Facoltà di Farmacia

DRUG DISCOVERY IN ACADEMIA

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DRUG DISCOVERY IN ACADEMIA

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INTRODUCTION

Discovering a new therapeutic agent and shepherding it through the chain of events that result in an approved new drug application (NDA) by the FDA is an arduous quest by even the largest and most successful pharmaceutical companies. While the process is primarily the purview of industry, the past few decades have seen an increase in academic discoveries being commercialized. Our goal is to provide readers with both the science and technology transfer issues involved with drug discovery in a academia setting and the process involved with linking the intellectual property with business. It is our hope that the take home lessons listed at the end of this article will help other academicians who are interested in seeing their discoveries translate to therapeutic agents. The chapter summarizes the role of various components that play a role in translating basic research to industry. While not all of these introductory topics are germane for every case, they are meant to provide an overview of what an academician might encounter if she/he wishes to pursue development of a promising agent beyond publication.

LINKING THE UNIVERSITY WITH BUSINESS AND DRUG DISCOVERY

Startup Companies

The first spin off company arising from academia is believed to been that created by Horace Darwin, youngest son of Charles Darwin in 1881. The result was the Cambridge Scientific Instrument Company supplying Cambridge University's research laboratories [1]. Drug discovery in an academic setting has played an increasingly important role during the last three decades. This was not true during the first three quarters of the twentieth century. There are only isolated examples during this period of drugs that were discovered in Universities that eventually became marketed therapeutic agents. The role model provided by Genentech, founded in 1976 by venture capitalist Robert A. Swanson and biochemist Dr. Herbert W. Boyer, and changes in state and federal laws (the 1980 Bayh-Dole Act) paved the way for universities and academicians to participate in fostering business alliances and creating new companies.

Startup companies emerge now from academic discoveries world wide. The largest numbers of companies are located in clusters. California (San Francisco/San Diego) is first, and Massachusetts (Boston Area) has the second highest number of startups, with North Carolina not far behind. Other notable clusters are found in Europe, i.e., Cambridge in the UK, and Rhineland, Rein-Neckar, Berlin- Brandenburg, and Munich in Germany (www.biojapan.de/features/sumi.ppt). Mehta has published a summary of the number of startups initiated by university technology transfer processes. There were over two hundred a year from 1994-1996, three hundred a year from 1997-1999 and over 454 in the year 2000[2]. Clearly universities are playing an increasing role in the biotechnology revolution.

Licensing

The Bayh-Doyle act gave patent rights to universities that arose from research supported by federal funding with the stipulation that any royalties be shared with the inventors. University intellectual property offices were established during this period to more effectively pursue licensing opportunities for professorial (and research group) inventions. The Bayh-Dole policy was then expanded by most universities to include non-federally funded research. According to the Association of University Technology Managers, between the years of 1980 and 2000, over 3,300 university spinout companies were formed in the US and Canada, including 454 in the year 2000 alone[3]. The AUTM report finds that since 1980 academic licensing has resulted in at least 3,376 new companies. The total sponsored research expenditures for 190 reporting academic institutions was $29.5 billion up 10%, sponsored research expenditures funded by federal sources were $18.1 billion up 8% and the total 2002 sponsored research expenditures funded by industry were $2.7 billion with no increase over 2001. Invention Disclosures (13,032), New U.S. Patent Applications (5,545) and Patents Issued (3,764) were also record highs. The continuing interest of academia in commercialization of research discoveries suggests that even more future industry leaders will come from university laboratories. The once proud fiscal output of academic athletic programs can be dwarfed by comparison, but, unfortunately, the athletic programs still have a much higher publicity position in universities.

Even with this technology transfer success, the learning curve for university licensing offices with biotechnology companies has apparently lacked expertise and know-how in structuring deals with businesses. Edwards, Murray and Yu recently reviewed university licensing to biotechnology companies for the past 25 years{[4]. They concluded that universities often neglected important economic aspects in licensing agreements with biotechnology firms. The Alliance Database used in the study sampled 119 research institutions and all known commercial alliances made by 122 biotechnology companies where 36 instances revealed both

the full upstream and downstream economic terms. One major conclusion was that only one license in five provided for maintenance fees while milestone payments are even more neglected.

Universities and colleges are limited in their ability to narrowly focus on the complex and difficult task of discovering and developing a new therapeutic agent from the bench to clinical trials. The reason for this is obvious. The long standing role of the academy has been directed to scholarship in all of its forms and not following a discovery to its commercial endpoint. Besides, academic institutions unlike industry can not afford to set up expensively tailor-made laboratories and employ a multitude of high salaried individuals that span the diversity needed. Another obvious difference is that university professors have autonomy that industrial scientists rarely obtain unless they have been well proven. Massive screening of large combinatorial chemical libraries is more the purview of industry obviously due to the cost of expensive robots and an integrated cadre of high paid specialists. And, as drug discovery professionals well know, scientific advances and new therapeutic agents arise more times than one would believe by serendipity from an unexpected finding [5].

Research Parks

Biotechnology research parks located near Universities have also emerged with a growing trend since the 1980's. These parks were developed for a duality of reasons

1. To attract businesses that might benefit from alliances with academia, provide and provide a source of well educated and trained workforce.
2. The parks could enable faculty to be entrepreneurs, starting companies while retaining faculty status.

In 2002 the Association of University Research Parks (AURP) contracted with Association Research Inc. (ARI) to develop a profile of U.S. and Canadian

Research Parks [6]. The ARI sent out questionnaires to 195 entities believed to be operating in research parks and received 87 written responses with 79 yielding sufficient information to be stored in a database. Research parks such as Research Triangle Park occupy vast tracks of land (7,000 acres) while others such as University City Science Center (16 acres) and Audubon (3 acres) are relatively compact. The average size of a research park in the survey was 628 acres and the median 180 acres. Employment ranged from 10 to 42,000 for 62 research parks.

Conflict of Interest Issues for Academicians

During the 1980's and early 1990's there were little or no official university regulations at most institutions regarding faculty participation in or sitting on boards of startup companies they founded. Much of the concerns since the middle 1990's arose from clinical trials being conducted in medical schools by faculty with financial interests in the sponsors of the trials. In one celebrated case, after a young patient died via a gene transfer procedure, it was discovered that there was massive under-reporting to the NIH of adverse events of related gene transfer experiments in settings where investigators and in some cases institutions held significant financial positions. In 1988, Congress passed new regulations concerning individual conflict of interest in federally sponsored research. According to David Korn, senior vice president for Biomedical and Health Sciences Research at the Association of American Medical Colleges, mandating the federal law proved much easier than its implementation, which took seven years [7]. During the years since the federal law for government sponsored research passed, a plethora of new university rules and regulations have appeared at our institution and others that govern almost all faculty involvement with industry interactions. Unfortunately, draconian regulations have evolved to such an extent that even the most basic of research efforts, far removed from clinical trials, are scrutinized for all industry and even government sponsored studies. Faculty conflict of interest committees have taken center stage in academia placing increased restrictive provisions in place to

assure that faculty ownership and/or interactions with companies are in compliance with state and federal laws. It is not clear that such committees have members with extensive experience in these areas and brings to mind the old adage “the blind leading the blind”. As Max Perutz (whose laboratory he founded fostered 12 Nobel Laureates) mentions in the preface of his book *I Wish I Had Made You Angrier[8],*…”because creativity in science as in the arts, cannot not be organized [….] but hierarchical organizations, inflexible bureaucratic rules, and mountains of futile paper work can kill it”.

In academia today, the appearance of a possible conflict of interest is enough to derail efforts to conduct industrial research in academic laboratories, as well as inhibit startup company formation while remaining in the university. This is especially true if the faculty member has a grant from her/his company to perform research in her/his laboratory, even in the non-clinically related research areas. In this writers opinion universities have come full circle separating any real entrepreneurship from the academy. The future might look brighter if David Korn’s thinking on conflict of interest can be successfully implemented. “Conflicts of interest are ubiquitous and inevitable in academic life, and the challenge for academic medicine is not to eradicate them, which is fanciful and would be inimical to public policy goals, but to recognize them and manage them sensibly, effectively, and in a manner that can withstand public scrutiny” [7].

Another issue of concern has been the tension created by university intellectual property management vs. the role of universities to discover basic knowledge. One of the most contentious issues now being debated is whether the role of a university is compromised by an imbalance or shift toward goal-oriented research and the creation of wealth[2]. There are those who see the universities losing there altruistic nature of education and the pursuit of knowledge for knowledge’s sake. In this regard, it should be remembered that even the pursuit of knowledge for knowledge’s sake is <u>not</u> primarily supported by the university but by federal government funding agencies such as NIH and NSF.

DRUG DISCOVERY IN ACADEMIA

Traditional drug discovery research in academia has focused on developing new methodologies, uncovering new agonists or antagonists, and isolating or cloning new targets. Methodologies employed in universities for drug discovery or method development include but are not limited to QSAR, structure based drug design, molecular modeling, computational chemistry, combinatorial chemistry coupled with high throughput screening, proteomics and natural products synthesis and screening. For comprehensive review chapters in these areas published in the sixth edition of Burger's Medicinal Chemistry and Drug Discovery see Table 1. The more rational the approach employed by academic researchers, the greater the chance to obtain NIH funding. For this primary reason, high throughput screening in academia is not in vogue, even if well funded by industrial grants, since screening without advancing theory can be viewed as lacking in high quality scholarship**.**

Clinical Trials in Academia

The availability of medical schools to conduct clinical trials on new drug entities funded by pharmaceutical companies has been ongoing during from the early years of the twentieth century until the present day. Indeed a university's research budget can be handsomely increased through such contracts. Advocates also point to the importance of the university published findings of clinical trials in the search for new therapeutic agents. Some clinical findings can produce information that will provide a basis for new drug discovery although this is rare.

Table 1. Comprehensive Reviews on Drug Discovery Methodology	**Authors, Burger's Medicinal Chemistry and Drug Discovery 6th edition, John Wiley and Sons Hobokin, N.J.**
History of Quantitative Structure Activity Relationships	C. D. Selassie, Pomona College, Claremont, California, USA
Recent and Developing Trends in QSAR: From Data Analysis and Model Validation to Drug Design and Discovery	A. Tropsha, University of North Carolina, Chapel Hill, North Carolina, US Vol. 1. Chpt. 2
Molecular Modeling in Drug Design	Garland R. Marshall, Washington University, St. Louis, Missouri, USA Denise D. Beusen, Tripos, Inc., St. Louis, Missouri, USA azs Vol. 1. Chpt. 3
Drug-Target Binding Forces: Advances in Force Field Approaches	Peter A. Kollman, University of California, San Francisco, California, USA David A. Case, The Scripps Research Institute, La Jolla, California, USA Vol. 1. Chpt. 4
Combinatorial Library Design, Molecular Similarity and Diversity Applications	Jonathan S. Mason, Pfizer Global Research & Development, Sandwich, United Kingdom Stephen D. Pickett, GlaxoSmithKline Research, Stevenage, United Kingdom Vol. 1. Chpt. 5
Combinational Chemistry and Multiple Parallel Synthesis	Leste A. Mitscher, and Apurba Dutta, U. Kansas, Lawrence KA Vol. 2. Chpt. 1
Virtual Screening	Ingo Muegge, Bayer Research Center, West Haven, Connecticut, USA Istvan Enyedy, Bayer Research Center, West Haven, Connecticut, USA Vol. 1. Chpt. 6
Structure-Based Drug Design	Larry W. Hardy, Aurigene Discovery Technologies, Lexington, Massachusetts, USA Donald J. Abraham, Virginia Commonwealth University, Richmond, Virginia, USA Martin K. Safo, Virginia Commonwealth University, Richmond, Virginia, USA Vol. 1. Chpt. 10
Natural Products as Leads for New Pharmaceuticals	D. Buss, MerLion Pharmaceuticals, Singapore Science Park, Singapore B. Cox, Medicinal Chemistry, Horsham, United Kingdom R. D. Waigh, University of Strathclyde, Glasgow, Scotland Vol. 1. Chpt. 20

The NIH and the pharmaceutical industry have formed a new partnership to overcome barriers to early phase clinical trials. The Secretary of Health and Human Services, Tommy Thompson, announced on July 9, 2003 grant awards for six cancer centers involved in a unique public-private partnership Five pharmaceutical companies together with NCI put forth a total of $5.7 million for this partnership. Institutions receiving funding include: Massachusetts General Hospital; University of Colorado Health Sciences Center; Washington University, St. Louis; University of Pittsburgh Cancer Institute; University of California, Davis Cancer Center; and Ohio State University Comprehensive Cancer Center (http://www.bms.com/news/other/data/pf_other_news_3855.html).

Molecular Modeling and Computational Chemistry

Academia has been rich in producing theoretical computational methodology that underpins molecular modeling. The following software arose from universities or private and publicly funded institutes; AMBER[9], INSIGHT [10;11], CHARMM [12], SYBYL [13], GRID [14], DOCK [15], and HINT[16]. All except AMBER were commercialized.

Structure Based Drug Design

Paul Ehrlich, in the early twentieth century, proposed that drugs interacted with receptors similar to the way a key fits a lock[17][*]. The scientists' great dream of viewing in three dimensions the structure of a biological lock and key was born into reality when Max Perutz solved the phase problem for determining at atomic resolution of large biological molecules using X-ray Crystallography[18-20]. The first suggestion to use X-ray crystallography of biological molecules for drug design purposes came from academia and was published in 1974 [21]. Structure based

[*] http://www.chemheritage.org/EducationalServices/pharm/chemo/readings/ehrlich/pabio.htm

design is perhaps the royalty of methodologies in drug discovery since it is the most rational approach and one suited in present day academia for federal funding proposals. A Google search using structure based drug design shows about **1,300,000** hits.

Structure based design as conducted in industry and academia is a cyclic process. One proceeds from an initial active compound whose complex with the protein or target receptor is determined. This is usually followed by design of a better binding molecule with an increase by biological activity. A new structure determination of the complex is then made and the cycle continued until a potential clinical candidate is obtained. The cyclic process refines each stage of discovery. The key to success for designing a clinical candidate in our experience involved selection of a structural scaffold for the initial lead molecule that possessed a low toxicity profile since toxicity had derailed successful drug discovery more than any other step in the process. When a plateau is reached in biological testing, one of the candidates can be forwarded for *in vivo* evaluation in animals.

Case Study: The Discovery and Development of Allosteric Effectors of Hemoglobin as Potential Therapeutic Agents.

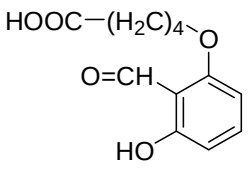
"Paul Ehrlich, the father of modern drug discovery, stated that scientists need the four German G's: Geshicht (Skill), Geduld (Patience), Geld (Money) and Glück (Luck)" [22]. The four German G's were all evident in this case study.

Our research group and associated colleagues have had the good fortune to have an allosteric effector of hemoglobin, RSR-13, discovered in our laboratories, proceed to an NDA submission which is being reviewed by the FDA as a radiation sensitizing agent for the treatment of breast cancer metastasis to brain. We have had a second agent proceed to a phase one clinical trial for the treatment of sickle cell anemia. The editor thought that a history of the discovery, design and development of a molecule that we have seen translated to clinical trials might provide readers with a case study that puts flesh on the preceding topics.

Seeing a chemical agent proceed through the maze required for FDA approval as a new drug entity makes one truly appreciative of the extensive expertise required at every stage. It is impossible to credit all of those involved in this 12 year quest. One thing is certain. Without collaborative skills at every level, a strong perseverance to continue despite the odds, a lot of money and some good fortune, RSR-13 would not have reached the NDA stage

GEDULD (PATIENCE)

The author started his academic career working in the cancer chemotherapeutics arena.* A switch was made to focus on Sickle Cell Anemia in 1975. The reason for the switch was straightforward. My long standing interest in drug discovery has always been directed toward structure based drug design [23]. In the early to mid 1970's, hemoglobin was the only large molecule drug target whose structure was determined at atomic resolution. At about the same time, another group headed by Peter Goodford at Burroughs Wellcome UK had the same idea. Goodford's group was the first to develop a structure based antisickling agent that reached clinical trials (BW12C, compound 1a) [24;25]. Later we confirmed the proposed BW12C binding site; however, BW12C interactions with the protein were different than those proposed [26]. BW12C was subsequently dropped from further study due to a short half-life and unfavorable route of administration for a chronic disease.



BW12C

Our own efforts languished from four failed NIH grants. At that time it was not clear to reviewers that structure based drug design using the native adult hemoglobin (HbA) coordinates would be productive. It would take five years, with four failed attempts, to win the first NIH grant and another decade plus to get an agent into clinical trials.

* When the author was ten years old he watched his maternal grandfather painfully die of cancer. He told his mother that one day he wanted to discover a drug to treat cancer. Thinking that MD's discovered new medicines, he enrolled in pre-medicine only to find that his real interest was in organic chemistry. After switching majors he was very pleased to discover that chemists usually discover new drugs.

GLÜCK (LUCK)

After four failed NIH grants and without funds, our group dwindled to only my oldest son William, who was in high school and volunteered to work for the summer. Serendipity and an unexpected ally saved the day. The ally was the goods delivery person in Salk Hall at the University of Pittsburgh, Mr. Robert Heflin. One day, while we were ridding together in the elevator, Bob asked why I looked so distraught and I told him it was due to the fact that I had lost all research funds since the NIH did not believe in my idea to design an antisickling agent from the structure of hemoglobin. Mr. Heflin told me that he could help and I almost said no thanks, thinking it not very likely. However, his sincerity was so appreciated, I took him to my office where a student and I had put together a lab-quip plastic model of HbA, comprising of 5000 atoms. I told Mr. Heflin to pretend the giant globe shaped tetramer was the moon and the potential drug was a space ship that had to land near the mutant binding site. Bob responded with a big smile on his face and repeated he could help. I asked how and he responded that he knew the famous first baseman for the Pittsburgh Pirates, Mr. Willie Stargell. Mr. Stargell was heading group of black athletes in the National Baseball league funding sickle cell anemia research. Mr. Stargell provided the University of Pittsburgh, where my laboratories were at that time, 18 thousand dollars for our first sickle cell anemia funding over the next few years. This seed money revived our research efforts to provide enough results and experience to get our first NIH grant to study sickle cell anemia, an RFP (Request for Proposal) from the NIH for sickle cell structure based drug design.

GESCHICK (SKILL)

Another problem our group had was to make the transition from small molecule to large molecule crystallography. At that time (1970 – 1975) only a few centers in the world were equipped to determine the structure of a large molecule using X-ray

crystallography. At that time, groups normally had to spend a decade or more to complete a single structure and the process was very labor intensive. It was clear after five years (1975-1980) that my small crystallography laboratory in neither the Pharmacy School* nor the well equipped Department of Crystallography at the University of Pittsburgh (under Professor George Alan Jeffrey and famous for small molecule structure determination) had the equipment and skills to progress rapidly with solving potential drug complexes with hemoglobin.

While reading a *Nature* commentary one afternoon, a plan of action crystallized. The author wrote that if you want to get hit by lightening you must go where it storms. It was clear that if we were to make rapid progress and learn hemoglobin crystallography there was only one place to go, Cambridge UK in Max Perutz's laboratory at the Medical Research Council Laboratory of Molecular Biology. Fortunately, a symposium was being held in honor of Max's 65th birthday at Airlee House in Virginia (1980), only a four hour drive from Pittsburgh. With some nervousness I approached Max during a poster session with my ideas, and to my great surprise he immediately invited me to join him in Cambridge UK at the Medical Research Council Laboratory of Molecular Biology (MRCLMB).

Mr. Robert Heflin again came to our rescue to link me up with funds to study in Cambridge via Ms. Ruth White, the then director of the Sickle Cell Anemia Society in Pittsburgh. In one day Ms. White arranged for me to get a Heinz Foundation grant to travel and work in Perutz's laboratory.

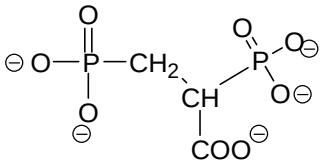
The author worked with Max Perutz and colleagues at the MRCLMB for the next eight years during periodic visits (1980-1988). During 16 trips to the MRCLMB we slowly worked out the structural parameters for a therapeutic agent that would bind strongly to hemoglobin. The overall template for binding to specific Hb sites that prevent polymerization of HbS were found to have an aromatic halogen ring

* My dark room for developing x-ray films was in my coat closet and to take cold temperature diffraction of crystals grown at 4 C°, I had to wait until winter to open the window.

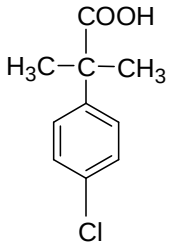
connected to a polar side chain. This information enabled our group in Pittsburgh to design and synthesize or select several potent antisickling agents but all failed as potential therapeutic agents due to red cell deformability and/or *in vivo* toxicity at the high *in vivo* dosage needed to interact with the approximately 650 grams of HbS in sickle cell anemia patients. The author asked Professor Don Witiak, a colleague from Ohio State University at that time, if there was a known drug with the halogen aromatic polar template that could be given in high doses. He told us yes, the antilipidemic drug Clofibrate that had as the active component clofibric acid (CFA). It was given to humans in 2 gram per day quantities.

We immediately tested CFA and found it to exhibit strong antigelling activity[27;28]. Just as important was the fact that CFA, when administered orally as the ethyl ester clofibrate (Clofibrate) could be given in high doses (2 gm/day). X-ray analyses of co-crystallized Hb and CFA showed two binding sites, one strong and one very weak, separated by several angstroms [29].

Since CFA might be a candidate to treat sickle cell anemia, we decided to determine what effect CFA might have on the oxygen binding properties of hemoglobin solutions. Many antigelling agents left shift the oxygen binding curve producing a high affinity oxy-Hb relaxed (R) state that does not incorporate into the polymerization of tense state (T) deoxy-HbS. It was a surprise when the antigelling CFA which inhibits sickle-cell Hb polymerization, was found to shift the Hb oxygen binding curve toward the right, i.e., toward T state Hb in a manner similar to that of the natural *in vivo* allosteric effector 2,3-Diphosphoglcerate (2,3-DPG) [27]. An agent that can produce an *in vivo* right shift in the oxygen binding curve had been long sought as a potential to treat human hypoxic conditions such as stroke and angina. It was obvious that 2,3 DPG, however could not be used as a therapeutic agent since its five negative charges prevent its transport across the hydrophobic red cell membrane.



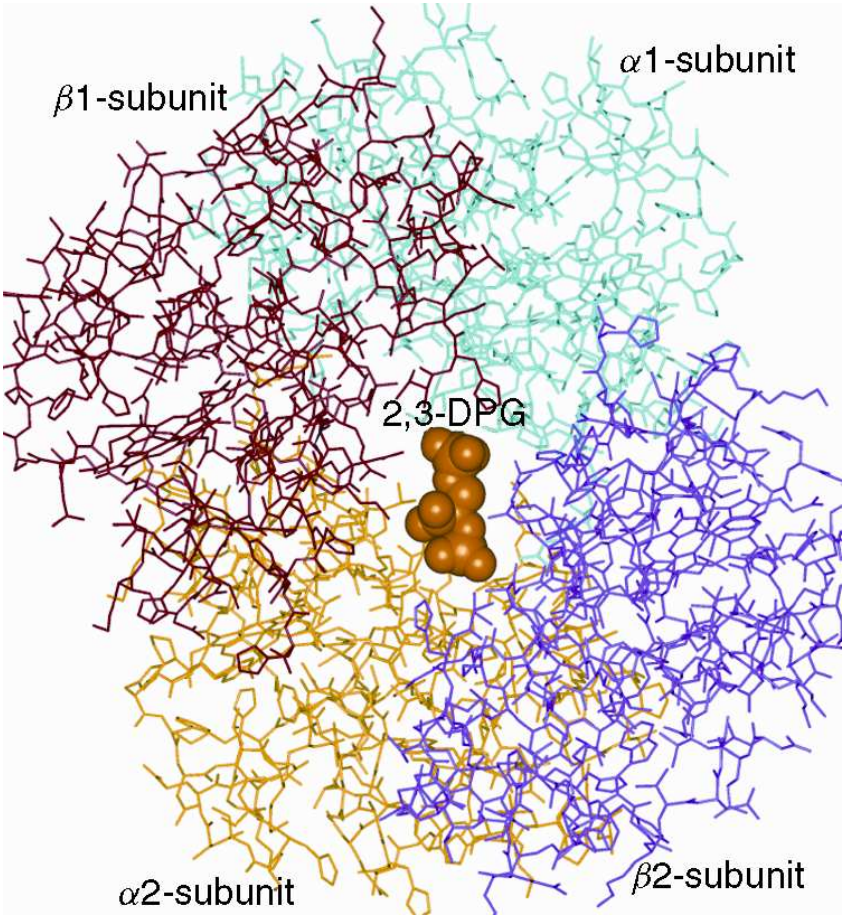
2,3 DPG



Clofibric Acid
CFA

The CFA binding site[27;30] was far removed from the 2,3-DPG site at the surface of the β subunits[31] (compare Figures 1 and 2c).

Figure 1. View of the 2,3-DPG binding site at the mouth of the β-cleft of deoxy hemoglobin[29].

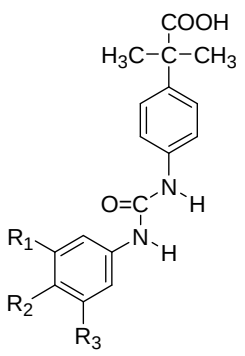


The determination of the CFA binding site on Hb was the first report of a tense state (deoxy state) non-2,3 DPG binding site. However, our excitement for using clofibrate to treat sickle cell anemia was quickly dashed when we checked the literature and discovered that CFA binds well over 90% to serum proteins regardless of dose. Later, we confirmed the serum protein binding in whole blood using oxygen equilibrium analyses that indicated very little CFA was transported into erythrocytes in quantities sufficient to interact with hemoglobin.

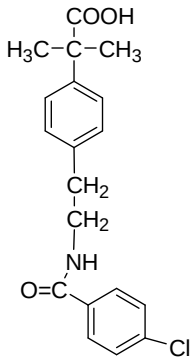
The shift in the oxygen binding curve in the opposite direction than that desired to treat sickle cell anemia opened new fields of potential treatment for ischemic disease states such as stroke and angina mentioned above as well as use for enhancing radiation of tumors, during transplant surgery to keep vital organs oxygenated, or even to greatly extend the shelf life of stored blood since aged blood is not effective due to 2,3-DPG depletion. When we published our CFA results[32], the first clinically oriented researchers to use the clofibrate discovery (to the surprise of Max Perutz and the author) were the radiation oncologists who were testing a theory that radiation treatment of hypoxic tumors was enhanced if oxygen levels were increased[33;34].

Perutz and Poyart tested another antilipidemic agent, bezafibrate (BZF), and found it much more effective than CFA[35]. It was the authors task on a subsequent visit to the MRCLMB to determine the binding site for bezafibrate and found that it linked both the high and low occupancy CFA sites as well as a new type of hydrogen bond between Asn 108β and the halogenated phenyl ring of bezafibrate [29;30]. Perutz gave a lecture at Harvard and pointed out the new type of hydrogen bond and this stimulated Burley and Petsko to look for this general type of hydrogen bond in protein structure [36;37]. This is one of the few examples where drug binding has helped elucidate and understand protein structure.

Lalezari and Lalezari synthesized urea derivatives of bezafibrate and with Perutz, determined the binding site of the most potent derivatives to be the same that we discovered for bezafibrate [38]. Although all of these compounds were extremely potent, they were not suitable clinical candidates being hampered again by serum protein binding[39;40].



Lelazari Series

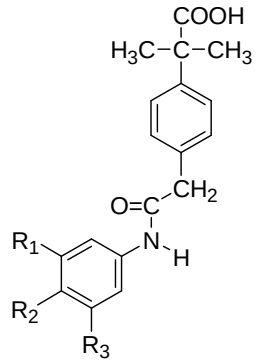


Bezafibrate

From this point our laboratory used a more classical structure activity approach to find the best clinical candidate. To get a structure activity spread we synthesized a series of *bis*-phenyl fibrate analogs making every type of substitution in the three atom linking chain between the aromatic rings (NH-CO-CH_2) shortening the four atom bezafibrate linking chain[40]. To our surprise and great delight, one class (RSR) produced a large shift of the oxygen binding curve of hemoglobin in the presence of whole blood, something we or others had not observed previously with any other classes of molecules. The two most active molecules were RSR 4, the 3, 5-dicholor derivative, and RSR 13, the 3,5-dimethyl derivative. RSR are the initials of Ram S. Randad, the excellent postdoctoral researcher who synthesized this

class of molecules*. Another postdoctoral researcher, Ahmed A. Mehanna returned for a second stay in our laboratory and played a significant role in overseeing the synthesis for all classes of molecules being made in our group. Seeing the RSR 4 and RSR 13 results in whole blood compared to the results for the Lalezari molecules and bezafibrate discussed below was the first time the author was confident enough to believe we had a discovery that might result in a real therapeutic agent[41].

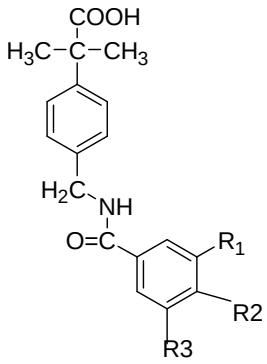
The substitutions that reversed the amide bond, derivatives of graduate student Mona A. Mahran (the MM series) were all weaker allosteric effectors. We spent the next few years sorting out the molecular reason for the behavior of this class of molecules.



RSR Series

RSR 13 R1,3 =CH3; R2 = H

RSR 56 R1,3 = OCH3; R2 = H



MM Series

MM 30 R1,3 = Cl; R2 = H

MM 25 R1,R3 = H; R2 = Cl

X-ray crystallographic analyses alone could not sort out the reason for differences between the RSR, MM and other weak acting series. **Figure 2a** is a stereo diagram showing the overlap of four allosteric effectors that bind at the same deoxy Hb site but differ in their allosteric potency. Only small differences in atomic

* In our research group all new compounds are coded with the initials of the person who synthesizes them. Ram Randad's initials appear now routinely in the chemical literature attached to widely studied RSR 13 allosteric effector.

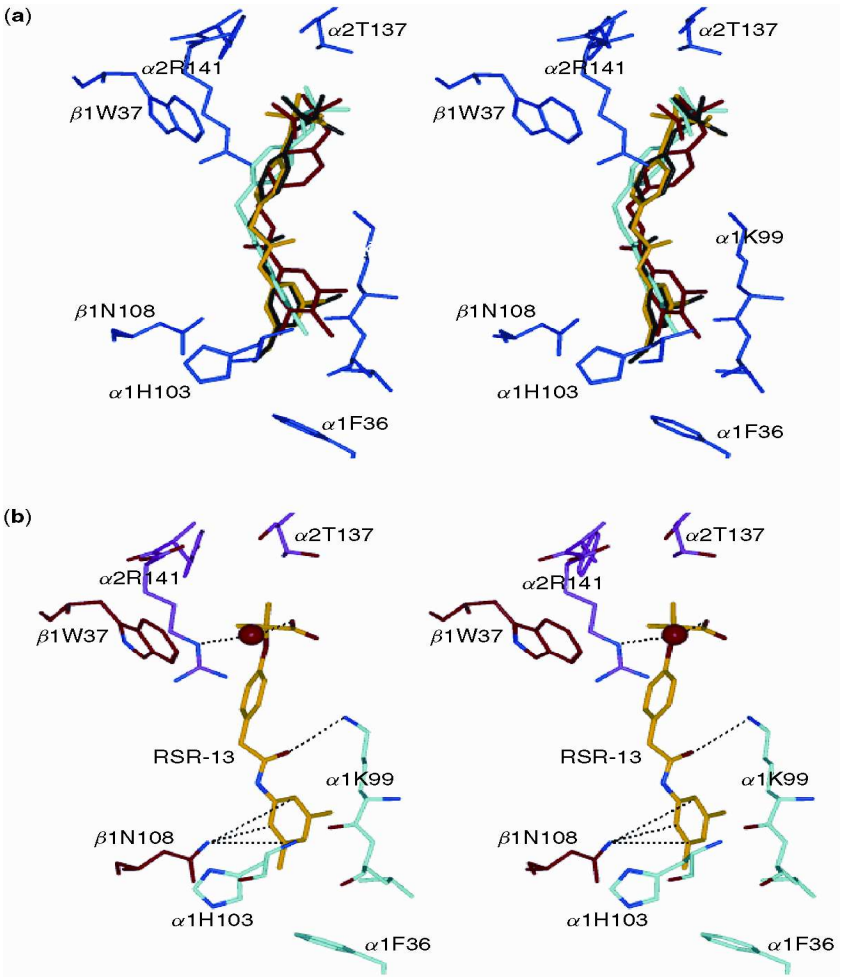
positions are apparent when comparing the strong RSR molecules with the MM molecules.

The computational program HINT* (Hydropathic INTeractions), developed in our laboratories[16], however provided invaluable information fingering the important interactions between the strong and weak acting allosteric effectors. The HINT analyses revealed that the amide linkage between the two aromatic rings of the compounds must be orientated so the carbonyl oxygen forms a hydrogen bond with the side-chain amine of αLys99[39;42]. [30;39] (**Figure 2b**). Three other important interactions were found. The first were the water-mediated hydrogen bonds between the effector molecule and the protein, the most important occurring between the effector's terminal carboxylate and the side-chain guanidinium moiety of residue αArg141. Second, a hydrophobic interaction involves a methyl or halogen substituent on the effector's terminal aromatic ring and a hydrophobic groove created by Hb residues Phe36α, Lys99α, Leu100α, His103α, and Asn108β. Third, a hydrogen bond is formed between the side-chain amide nitrogen of Asn108β and the electron cloud of the effector's terminal aromatic ring [39;42;43]. The author first observed the new hydrogen bond while contouring the Hb binding site of bezafibrate [29; 35].

Figure 2. Stereoview of allosteric binding site in deoxy hemoglobin. A similar compound environment is observed at the symmetry-related site, not shown here. (a) Overlap of four right-shifting allosteric effectors of hemoglobin: (RSR13, yellow), (RSR56, black), (MM30, red), and (MM25, cyan). The four effectors bind at the same site in deoxy hemoglobin. The stronger acting RSR compounds differ from the much weaker MM compounds by reversal of the amide bond located between the two phenyl rings. As a result, in both RSR13 and RSR56, the carbonyl oxygen faces and makes a key hydrogen bonding interaction with the amine of αLys99. In contrast, the carbonyl oxygen of the MM compounds is oriented away from the αLys99 amine. The

* Glen Kellogg came into my group as a post doctoral researcher and wrote the code for HINT based on ideas published by Al Leo and myself [62] . Professor Kellogg deserves credit for the development and use of HINT in industrial and academic laboratories world wide.

αLys99 interaction with the RSR compounds appears to be critical in the allosteric differences. (b) Detailed interactions between RSR13 and hemoglobin, showing key hydrogen bonding interactions that help constrain the T-state and explain the allosteric nature of this compound and those of other related compounds



As mentioned above, Burley and Petsko subsequently pointed out this type of hydrogen bond existed in a number of proteins [36;37]. Perutz and Levitt estimated this bond to be about 3 kcal/mol, much stronger than we originally thought [44].

Dr. Gajanan Joshi in our group next measured with painstaking experiments the binding constants of over thirty allosteric effectors and compared them with number of binding sites and found that all agreed with the number of crystallographic binding sites found[45]. The degree of right shift in the oxygen-binding curve produced by these compounds was not solely related to their binding constant, providing a structural basis for E. J. Ariens' theory of intrinsic activity. These studies enabled us to get information directly related to the atomic mechanism of action and reasons for the allosteric shift. The conclusions from our structural studies provide a near complete understanding for the observed structure activity results.

1. All of these derivatives bind and overlap in the bezafibrate binding site. Direct analysis from the crystal structures did not reveal the reasons for the observed maximum activity.
2. HINT clearly diagnosed that the amide bond oxygen must face and hydrogen bond with Lys 99α.
3. The interactions between the allosteric effectors and hemoglobin add hydrogen bonds to the Hb tense state, similar to DPG, and therefore stabilize that state resulting in an increased delivery of oxygen.
   Hb O_2 (R state) + Allosteric Effector ↔ Hb (T-state)· Allosteric Effector + O_2
4. A single substitution on the terminal aromatic ring at the 3 (meta) position of the terminal aromatic ring in the RSR Series oriented the substituted group away from the alpha helix containing Lys 99α (G helix) and toward the sterically more accessible Hb central water cavity. The mono substituted phenyl derivatives were all much weaker allosteric effectors than the 3,5 di- or 3,4, 5 tri- substituted molecules. However, the di- or tri- substitutions at 3- and 5- or 3,4,5- positions forced one methyl group into the G helix and we believe it acts like a screwdriver wedged in to a gear preventing it from undergoing

the allosteric transition to the R state due to restriction of movement of the G helix.

5. The extra binding affinity by the addition of the second or third substitution was not in proportion to the degree of shift in the oxygen binding curve. There were some derivatives with lower activity with binding constants equal or close to the most active analogs. The difference for the increased activity was the entropic placement of the second substituent into the G helix hindering its movement during the allosteric transition to the tense state[45;46].

GELD (MONEY)

ADVANCING A LEAD COMPOUND TO CLINICAL TRIALS

Preclinical Studies at Virginia Commonwealth University's Medical School on the Medical College of Virginia Campus.

The following two **Figures: 3 a** (university units) and **3b** (individuals) show the scheme we used to link the university together to forward the new molecule to phase one clinical trial. We were fortunate to link a number of Virginia Commonwealth University (VCU) medical professors on the Medical College of Virginia campus to test RSR 13 in preclinical evaluation for its efficacy in treating hypoxic diseases. The first radiation oncology animal investigations were performed by Prof. Rupert Schmidt-Ullrich's group [47;48] and the first investigations of RSR 13 for potential use in stroke were performed by Prof. Hermes Kontos' [49], and Prof. Ross Bullock's groups [50]. Other VCU professor's and their colleagues looked at RSR 13 to counter hypoxia in brain injury and for surgical uses. Professors Albert Munson and Jean Meade (VCU Department of Pharmacology and Toxicology) performed the first toxicology evaluations that were key to the initial venture capitalization. Linking well funded university professors together across disciplines who wanted to study RSR 13 in their areas of specialty resulted in numerous publications indicating an excellent

prognosis for efficacy and safety *in vivo*. Allos Therapeutics Inc. was then able to proceed through a phase one

Figure 3a The linked departments and institutes at VCU that were associated with moving both Hemoglobin allosteric effectors from the bench to phase one clinical trials. The CIT box is the State of Virginia Center for Innovative Technology that held the original VCU patents and matched funds with industry to develop new drugs. The Institute for Structural Biology and Drug Discovery is located in the Virginia Biotechnology Research Park.

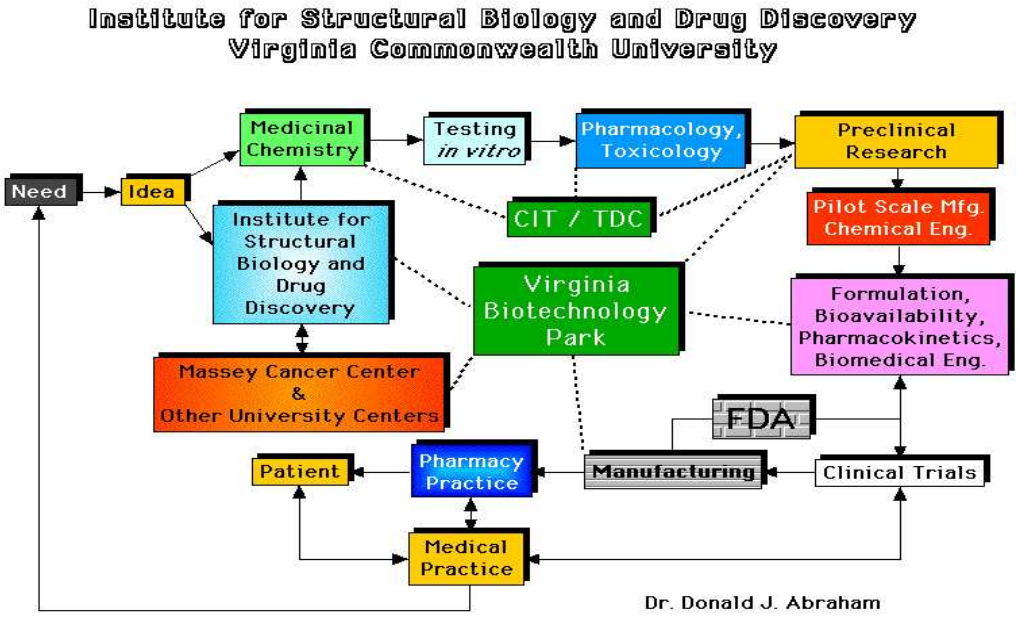
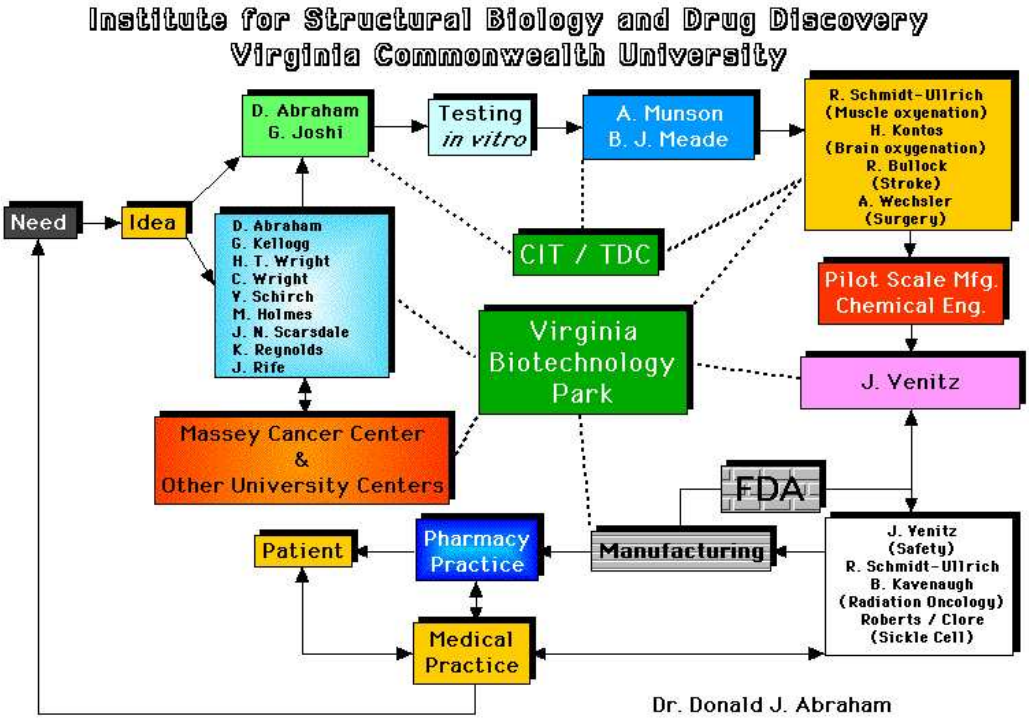


Figure 3b VCU professors in the Institute for Structural Biology and Drug Discovery and professors from the associated departments in Fig. 1a. who also performed translational research that performed basic and/or clinical research in advancing RSR -13 and (vanillin) an antisickling molecule to clinical trials.



study based on the basic and preclinical studies for only 2 million dollars. This is perhaps a record for a new drug. The following graph prepared by former Allos CEO Stephen Hoffman compared the cost of development of RSR-13 to the blood substitute companies who also sought to increase oxygen delivery *in vivo* (**Figure 4**).

The role of the Virginia Biotechnology Research Park in our drug discovery efforts:

Advancing any new agent from the laboratory bench to clinical trials is rare. Advancing it through an academic network is even rarer. The advent of the

Virginia Biotechnology Research Park (VBRP) adjacent to the Medical College of Virginia campus of Virginia Commonwealth University turned out to be a key player in the process toward commercialization of the first hemoglobin allosteric effector RSR-13. VCU President Eugene Trani and also Chairman of the Board for the VBRP encouraged faculty to initiate biotechnology companies for the research park. Several faculty initiated companies sprang to life, including what was first named HemoTech Inc. and later named Allos Therapeutics, a company the author was encouraged to found by VCU President Eugene Trani's office. Mr. James Farinholt, President Trani's lead person for business development at VCU at that time, helped tremendously in sorting out the details needed to tie the university and state with any future funding by venture groups or pharmaceutical houses.

Figure 4: The comparison of the costs of several blood substitutes companies to develop an oxygen carrying molecule vs. the Allos Therapeutics Inc. allosteric effector RSR 13 to an IND and phase one clinical trial.

Surgical Blood Loss: Development Comparison

	Research	Preclinical	IND	Phase I	Phase II
Somatogen					
Project	Genetically linked Hb with Hb Presbyterian substitution (Asn108β->Lys)				
Cum. Spending (million $)		$18.7	$18.7	$75.1	$119.6
Valuation (million $)		$30.0	$102.0	$122.0	$445.0
Date		7/89 - 6/91	6/91	2/92 - 8/93	9/93 - 9/95
Duration / Cum. Time (mos.)		24/48	7/55	19/74	24/98
Northfield					
Project	Glutaraldehyde crosslinked multimer Hb				
Cum. Spending (million $)				$36.4	$56.1
Valuation (million $)				$90.0	$231.0
Date				87 - 2/94	3/94 - 9/95
Duration / Cum. Time (mos.)				93/98	16/114
Alliance Pharmaceuticals					
Project	Perfluorocarbon				
Cum. Spending (million $)				$48.3	$70.7
Valuation (million $)				$297.0	$305.0
Date				2/92 - 5/93	6/94 - 6/95
Duration / Cum. Time (mos.)				15/unk	12/unk
Baxter					
Project	Crosslinked Hb with diasperins				
Allos Pharmaceuticals					
Project	Allosteric effectors				
Cum. Spending (million $)			$2.2	$2.5	
Valuation (million $)			$8.5	$8.5	
Date			6/95	7/95 - 9/95	
Duration / Cum. Time (mos.)			12	3/15	

The name chosen for Allos Therapeutics Inc. was taken from the scientific terminology for **allos**teric equilibriums modulated by allosteric effectors. Allosteric proteins regulate some of the most important pathways for life. Allos' initial focus was to discover and develop allosteric effectors as therapeutic agents. Having associated with a number of large pharmaceutical houses in drug discovery efforts toward finding an antisickling drug, it was clear that nothing was advanced through the complicated maze of large pharmaceutical house's drug development teams without a champion overseeing every phase. We had no such champion and the concept of an allosteric effector as a drug was a hindrance. Few if any in the drug discovery world understood the theory of allosteric regulators and their potential as a new type of drug action.

On the other hand, small start up companies focused intensely and entirely on what they were created to do with the incentive that early and even later employees would be well rewarded if the venture was successful. It was clear to the author, in this case, that if the translation from basic and preclinical research to clinical trials were to occur, our best chance was to initiate a company. For the most part, big pharmaceutical houses have stayed clear of allosteric effectors as drugs. We had the enormous advantage that far more is know about hemoglobin as an allosteric protein than any other.

Venture Capital and New Companies:

The best discoveries can lay dormant without recognition of their importance by the investigator(s). Medicinal chemists involved in drug discovery in academia must consider patenting as well as publishing. Publishing the results of a new compound without patenting assures that it will not be translated to a new drug. Publications and accompanying patents are not just necessary but critical to attract venture groups who can provide startup funds for a new company.

HemoTech Inc. went without major funding for several years and no one seemed interested. The director of the VBTRP had a contact with a Wall Street Journal reporter who was interested in early stage discoveries. The Wall Street Journal published an article[51] on the same day we published the first *in vivo* results combined with the crystallographic binding site for RSR-13 and RSR-4[39]. This drew much attention from the venture capital groups and large pharmaceutical houses. While Ortho Biotech, a Johnson and Johnson subsidiary wished to license the technology, Johnson and Johnson's venture outlet decided a startup company would be better and a conglomerate of Venture groups with them, called Med Vest, provided funds for the toxicology and phase one trial for RSR-13. The due diligence performed by venture groups can be much more extensive than imagined. After the company was funded the author was provided with the due diligence including letters of approval and extensive review of our basic science studies by colleagues in a host of academic institutions. The process made NIH reviews for research grants look trivial, which those of us who apply routinely for NIH funding know is not the case.

Essential was the selection of Stephen Hoffman, MD, Ph.D., as CEO for HemoTech Inc.[*] Stephen, whose Ph.D. was in Organic Chemistry had both a firm hand on the science and the medical aspects. Dr. Hoffman's experience as the scientific founder of Somatogen, the first blood substitute company to go public, provided him with a firm understanding of the chemistry and physiology of oxygen delivery. Stephen was also able to link our new company with a venture investment group headed by Mr. James Daverman of Marquette Ventures, who had invested in Somatogen and therefore had considerable knowledge in the hemoglobin field. Mr. Daverman was fortunately for us a member of the Medvest Inc. conglomerate venture group who funded Allos Therapeutics Inc. Overall, the understanding of allosteric regulation processes and their potential by RSR 13

[*] The name was changed later to Allos Therapeutics Inc

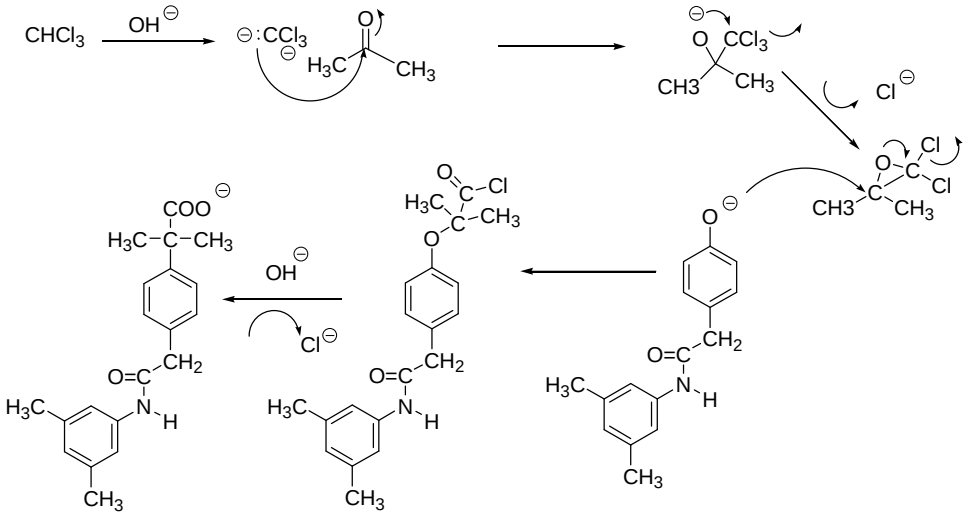
investors was not only a surprise, but made all the difference in their decision to provide the first two million dollars

Initial Toxicology and Venture Capital Funding

The choice of which allosteric effector to forward to clinical trials was made intuitively. A crucial decision had to be made since the potential venture capital group, Med Vest, headed by hematologist turned entrepreneur, Dr. Geoffrey Brooke, would only pay for one toxicity study before a decision was made on the commitment of the first two million dollars to fund our new company. The author had to chose from the top two candidates, either the weaker acting 3, 5 di-methyl derivative (RSR 13) or the more potent 3, 5 di-chloro RSR 4 molecule. Both molecules completely overlapped in their binding sites with only the longer bond lengths for the chorine atoms. We had considerable experience testing halogenated aromatic acids in our sickle cell assay and almost all were unsuitable due to toxicity issues. The author also believed that, in general, in drug discovery, alkane derivatives had less toxicity issues than the corresponding halogen derivatives. Therefore the author chose the 3, 5-disubstuted methyl groups (RSR 13) over the 3, 5-disubstituted chlorines (RSR 4). The whole advance of this project would be determined from the choice made. Fortunately, this choice of RSR 13 was well founded. The di-chloro derivative RSR 4 tended to lyse erythrocyte membranes and was not suitable for *in vivo* study at the doses needed. To our further surprise the di-methyl derivative, RSR13, was even more active *in vivo* in animal studies than RSR 4, opposite of the *in vitro* tests.

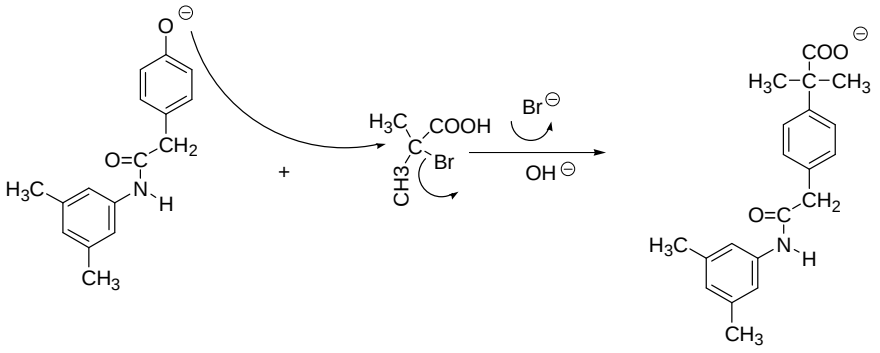
Synthetic Chemistry and Purification

Both the two step synthetic scheme we had used for RSR 13 and the method for purification of the sodium salt of RSR 13 had to be altered for large scale preparations to be used in clinical trials. The normal two step synthesis involved a standard carbene reaction run in chloroform.



RSR 13 via carbene reaction

Due to the environmental restrictions on the use of chloroform in industrial chemistry, an alternate synthesis was devised by the scale up chemists. It involved a seemingly prohibitive step, at least as professors instruct students in organic chemistry: an SN_2 reaction on a tertiary carbon.

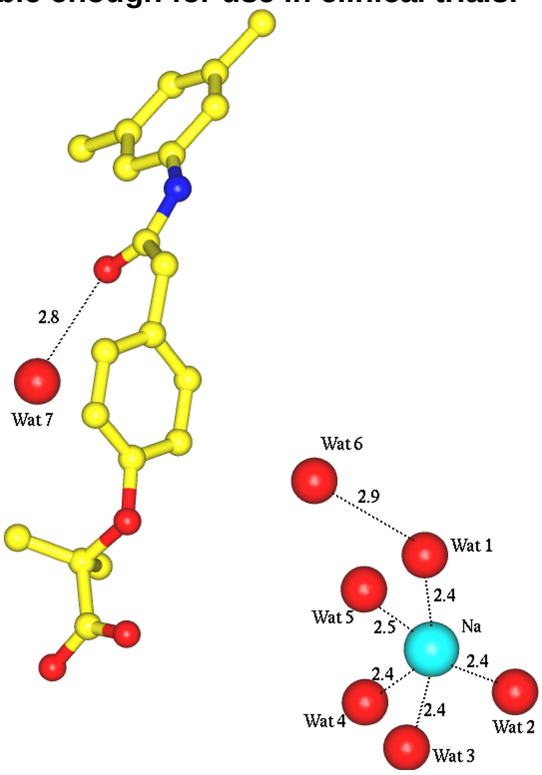


RSR 13 Industrial synthesis

The other change that needed to be made in the synthesis of RSR 13 for *in vivo* administration was the method of purification. RSR 13 is used *in vivo* as the sodium salt. The author prepared the first batch for *in vivo* toxicology triturating RSR 13 sodium salt with acetone to remove any vestiges of water. However, the first industrial scale up procedure called for crystallization of the salt from ethanol-water. The ethanol-water crystals were not as soluble as the acetone triturated method and could not be formulated at a reasonable volume. We performed the crystal structure of the ethanol water crystals and found that it was a hepta-hydrate **(Figure 5)** [52]. The problem for large scale production of RSR 13 was solved eventually by industrial the producers of RSR 13.

A recent Chemical and Engineering News article highlighted the Allos Therapeutics Inc. outsourcing of RSR 13 along with three other pharmaceutical companies of different sizes. In particular, Allos Therapeutics Inc. contracted Hovione, a Portuguese pharmaceutical chemical company to synthesize RSR 13 under cGMP conditions for clinical trials. While the synthesis was not difficult, Douglas G. Johnson VP for manufacturing at Allos reported that an unsuspected impurity profile showed up upon the scale up and could only be remedied by equipment changes. Hovione were committed to this project and agreed to purchase special equipment. The C & E News article concludes that good service leads to repeat business and Hovione remained committed to Allos and purchased the needed equipment. [53]

Figure 5: RSR 13 Heptahydrate Sodium Salt. The sodium atom is in light blue, water atoms are large red spheres, carbons yellow, nitrogen dark blue, and oxygens red. This particular RSR 13 salt was not soluble enough for use in clinical trials.



Allos Therapeutics Inc. and Clinical Trials with a submission of an NDA.

The phase one safety study was performed by Professor Jurgen Venitz in our Department of Pharmaceutics in the School of Pharmacy. Jurgen is one of the best qualified phase one clinical trials scientists in the country. Being convinced that RSR 13 was in deed safe, the author volunteered to be the first subject, but was not allowed to be enrolled in the study due to my age and the fact that RSR 13 came from my laboratories. The author was allowed to be present at the first

injection of RSR 13 in humans. It was a nervous time, even with the firm belief that safety issues with RSR 13 should not be an issue.* And it was not.

CEO Stephen Hoffman and VP for clinical studies Michael Gerber of Allos Therapeutics guided RSR-13 through a series of phase one and two clinical trials for radiation treatment of brain tumors and for potential use in cardio pulmonary bypass surgery [54;55;56;57;58;59]. Considering the cost of running a phase three clinical trial, only one was possible. The very positive phase two results for use of RSR 13 to treat metastatic brain cancer provided the impetus for selecting that indication for a phase three trial.

Stephen Hoffman as CEO of Allos raised about 40 million dollars of private funding before he and the current CEO Michael Hart did a tremendous job of taking the company public and raising 90 million dollars. The public offering just made the window by a few dozen hours for an IPO, as the window quickly closed after President Clinton announced that the human genome sequence could not be patented. This news sent the biotechnology stock market plummeting.

The total sum of 140 million and subsequent private funding of around 12 million dollars enabled the completion of the phase three clinical trials with about enough remaining to conduct a second phase three trial if the FDA does not approve the current NDA.. The total amount spent if the RSR 13 is approved is greatly reduced compared to the estimated current average cost for a new therapeutic agent, over one half billion dollars.

The phase three results were un-blinded in March of 2003 and overall, for all types of metastatic brain cancer, were reported as non-positive showing the drug to have efficacy but not at the statistical level set at the beginning of the trial by |Allos Therapeutics Inc for a successful phase three trial. The subset analysis for the

* The author felt like he was watching the troops, in this case the first medical students who took RSR 13, go ashore at the battle of Normandy while being safely sequestered on a ship watching the invasion and praying no one would be injured.

different types of metastatic cancer to brain, however, showed that breast cancer had a 50% increase in survival. The 500 plus patient trial also demonstrated RSR 13 to be extremely safe confirming our early design feature of using a known antilipidemic drug, clofibrate, as a substructure. An NDA for RSR 13 with trade name **efaproxaril** is being reviewed by the FDA on a fast track review for use in treatment of metastases of breast cancer to brain with the decision to be made by June 2004. As stated above, Allos has started another phase three trial specifically for the use of efaproxaril to treat metastatic breast cancer to brain.

Just the Beginning

Normally, the body only uses about 25 % of Hb's oxygen for normal biological processes. The potential magnitude of having a therapeutic agent that delivers stored oxygen cannot be underestimated. To date, no such agent has been available to study the physiology of numerous *in* vivo pathways that might be regulated with an increase or decrease in oxygen pressure.

It is the author's opinion that if RSR 13 is approved, its potential use in medicine could be far beyond the radiation treatment of tumors. RSR 13 is the first molecule with human efficacy to safely shift the oxygen levels to tissues. RSR 13 has a short half life with IV infusion and therefore will be limited to acute uses. The potential acute use for cardiovascular areas might prove to be the largest target. RSR 13 is also a potential drug that coulb be used illegally in sports to increase VO_2 max, the volume and velocity of oxygen released to tissue during exercise. Apparently RSR 13 was credited for a disqualification of a bike rider in the Italian version of the Tour de France, the 2001 Giro d'Italia, see http://www.totalbike.com/news/article/556/, and http://perso.wanadoo.fr/jc.auriol/histoires.htm. Allos Therapeutics Inc. has been working with the Olympics committee on how to detect any illegal uses of RSR 13.

Another Allosteric Effector for Treatment of Sickle Cell Disease.

In the 1990's we advanced another Hb allosteric effector to clinical trials, vanillin [60]. Dr. Martin Safo in our group has recently extended our search for a non-toxic aldehyde food substance as a potential antisickling agent. The latest molecule 5-HMF (5-Hydroxymethyl-2-furfural) just completed *in vivo* tests in sickle cell transgenic mice with outstanding results [61]. We hope our experience with RSR 13 will aid us in forwarding 5-HMF through clinical trails.

Take Home Messages

1. Collaborative efforts across academic units were a vital key for successful translation from the bench to the bedside.
2. Use of a known low toxicity scaffold (drug) for building molecular specificity was the most important advance permitting us to overcome toxicity issues due to the large doses required to treat almost a pound and a half of the *in vivo* receptor (hemoglobin).
3. When in doubt as to which molecule to forward as a clinical candidate, consider metabolism and toxicity profiles as well as biological activity.
4. Having a dedicated startup company to champion moving a molecule through all the steps to an NDA made the difference as large pharmaceutical companies are less likely to champion a compound from academia.
5. Serendipity still continues to play its important role in drug discovery. All four German G's of Paul Ehrlich[8] that scientists need were evident in our case study.

Acknowledgements: The author acknowledges the financial support for the Hb basic research and preclinical studies from our long standing NIH grant: 5 RO1HL32793, NIH grant NO1-HB-1-301, Allos Therapeutics Inc. grants, The H.J. Heinz Company, The Willie Stargell Foundation, and The C. Usher Foundation and The European Molecular Biology Organization (EMBO) and the Max Perutz Fund for short term fellowships to visit the MRC Laboratory of Molecular Biology. The author also wishes to thank the help of Mrs. Ruth White and the Sickle Cell Society of Pittsburgh, the Deans and Administrators at the University of Pittsburgh and Virginia Commonwealth University for the freedom to pursue this work as needed and for financial contributions. Last, but not least, we thank our many colleagues and collaborators in Europe and the USA who put their shoulder to the till to provide the strong science contributions that built a solid foundation for advancing two agents to clinical trials. A very special thanks to my wife Nancy L. Abraham who also provided me the freedom and support to spend whatever time was needed at our universities or abroad to bring this work to fruition.

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