Engineered Antibodies for Monitoring of Polynuclear Aromatic Hydrocarbons

Alexander E. Karu [1], Qing X. Li [2], and Victoria A. Roberts [3]

[1]Div. of Nutritional Sciences & Toxicology,
University of California, Berkeley CA 94720,

[2]Dept. of Environmental Biochemistry,
University of Hawaii, Honolulu HI 96822, and

[3]Dept. of Molecular Biology,
The Scripps Research Institute, La Jolla, CA 92037

GRANT DE-FG07-96ER62316

October 1996 — Sept. 1999

Poster presented at the DOE Environmental Science Management Program Workshop, Chicago, IL, July 27-30 1998

Table of Contents

Objectives 3
Specific Aims 4
Potential Applications in EMSP 5
Components of the project 7

Major accomplishments to date
- Karu Laboratory 8
- Li Laboratory 9
- Roberts Laboratory 10

PAH analytes and haptens synthesized for this project 11

Two ways to derive improved recombinant antibodies 13
- Selecting and enriching phage from a large combinatorial library 14
- Engineering new PAH specificities 15

PAH immunoassay development
- Affinity measurements 16
- Cross-reactivities with different competing haptens 17
- Indirect ELISA standard curve for benzo[a]pyrene, and correlation between ELISA and gas chromatography 18
- Recoveries of benzo[a]pyrene spikes from tap water, canal water, and urine 19

Molecular modeling of PAH-antibody binding
- Antibody and PAH modeling methods 20
- There are four energetically favored orientations for benzo[a]pyrene in the binding site 21
- The PAH binding site of rFab 4D5 22
  - Shape of the binding pocket 23
  - Position of key tryptophan side chains 24
- Alternate loci for PAH-antibody interactions outside of the primary binding site identified by theAUTODOCK simulation program 25
- Figure showing alternate PAH binding sites 26

Summary 27
Participating researchers 28

Objectives

ä The long-term goal of this project is to develop antibodies and antibody-based methods for detection and recovery of polynuclear aromatic hydrocarbons (PAHs) and PAH adducts that are potential biomarkers in environmental and biological samples. The inherent cross-reactivity will be exploited by pattern recognition methods.

Specific Aims

- ä Use genetic engineering to improve recombinant antibodies that detect key PAHs for environmental monitoring and risk assessment
- ä Gain insights into the mechanisms of PAH recognition by the antibodies
- ä Devise robust, versatile analytical methods to recover and measure PAHs in environmental and biological samples

Potential Applications in EMSP

Enabling Technology

Discrete or real-time high-throughput monitoring

—Sensors and rapid tests for site evaluation and surveillance of soil, water, and waste

—Immunoaffinity methods for PAH residue recovery before instrumental analysis

DOE Problem Area

– Implement inexpensive, science-based sampling and analysis plans

– Fulfill project-specific data quality objectives

– Prevention and emergency response

– Detect leaks of solvents, fuels, and lubricants

– Identify waste spill ‘hot spots’

– Find perimeter, expansion rate of plumes

–Monitor progress of remediation

Potential Applications in EMSP (cont'd.)

Enabling Technology	DOE Problem Area
Recognition of immunologic cross-reactivity patterns	Identify substances by their PAH ‘signatures’ Characterize raw materials, formulations, complex pollutants Monitor chemical conversion as an indicator of aging, wear, etc.
Detection and identification of biomarkers of PAH exposure and effect	Dosimetry Molecular epidemiology Ecotoxicology

Components of the project

- **Dr. Karu's laboratory uses new haptens representing key PAHs to derive recombinant Fab (rFab) and single-chain Fv (scFv) antibodies from hybridoma lines and combinatorial phage display libraries.**
- **Dr. Roberts's laboratory makes computational models of the antibody binding sites and the PAH haptens and parent compounds to guide antibody engineering by mutagenesis.**
- **Dr. Li's laboratory develops haptens, enzyme immunoassays (EIAs), sensors, and immunoaffinity methods that make use of the novel antibodies for practical analytical applications in support of DOE's mission.**

Major accomplishments to date

- **Karu Laboratory**
  - **Corrected amino acids that affect affinity of rFab 4D5 by site-directed mutagenesis**
    - **Derivation of custom PAH libraries by combinatorial mutagenesis and phage panning is in progress**
  - **Recovered single-chain Fv antibodies that bind naphthalene and phenanthrene from combinatorial phage display library**
    - **Characterization and derivation of scFvs with other PAH specificities is in progress**

Major accomplishments to date

- **Li Laboratory**
  - **Twenty PAH haptens and conjugates synthesized**
    - **represent all major 2, 3, 4, and 5-ring PAHs amenable to immunoassay**
      - **spacer arms of different lengths at different positions**
      - **only two generic synthesis schemes needed**
  - **Characterized two monoclonal antibodies (MAbs) and recombinant Fabs (rFabs) derived from them by direct and indirect enzyme immunoassay (EIA)**
    - **Different PAH sensitivities and cross-reactivity patterns obtained with different competing haptens**
  - **Developed supercritical fluid extraction and immunoaffinity methods for PAH residue recovery**
  - **EIA method for benzo[a]pyrene (BaP) in water samples**

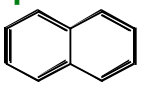
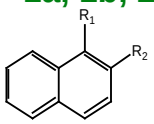
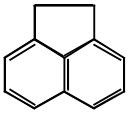
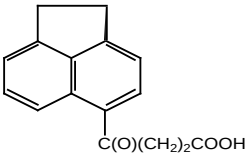
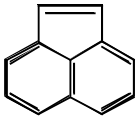
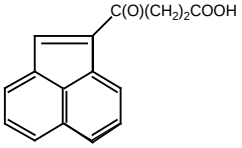
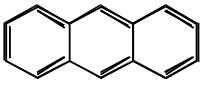
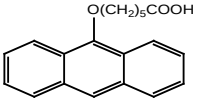
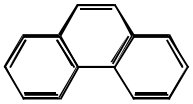
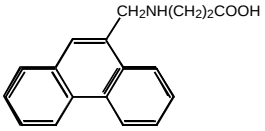
Major accomplishments to date

- **Roberts Laboratory**
  - **Developed 3-D structure models of the PAH binding site**
  - **Identified the major PAH bonding interactions**
    - **Binding may involve cation - π interactions with PAHs**
  - **Prioritized sites for directed and combinatorial mutagenesis**
  - **Performed automated docking of PAHs and haptens**
    - **Validated experimental observation of BaP binding in both C1 and C6 orientations**
    - **Identified PAH-binding domains outside of the primary binding site**
      - **These may account for some "cross-reactivity"**
      - **Can the sites be abolished by antibody engineering?**

PAH Analytes and the Haptens Synthesized for This Project

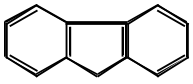
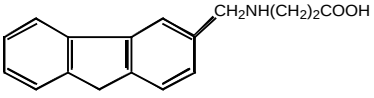
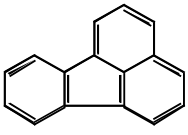
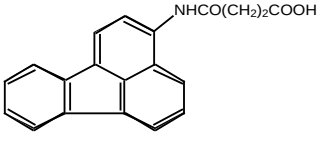
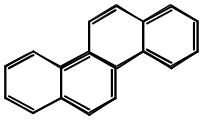
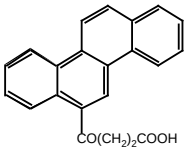
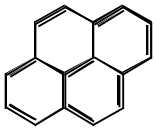
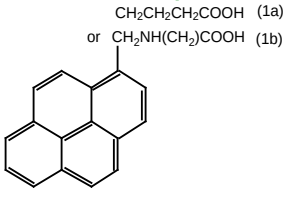
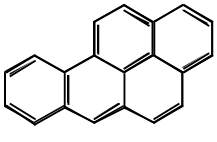
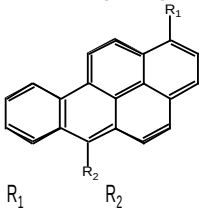
We synthesized 11 haptens representing the major 2- to 5-ring PAHs that are found in a wide variety of biological and environmental matrices and have aqueous solubilities and vapor pressures amenable to immunoassay. These haptens are being used to format PAH immunoassays, and select antibodies with new cross-reactivities from phage display libraries.

Metabolites of chrysene, pyrene and benzo[a]pyrene (BaP) form adducts that are biomarkers of PAH exposure.

PAH	Aqueous solubility (µg/mL; ppm)	Hapten	Major Occurrence †	Bioadducts
Naphthalene 	30	NAP-1a, 1b, 2a, 2b 	A,C,O,T,W	
Acenaphthene 	3.47	AC-5a 	A,O,T,W	
Acenaphthylene 	3.93	ACN-2a 	A,T,O	
Anthracene 	0.07	ANT-9a 	A,C,F,T,W	
Phenanthrene 	1.29	PHE-10a 	A,C,F,O,T,W	Thiols, Glucuronides

†Major occurrence: A= air, C = coal and byproducts, D, diesel exhaust, F = foods, G = gasoline exhaust, O = oils and lubricants, T= tobacco and other smoke, W = ground, surface, or sea water

PAH Analytes and Haptens for This Project (cont'd.)

PAH	Aqueous solubility (µg/mL; ppm)	Hapten	Major Occurrence †	Bioadducts
Fluorene 	1.98	FLR-3a 	A,T	
Fluoranthene 	0.26	FLA-3a 	A,F,O,T,W	
Chrysene 	0.002	CHR-6a 	A,C,F,T,W	Protein, DNA
Pyrene 	0.14	PYR-1a, 1b 	A,C,D,F,G, O,T,W	Protein, DNA, Glucuronides
Benzo[a]pyrene (BaP) 	0.004	BaP-2a, 6a, 6b 	A,C,D,F,G,T	Protein, DNA

R_1	R_2	
$CO(CH_2)_2COOH$	H	BaP-1a
H	N=C=O	BaP-6a
H	$CH_2NH(CH_2)_2COOH$	BaP-6b

†Major occurrence: A= air, C = coal and byproducts, D, diesel exhaust, F = foods, G = gasoline exhaust, O = oils and lubricants, T= tobacco and other smoke, W = ground, surface, or sea water

References:

Pucknat, A. W. Ed..; Health impacts of polynuclear aromatic hydrocarbons. Noyes Data Corp.: Park Ridge, NJ, 1981; 271 pp.

Neff, J. M. Polycyclic Aromatic Hydrocarbons in the Aquatic Environment; Applied Science Publishers, Ltd.: London, 1979.

Lee, M. L.; Novotny, M. V.; Bartle, K. D. Analytical chemistry of polycyclic aromatic compounds; Academic Press: New York, 1981; 462 pp.

Schnell, F. C. Protein adduct-forming chemicals and molecular dosimetry: potential for environmental and occupational biomarkers. In Reviews in Environmental Toxicology; Hodgson, E. Ed.; vol. 5); Toxicology Communications, Inc.: Raleigh, NC, 1993; pp 51-60.

Two ways to get improved recombinant antibodies

Very large, diverse combinatorial library

↓

Multiple rounds of panning and amplification

↓

Specific binders

Small set of known binders

↓ Mutagenesis & selection

Improved binders (1)

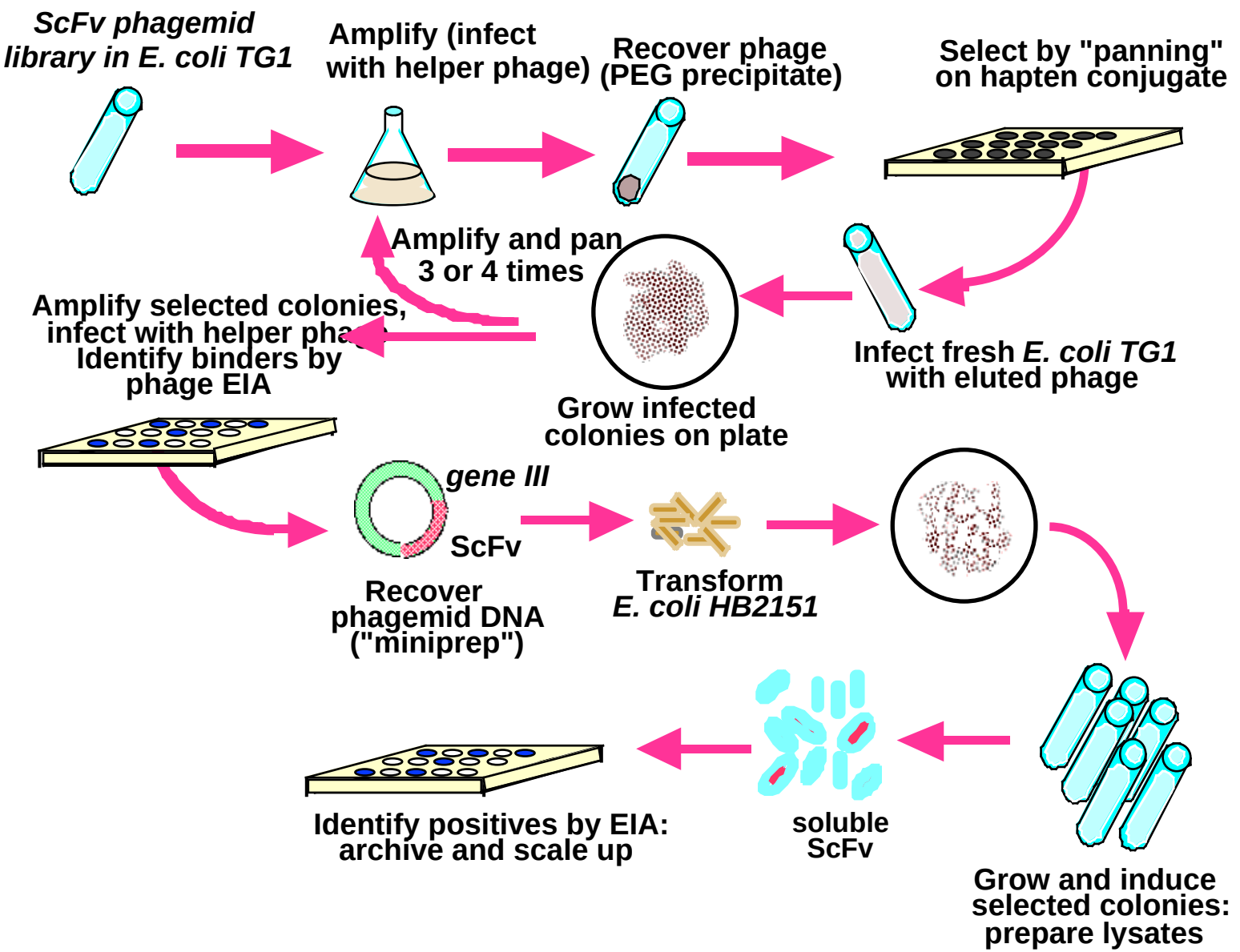
↓ Mutagenesis & selection

Improved binders (2)

↓ Mutagenesis & selection

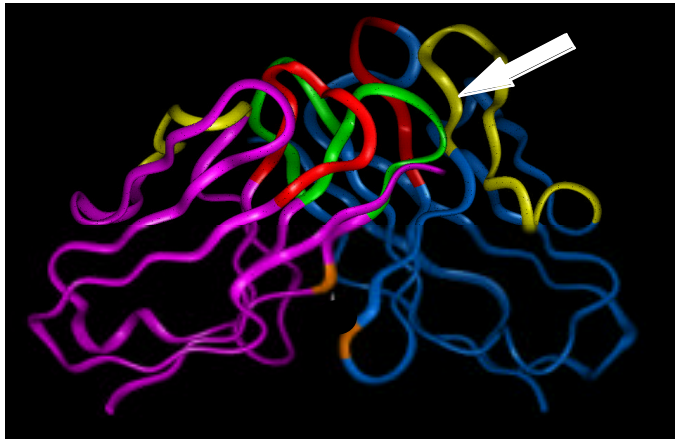
Improved binders (3)

This flow chart depicts the process of selecting and enriching phage that display desired antibodies from a large combinatorial library, and expressing the antibodies as soluble single-chain Fvs.

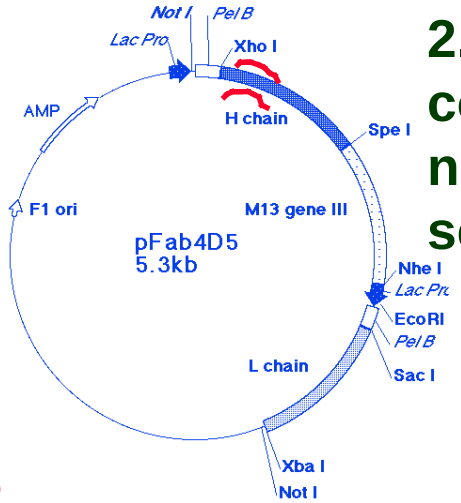


Engineering New PAH Specificities

This flow chart shows how we derive new antibodies by specific and combinatorial mutagenesis.



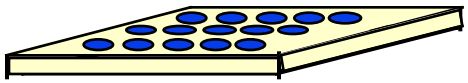
1. Select region to alter, based on structure model

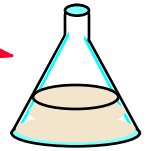


2. Design degenerate complementary oligo-nucleotides for the sequence to be altered

3. Perform combinatorial mutagenesis — express mutants displayed on phage

4. Select variants by panning on different haptens





4. Express variants as soluble Fabs

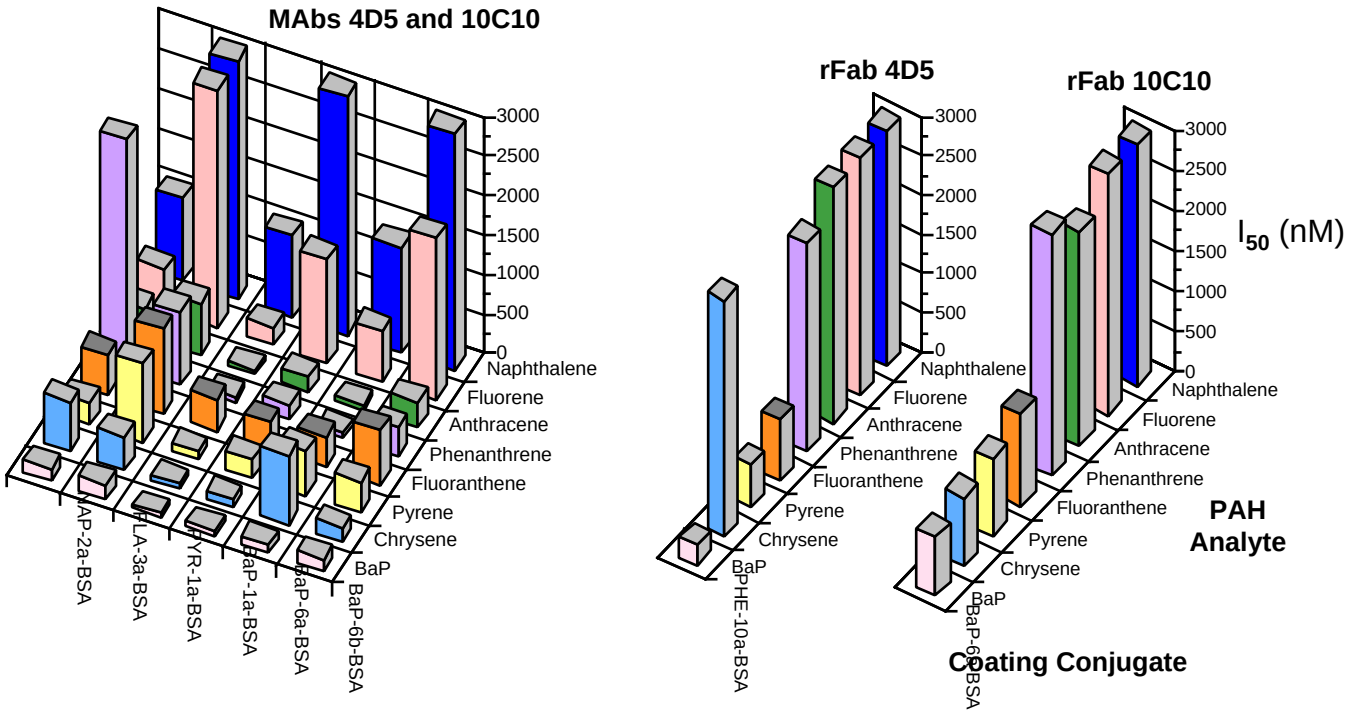
Affinities of the Antibodies

		Equilibrium dissociation constant K_D (μM)		
Analyte	Antibody	Fluor. Quench	KinExa	Competition EIA*
Benzo[a]pyrene	MAb 4D5	0.38	0.16	0.36
	rFab 4D5			0.99 — 1.52
	Mab 10C10	0.33	0.39	0.44 — 0.56
	rFab 10C10			3.67
Phenanthrene	MAb 4D5	2.05	1.14	4.5
	rFab 4D5			5.9 — 8.7
	Mab 10C10	1.01	0.44 —0.94	2.66
	rFab 10C10			11.2

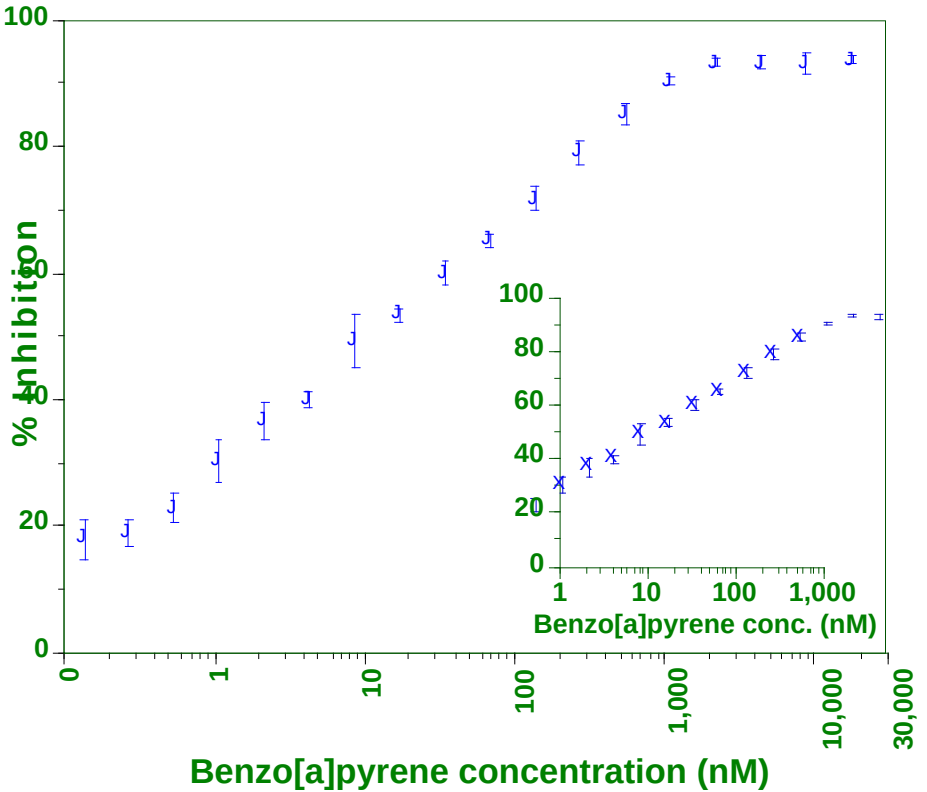
* Indirect EIA;
wells coated with
benzo[a]pyrene—BSA

These bar graphs compare the relative sensitivities of the indirect competition EIA for different PAHs when various PAH haptens are used as competing conjugates.

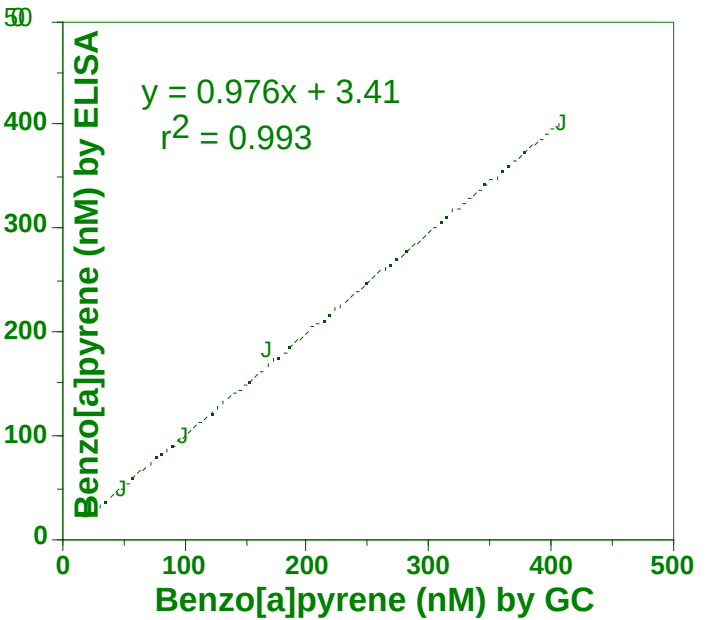
The half-maximal inhibition (I_{50}) values are shown. The shorter the bar, the more sensitive the assay. The response pattern for PAHs is different with each competing hapten.



Dose-response of indirect competition ELISA for benzo[a]pyrene with MAb 10C10 and BaP-6a-BSA coating conjugate



Correlation between ELISA and gas chromatography (GC) for canal water samples fortified with different amounts of BaP

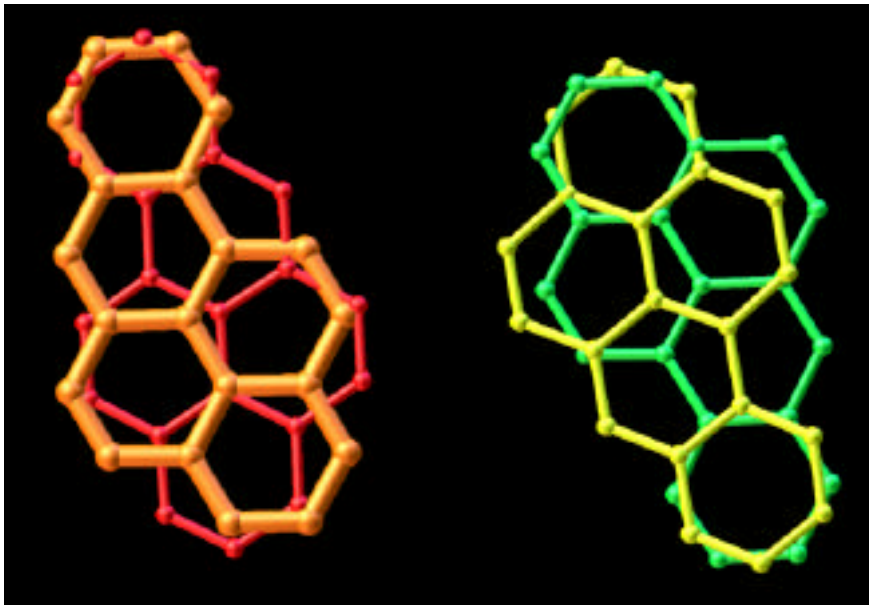


Recoveries of benzo[*a*]pyrene spikes from tap water, canal water, and urine.

Spike (nM)	Recovery (% ± SD) determined by ELISA			
	Buffer	Tap water	Canal water	Urine
2	84 ± 8	96 ± 12	94 ± 10	118 ± 17
4	96 ± 7	86 ± 9	96 ± 9	113 ± 11
20	105 ± 7	97 ± 5	86 ± 7	85 ± 10
40	83 ± 9	81 ± 9	92 ± 5	89 ± 10
200	85 ± 7	84 ± 8	106 ± 5	127 ± 19
400	98 ± 5	85 ± 8	100 ± 7	127 ± 9
600	117 ± 15	87 ± 9	80 ± 9	118 ± 13
Average	95	88	93	111

Molecular modeling methods

- ä **Binding sites were modeled using the Antibody Structural Database (ASD) of crystallographically determined structures, and visually analyzed with INSIGHT II.**
- ä **Geometry and partial atomic charges of PAHs and haptens were calculated with the program Gaussian94.**
- ä **Electrostatic potentials were calculated with DelPhi and mapped onto molecular surfaces.**

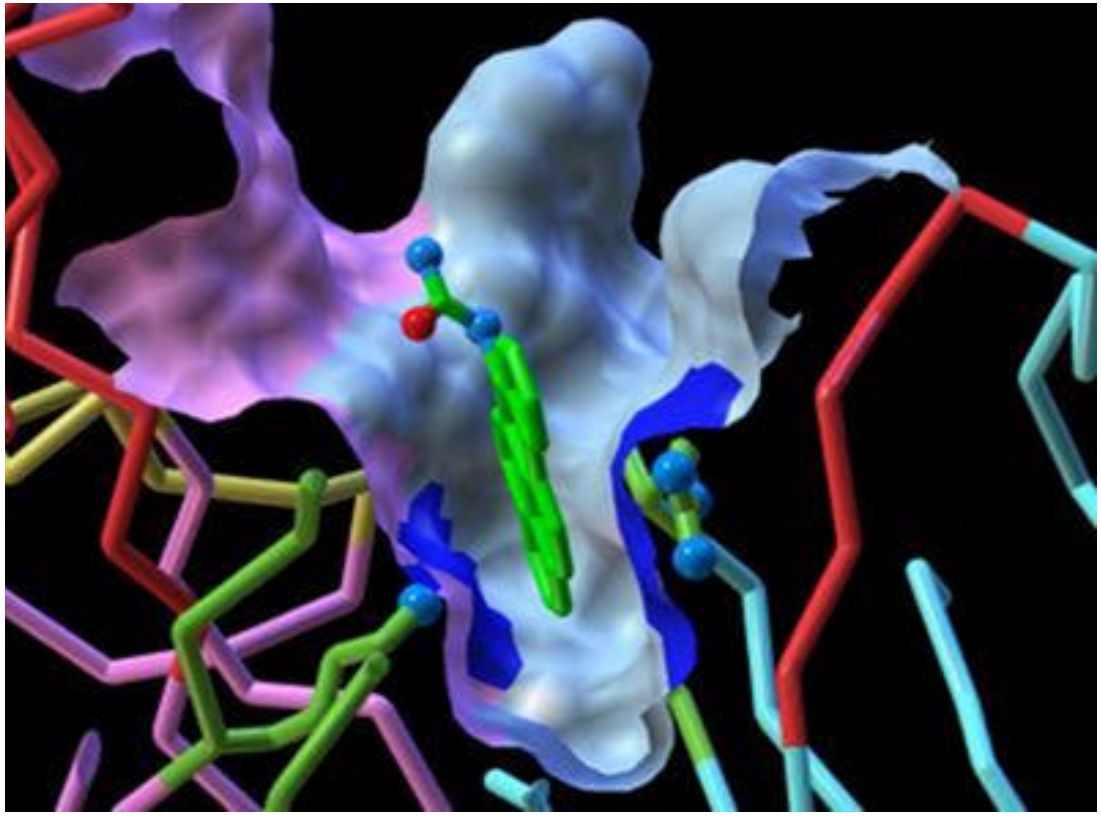


The four most favorable energy modes of BaP binding in the 4D5 binding pocket, oriented so that the bottom is surrounded by the antibody and the top is solvent-exposed. Color indicates energy from most favorable (red) to least favorable (green). Thickness represents the number of docked structures that fell into each cluster. The orange cluster matches the orientation in the energy-minimized model, and is the most likely.

In the two most favored structures (left), the pyrene moiety is deep in the pocket, and the 6-position, where the linker is attached, is solvent-exposed. In the other two structures (right), the pyrene moiety up so that the 1-position is solvent exposed.

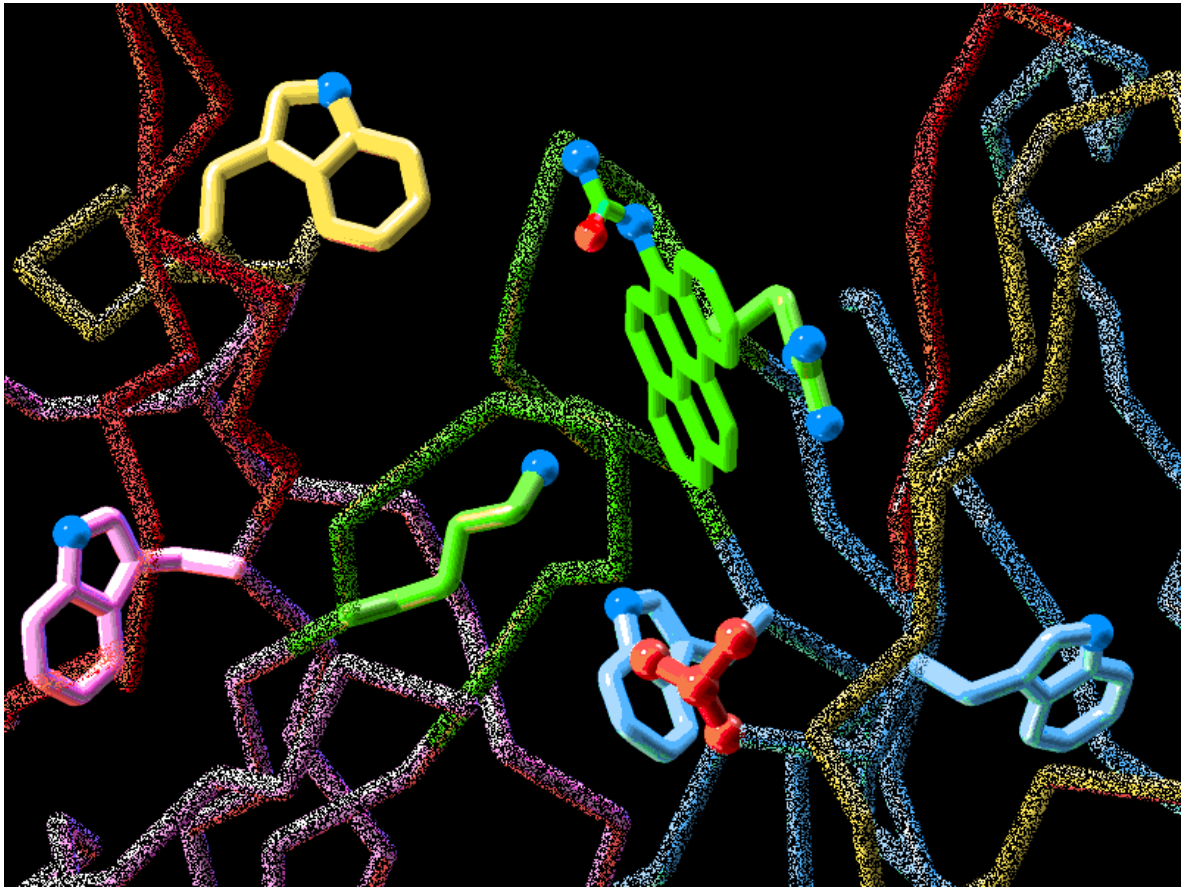
The PAH binding site of rFab 4D5

- ä **Binds BaP C1 and C6 haptens about equally well**
- ä **Val instead of Trp at H47 forms a very deep binding pocket**
- ä **Strong shape and charge complementarity of pocket and BaP**
- ä **Lys^{L89} and Arg^{H95} cationic groups may interact with π electrons of BaP**



Cutaway view of the solvent-accessible surface of rFab 4D5, with BaP-6-isocyanate positioned by the best fit of its van der Waals surface (not shown) in the binding site. The portion of the surface contributed by the light-chain variable region is magenta; the light blue portion is contributed by the heavy chain.

Dark blue patches denote regions of positive charge contributed by the side chain amino group of Lys^{L89} (green with blue tip on left) and the guanidinium group of Arg^{H95} on the heavy chain (green with two blue tips on right). The large bulge in the surface (top, center) is the CDR H3 loop, which appears not to play a major role in forming the binding pocket.



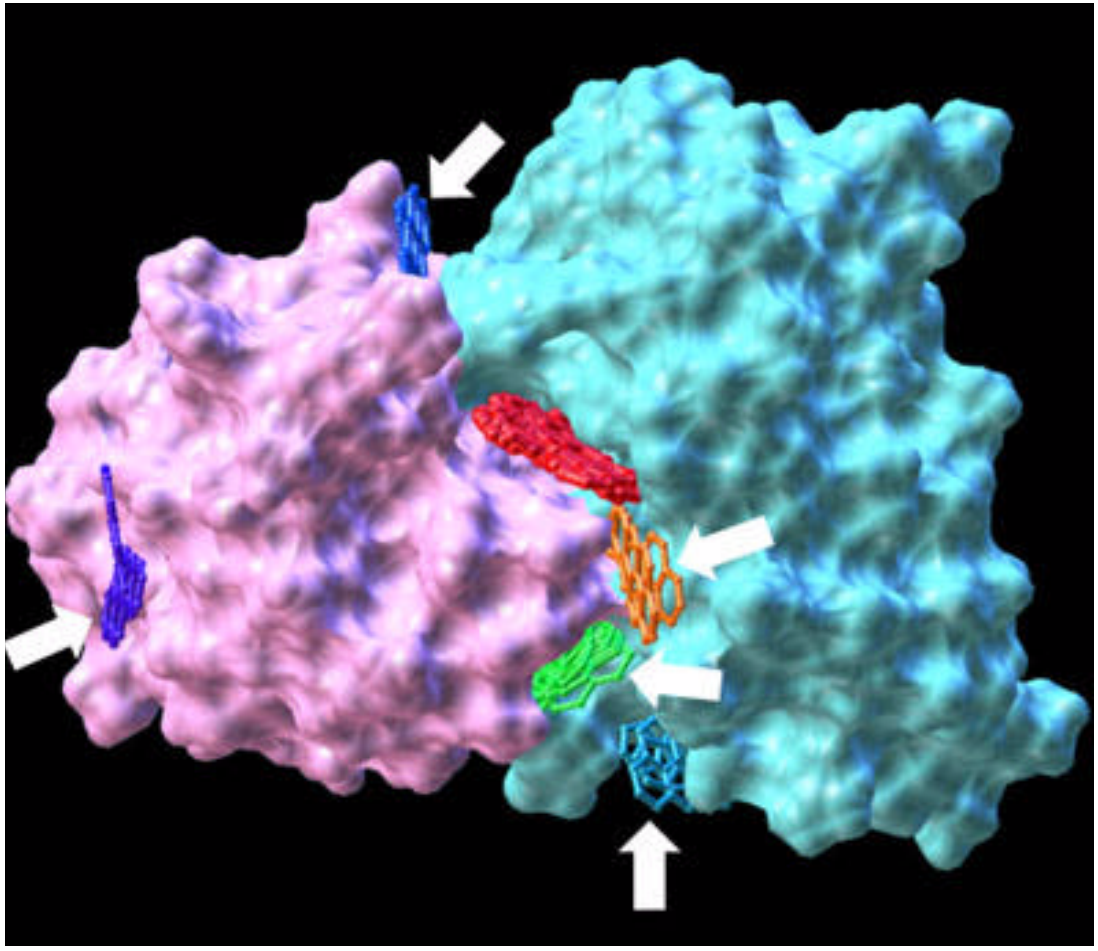
Relative positions of lysine 89 on the light chain (green with blue tip on left) and the guanidinium group of arginine 95 on the heavy chain (green with two blue tips on right), with BaP-6-isocyanate docked in the binding site of rFab 4D5. The four tryptophan side chains that help to form the binding pocket are also shown.

The side chain of valine H47 (orange) forms the bottom of the pocket. This position is occupied by a tryptophan in all other known antibodies. The much smaller valine side chain provides the space needed for BaP to bind.

Possible PAH-antibody interactions outside of the primary binding site

The automated docking studies revealed potential, although less energetically favorable, binding sites for BaP that are near, but not in, the binding pocket. These provide an explanation for the relatively strong binding of PAH hapten conjugates and PAH metabolite-protein adducts. One molecule of a conjugated protein may present more than one hapten or adduct residue to a single antibody molecule, resulting in binding external to, as well as within the specific primary binding site.

The secondary loci may also account for the large percentage of weak non-specific hydrophobic binders that Dr. Karu's group recovered when they used PAH-hapten conjugates to pan Fab and scFv display phage from large combinatorial libraries.



Putative binding sites for BaP on the antibody surface, determined by the program AUTODOCK. The four most favorable energy clusters (red) are closely superimposed in the binding pocket.

Additional binding sites (orange to purple) that are possible but less energetically favored are found in the groove formed between the light chain (light purple) and the heavy chain (light blue), and elsewhere on the solvent-accessible surface (white arrows).

Summary

At the midpoint of this project, substantial progress has been made on <u>all</u> of the originally proposed aims.

- **Haptens were synthesized that represent all of the major PAHs amenable to immunodetection.**
- **Two rFabs are being engineered to produce variants with different PAH-binding properties.**
- **ScFvs that bind naphthalene and phenanthrene have been obtained from a large semi-synthetic combinatorial library, and scFvs for other PAHs are now being recovered.**
- **Development of immunoassays and immunoaffinity recovery of PAH residues is in progress.**
- **Unique new information has been obtained on the mechanisms of PAH-antibody binding.**

Participating Researchers

- **Karu Laboratory**
  - Christopher W. Bell (Postdoc)
  - Tina E. Chin (Asst. Specialist)
  - Bitao Zhao (Asst. Specialist)
  - Hui-I Kao (Lab Assistant)
  - Nancy Elser (Grad. student)
- **Li Laboratory**
  - Rongliang Chen (Postdoc)
  - Kai Li (Postdoc)
  - Mei Liu (Grad. student)
- **Roberts Laboratory**
  - Jean-Luc Pellequer (Postdoc)