound has $\lambda_{\max}$ at 420 nm and $\epsilon = 7200 \text{ M}^{-1}\text{cm}^{-1}$. Preliminary photolysis results indicate a very low quantum yield.

PALs with promising properties

We have under investigation two commercial probes which show some promise.

- a. 9-azido acridine: This compound appears to have many of the characteristics which would be useful for a PAL. It has $\lambda_{\max}$ at 357 nm and $\epsilon = 6000 \text{ M}^{-1}\text{cm}^{-1}$. In addition, photolysis of the compound gives a high quantum yield for destruction. Unfortunately, we have been unable to quantitatively separate the non-specifically bound (NSB) probe from the model protein (HSA) that we have been using for the probe development studies.
- b. N-(2-aminoethyl)-4-azido-2-nitroaniline: This derivative of the nitrophenylazide has $\lambda_{\max}$ at 460 nm and $\epsilon = 4500 \text{ M}^{-1}\text{cm}^{-1}$. In addition to favorable photochemical properties, it has an active side chain for attachment of the radiolabel.

4. Binding characteristics of PALs to HSA

It was assumed in the proposal that hydrophobic PALs in aqueous solutions would associate readily with hydrophobic pockets in proteins. This has been found to be true using HSA as a model protein for these studies. For example, we showed that acridine and 9-azido acridine have a high association constant with HSA in which >98% of these lipophilic molecules rapidly associate with the protein upon mixing.

Separation with some PALs is achieved by denaturation with SDS and subsequent elution by Sephadex gel filtration. Removal of some non-conjugated PALs from HSA can be accomplished using a C-8-reversed phase by a simple Sep-Pak system. For example, acridine non-specifically bound (NSB) to HSA is quantitatively removed from the protein by this technique without denaturation of HSA. Preliminary work with 9-azido-acridine indicates that a longer reversed phase column must be used to separate this NSB PAL. Work on developing appropriate technologies for separation of PALs from MAb and other proteins is continuing.

5. Nd-YAG Laser

This laser was purchased and is now in operating condition. This laser can be used with or without a dye laser to provide the high energy, monochromatic, short pulse needed for selective excitation of the probe in the presence of the antibody. Currently, the laser is in the dye-pumping mode which can provide excitation at 530 nm and above. In view of the likelihood of probe absorptions near the 353 nm third harmonic, it will be worthwhile to have access to the third harmonic beam without removal of the dye laser from the Nd-YAG beam. Therefore, we are in the process of purchasing additional optics to divert the third harmonic from the YAG beam without disrupting the dye laser arrangement. This will lead to greater efficiency in future studies of the reactive photochemical probes that have a high extinction coefficient in the 353 nm range.

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