GLAXO CARDIOVASCULAR DISCOVERY GRANTS

RESEARCH AGREEMENT

THIS AGREEMENT, dated as of July 1, 1989, by and between **GLAXO INC.**, Five Moore Drive, Research Triangle Park, North Carolina 27709, a North Carolina corporation ("Glaxo"); and **VANDERBILT UNIVERSITY SCHOOL OF MEDICINE**, Nashville, Tennessee 37232 ("University"); and **JACKIE D. CORBIN, Ph.D.** ("Principal Investigator").

WITNESSETH:

WHEREAS, the Principal Investigator is an applicant for a research funding grant from Glaxo under its program entitled "Glaxo Cardiovascular Discovery Grants," and the Principal Investigator has been recommended as an awardee for such grant; and

WHEREAS, the University wishes to have the Principal Investigator conduct the program of research defined below at the University, which program will be of benefit to the University and will further the University's goals of research, teaching, education and public service; and

WHEREAS, Glaxo wishes to sponsor the research and to obtain an exclusive license to inventions arising from such research;

NOW, THEREFORE, in consideration of the mutual benefits to be derived therefrom, the parties hereto agree as follows:

I. Statement of Work

(a) The research program to be conducted at the University under the direction of the Principal Investigator shall be as described in the proposal attached hereto as Exhibit A and incorporated into the Agreement by reference and such other work as may be undertaken by the Principal Investigator upon prior agreement by the Principal Investigator and Glaxo (the "Project"). The Project shall be subject to reasonable modification by the Principal Investigator as deemed necessary to fulfill the objects of Exhibit A, without the prior approval of Glaxo.

(b) The University, under the direction of the Principal Investigator, shall exercise reasonable efforts to carry out the Project in accord with accepted standards for the conduct of scientific research.

II. Period of Performance

The Project, which shall be funded for a three-year period or such other period as indicated in Exhibit A or B,

shall commence on or about July 1, 1989, and will terminate on June 30, 1992. This Agreement may be renewable for additional periods upon the mutual consent of the parties.

III. Payment

Payment for the Project will be made to the University by Glaxo in accordance with the schedule attached as Exhibit B hereto. Title to any and all supplies and equipment purchased by the University for use in the Project shall be in the University and shall be free of all claims, liens or encumbrances of Glaxo or anyone claiming under or through Glaxo. A final financial accounting of all costs incurred and all funds received by the University under this Agreement, together with a check for the unexpended balance, if any, will be submitted to Glaxo within ninety (90) days following the termination of Project.

IV. Rights in Inventions and Know-How

(a) Ownership of Inventions: Any invention in the Field, below defined, made by the Principal Investigator or any other person who participates in the Project at the University (other than Glaxo employees) and conceived or reduced to practice during the course of and under the Project shall be the property of the University subject to the rights agreed herein to be granted to Glaxo under a

License Agreement to be entered into between the parties. "The Field" shall be the subject matter of the research described in the Project protocol and amendments thereto and attached as Exhibit A. The University shall promptly and confidentially disclose any invention in the Field to Glaxo in writing after disclosure has been made to the University's Office of General Counsel by the investigators working on the Project. All inventions in the Field conceived or reduced to practice during the course of and under the Project which are made, in whole or in part, by any non-Glaxo employee participating in the Project, shall be referred to hereinafter as "Inventions."

All patent applications covering Inventions, together with any patent resulting therefrom, including divisionals, continuations, continuations-in-part, reissues, extensions of term, registrations and confirmations thereof shall be referred to collectively hereinafter as the "Patents." So defined, the term "Patents" does not include patents on inventions reduced to practice prior to the commencement date of the Project or not reduced to practice or conceived during the course of and under the Project, which patents (if any) shall be referred to herein as the "Underlying Patents."

(b) Exclusive License: Glaxo shall have an option to a License of patent rights to an Invention made pursuant to

this Research Agreement. The option shall expire six months from the date a patent application is filed covering such Invention unless exercised in writing by Glaxo.

The License Agreement shall be an exclusive worldwide license, with right to sublicense the Invention, subject to agreement by the parties, following good faith negotiations of specific terms, including royalty rates, and subject to the rights of the Federal Government, as required by applicable law and regulation. Glaxo's license shall encompass a non-exclusive right to use and disclose all Know-how arising from the Project, and such shall be royalty free. Know-how shall include, without limitation, all technical data, information, materials and non-patentable or non-patented discoveries arising out of the Project. Additionally, Glaxo shall have the unlimited right, without payment, to use non-patentable know-how in the furtherance of its own research. To the extent the University may legally do so, it shall grant to Glaxo a non-exclusive license under any or all Underlying Patents to the extent necessary to practice the Inventions covered by the Patents to be licensed pursuant to this Agreement and subject to any then-outstanding licenses or options previously granted by the University to third parties. The parties agree to negotiate in good faith the terms of any such non-exclusive

license including any royalty to be paid.

Within three months after Glaxo exercises its option to License, Glaxo and the University shall negotiate in good faith the terms of a license agreement. In such terms, a reasonable royalty shall not exceed seven percent (7%) for patents covering such Invention, based upon Net Sales (hereinafter defined) of any product embodying the Invention, provided, that royalties otherwise payable may be offset by Glaxo's worldwide reasonable costs of patenting the Invention (pursuant to subparagraph IV(c) hereof) and maintaining and enforcing the patent; and provided, however, that the amount of such offset shall be mutually agreed upon but in no event shall exceed one-half of the royalty otherwise payable. "Net Sales" shall mean the gross invoice price of the sold product less (i) charges for transportation, insurance, taxes and duties or governmental charges shown on the invoice, or otherwise capable of documentation, (ii) credits for trade, cash or other discounts actually granted, and (iii) amounts repaid or credited by reason of rejections, recalls or return of goods or because of retroactive price reductions. The royalty terms to be negotiated in good faith may include an initial license fee not to exceed One Hundred Thousand Dollars ($100,000.00), payable to the University upon execution of the License Agreement. Annual minimum royalties

may also be included in the royalty terms to be defined in the License Agreement.

Criteria of due diligence to be met by Glaxo in commercializing a Licensed Invention shall also be negotiated in good faith and set forth in the License Agreement.

If the parties fail, after good faith negotiation, to conclude a license agreement within three (3) months of beginning negotiations, Vanderbilt shall be free to undertake and conclude licensing negotiations with a third party, with no further obligation to Glaxo except as provided in IV(c)(i).

(c) Patenting

(i) University shall, at its discretion, have the right to seek patent protection for Inventions at Glaxo's expense and in the name of the University, using independent patent counsel satisfactory to Glaxo, but chosen by the University to represent it. University shall provide regular reports on the progress of patent applications. Glaxo shall inform the University in which countries it desires to seek patent protection for such Inventions, and University shall make such filings at Glaxo's expense. The University shall provide Glaxo sufficient opportunity to review and approve any patent applications or other filings and correspondence prior to their submission by University to the appropriate

Patent Office, and shall work in good faith with Glaxo's patent counsel to resolve any disputed provisions before submission to the Patent Offices. University shall also promptly provide Glaxo with copies of all documents and correspondence received by University from the Patent Offices. The University and the Principal Investigator and any other inventors will execute all documents and perform all acts, at Glaxo's expense, reasonably necessary to prosecute, maintain and enforce the Patents. Glaxo shall be reimbursed for expenses incurred by it in the filing of a patent application covering an Invention, on which it determined not to exercise its option to License, or for which no license agreement was entered into, but such reimbursement shall only be made by University to Glaxo from monies received by University either as a license fee or royalty payments from licensing of the Invention to a third party.

(ii) Glaxo shall promptly advise the University of any decision not to finance the filing, prosecution, or maintenance of a Patent in adequate time to allow the University, at its own cost, to effectuate such filing, prosecution, or maintenance if it so desires; and shall, at the request of the University, take whatever steps may be necessary to return to the University all rights which Glaxo

may have thereto.

(iii) Although the party seeking patent protection shall use reasonable efforts to obtain patent protection, neither party warrants that it will be able to do so and it shall not be a breach of this Agreement and neither party shall have liability hereunder with respect to the patenting including, without limitation, if the United States Patent Office or any foreign Patent Office refuses to grant a patent.

(d) Affiliates: Any right of Glaxo pursuant to this Article IV shall also be vested in any Affiliates of Glaxo. As used herein, "Affiliate" shall mean any company controlling, controlled by, or under common control with Glaxo.

V. Warranty

The University warrants that it has not previously and is not currently receiving grant support and is not consulting with any other company or government agency, which support or consultancy would or could entitle such company or government agency to rights to any Invention, subject to any rights acquired by the Federal Government from previously funded research. Glaxo agrees that the University may supplement the Project funds by seeking and securing monies by means of grants from agencies of the United States

Government. University will grant Glaxo an exclusive license to any Invention in the Field developed from the joint support of Glaxo under this Agreement and any Federal funds to be applied in the future to the Project, subject to the reservation to the Government of a non-exclusive, fully paid-up license to practice the Invention. The University shall receive Glaxo's written permission before performing services for non-governmental entities which may directly or indirectly conflict with Glaxo's interest. By entering into this Agreement, it is understood that the University presently has no such conflicting interests, agreements or obligations with any other party, other than to the Federal Government as aforesaid.

VI. Confidentiality/Publication

(a) For purposes of expediting the progress of the Project, it may be desirable or necessary to each party, during the life of this Agreement, to disclose to the other party certain confidential or proprietary information so identified and in its possession relative to the Project. In such event, the receiving party agrees to preserve such information as confidential. The obligation of confidentiality shall not apply to any information which:

(1) is now in the public domain or which becomes generally available to the public through no

fault of the receiving party; or

(2) is already known to, or in the possession of, the receiving party as can be demonstrated by documentary evidence; or

(3) is disclosed on a non-confidential basis from a third party having the right to make such disclosure; or

(4) is independently developed by the receiving party as can be demonstrated by documentary evidence.

Glaxo shall direct all such confidential information to the Principal Investigator, unless otherwise requested by the University, and the Principal Investigator and the University shall direct all such confidential information to Don F. Kirksey, Ph.D., or another Glaxo designee.

(b) The University and its employees shall have the right, consistent with academic standards, to publish or present the results of research performed under this Agreement, provided such publication or presentation does not disclose proprietary trade secrets or confidential information of Glaxo. The University agrees to submit all publications and presentations describing the methods or results of the Project to Glaxo thirty (30) days prior to submission of such proposed publication or presentation to a

journal, editor or other third party. Glaxo shall have fifteen (15) days after the receipt of the publication or presentation to review it. Upon notice by Glaxo that Glaxo reasonably believes a patent application relating to an Invention should be filed prior to the publication or presentation and if Glaxo and the University cannot agree on revising the publication or presentation such that in Glaxo's opinion it can be published or presented prior to the filing of a patent application, submission of the publication or presentation shall be delayed for up to three (3) months from the date Glaxo so notifies the University or until a patent application or applications are filed, whichever comes first.

(c) Glaxo shall have the right to request modification of any manuscript to be published if such manuscript will jeopardize a patent application or patent. If the University does not agree with such modification, Glaxo and the University will, at Glaxo's expense, consult independent patent counsel satisfactory to both parties. The University shall have the right to make the final determination regarding modifications after receiving patent counsel's opinion, subject to subparagraph VI(a) above and any confidential disclosure agreements between Glaxo and the Principal Investigator.

VII. **Reports**

The University will provide Glaxo with progress reports on the Project at such times and in such form as Glaxo may reasonably request, with a final report on the Project due sixty (60) days after termination or expiration of this Agreement. Glaxo shall have the right at reasonable times to discuss the reports and the progress of the Project with and suggest avenues of research to the Principal Investigator.

VIII. **Termination**

(a) If either party breaches any of the warranties, terms or conditions of this Agreement and fails to remedy that breach within thirty (30) days after receipt of notice of such breach from the other party, the party giving notice may, at its option and in addition to any other remedies it may have in law or in equity, terminate this Agreement by sending written notice of termination to the other party.

(b) If the Principal Investigator is unable or unwilling to continue to serve as Principal Investigator, or if in Glaxo's judgment the progress of the Project significantly fails to meet the specific aims indicated in Exhibit A, Glaxo may terminate this Agreement and its funding thereunder on thirty (30) days prior written notice.

If the Principal Investigator ceases employment at Vanderbilt and becomes employed at a different institution in

which he is able to continue the research Project undertaken pursuant to this Agreement, Glaxo will continue to support the Project at the other institution, subject to an agreement between Vanderbilt and the other institution as to ownership of any inventions and patents developed from the research begun at Vanderbilt and an agreement between Vanderbilt and the other institution concerning negotiation of a license agreement with Glaxo, including an equitable sharing of royalty payments.

(c) Upon notice of termination pursuant to this Paragraph VII, the University will proceed in an orderly fashion to terminate any outstanding commitments and to phase-down the Project, unless it desires to proceed further at its own expense or with the support of a third party. Glaxo shall reimburse the University for its reasonable costs to the date of termination, including irrevocable commitments existing at the time the notice of termination is received. In no case will reimbursement under this provision exceed the total Project cost set forth in Exhibit B.

(d) The provisions of Paragraphs IV, V, VI, VII, XII and XVI-XVIII will survive the termination or expiration of this Agreement.

(e) Upon termination of this Agreement, the University shall, if requested to do so by Glaxo, (i) provide a copy of

all Know-how, as defined in Article IV(b), arising from the Project to Glaxo, and/or (ii) return all confidential information disclosed to the University by Glaxo, except one copy which may be retained by the Counsel's office or similar equivalent for archival purposes.

IX. Use of Name

Neither party shall utilize the other's name in any promotional or advertising context or press release without the prior written consent of the other party. Glaxo may request that the University include an appropriate acknowledgement of Glaxo's sponsorship of its research program in any University publication discussing such research.

X. Notice

Any notice or other communication required or permitted under this Agreement shall be in writing and will be deemed given as of the date it is (a) hand delivered, or (b) mailed, postage prepaid, first-class, certified mail, return receipt requested, or (c) sent, shipping prepaid, receipt requested, by national courier service, to the party at the address listed above or subsequently specified in writing.

XI. Independent Contractor

The University's relationship to Glaxo under this

Agreement shall be that of an independent contractor and not an agent, joint venturer or partner of Glaxo.

XII. Liability/Insurance

(a) The University agrees that Glaxo shall not be liable for, or need to indemnify University for, damage to or loss of property or injury to or death of any persons to the extent arising out of or in connection with the University's performance of this Agreement to the extent such damages, losses or injuries occur on University premises and result from the negligence or intentional acts of University faculty, staff or students.

(b) Glaxo agrees to indemnify and hold harmless the University, Principal Investigator, members of his research team and other of the University's employees, trustees, officers, affiliates and their agents (hereinafter referred to collectively as the "Indemnified Persons") from and against any and all liability, claims, lawsuits, losses, damages, costs or expenses (including attorney's fees), which the Indemnified Persons may hereafter incur, suffer or be required to pay by reason of the manufacture, use, sale, performance, lease or other commercialization or distribution by Glaxo, its Affiliates, licensees or agents of products, devices, processes or procedures first conceived or reduced to practice at the University, by the Principal Investigator

and the Research Team under the Project, except to the extent that such liability, claims, lawsuits, losses, damages, costs or expenses (including attorney's fees) result from the negligence or intentional wrongful acts of the University, Principal Investigator or Research Team. The University shall notify Glaxo upon learning of the institution or threatened institution of any such liability, claims, lawsuits, losses, damages, costs and expenses, and the University shall cooperate with Glaxo in every proper way in the defense or settlement thereof at Glaxo's request and expense.

(c) Glaxo shall, upon the entry of any product, device, process or procedure resulting from the Project into FDA Phase III clinical trials and for so long as Glaxo or its Affiliates or any of their sublicensees or agents shall sell any products, devices or procedures which were invented or developed during the Project or licensed under a License Agreement to be entered into between the parties, or shall practice any process or method which incorporates the Project, Licensed Know-how or an Invention, secure and maintain in full force and effect a policy or policies of general liability insurance (with product liability endorsements) with limits of not less than One Million Dollars ($1,000,000) per person and Three Million Dollars

($3,000,000) per occurrence with no aggregate annual limit, or adequately self-insure. Such coverage(s) shall be purchased from a carrier or carriers reasonably acceptable to the University, which carrier(s) shall provide certificates evidencing the coverage(s) maintained and specifying that the University will be given no less than thirty (30) days prior written notice of any change in or cancellation of such coverage(s).

XIII. University's Disclaimers

(a) Disclaimers: Neither University nor any of its faculty members, researchers, trustees, officers, employees, or agents assume any responsibility for the manufacture, product specifications, or the use or administration of drugs, compounds or other substances which are manufactured by or for or which are sold by or for Glaxo or by any of its affiliates or sublicensees and which are the result of research undertaken pursuant to this Agreement. All warranties in connection therewith shall be made by Glaxo (or the particular Glaxo affiliate or licensee or sublicensee that is involved) as the manufacturer, seller, or administrator thereof and none of such warranties shall directly or by implication in any way obligate the University or any of its faculty members, researchers, trustees, officers, employees, or agents.

(b) Disclaimer of Warranty: THE UNIVERSITY MAKES NO WARRANTIES OR REPRESENTATIONS INCLUDING, BUT NOT LIMITED TO, WARRANTIES OF FITNESS OR MERCHANTABILITY, REGARDING ANY DRUG, COMPOUND OR OTHER SUBSTANCE PRODUCED BY OR RESULTING FROM THE PROJECT.

XIV. Assignment

Neither party may assign, delegate or otherwise transfer any of its rights or obligations under this Agreement without the prior written consent of the other party.

XV. Entire Agreement

This document contains the entire agreement and understanding between the parties as to its subject matter. This Agreement can only be modified by written agreement duly signed by persons authorized to sign agreements on behalf of Glaxo and the University.

XVI. Severance

Each clause of this Agreement is distinct and severable. If any clause is deemed illegal, void or unenforceable, the validity, legality or enforceability of any other clause or portion of this Agreement will not be affected thereby.

XVII. Waiver

The failure of a party in an instance to insist upon the

strict performance of the terms of this Agreement will not be construed to be a waiver of any of the terms of the Agreement, either at the time of the party's failure to insist upon strict performance or at any time in the future, and such terms will continue in full force and effect.

XVIII. Governing Law

The construction and performance of this Agreement will be governed by the laws of the State of Tennessee.

IN WITNESS WHEREOF, the parties hereto have caused this Agreement to be duly executed as of the day and year first above written.

"GLAXO"

By: Thomas W. D'Alonzo

Name: Thomas W. D'Alonzo

Title: President, Glaxo Inc.

Date: 7/24/89

APPROVED AS TO LEGAL FORM BSL LAW DEPT.

APPROVED BY FINANCE M.A.G. Name 7/14/89

"UNIVERSITY"

By:

Name: Eugene W. Fowinkle, M.D.

Title: Associate Vice Chancellor for Health Affairs

Date: JUNE 27, 1989

"PRINCIPAL INVESTIGATOR"

Jackie D. Corbin

Jackie D. Corbin, Ph.D.

Date: June 26, 1989

APPROVED BY:

Jeff Carr

Jeff Carr, Vice-Chancellor
for University Relations
and General Counsel

Date: JUNE 27, 1989

VANDERBILT UNIVERSITY

Office of Technology Transfer and Enterprise Development

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Of: Miles & Stockbridge

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Phone Number: (703) 610-8672

Number of Pages (including cover sheet): 14

Subject: Research Proposal Glaxo

Message:

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GLAXO.JDC/P0009/P. 7

3. ABSTRACT

RELAXATION OF VASCULAR SMOOTH MUSCLE BY cGMP ANALOGS

Angina pectoris and hypertension are serious health problems. Heart function, blood pressure and regional blood flow are largely regulated by the contraction and relaxation of smooth muscles surrounding blood vessels. Several natural agents (e.g., atriopeptin) and drugs (e.g., nitroglycerin) that lower blood pressure or relieve angina pectoris are known to be relaxants. These substances are believed to act directly on the smooth muscle cells to generate the second messenger cGMP. This messenger is thought to cause relaxation by activating the enzyme cGMP-dependent protein kinase, which phosphorylates a cellular protein(s) involved in regulating muscle contraction.

The objective of the proposal is to develop improved cGMP analogs to relax contracted pig coronary artery strips. Effectiveness will be assessed by specificity, potency and rate of reversibility of analog actions. The rationale is that the cGMP analogs will penetrate the smooth muscle cells of the artery wall, activate intracellular cGMP-dependent protein kinase and relax the artery strip due to specific protein phosphorylation events.

A key feature of this proposal is the synthesis of new cGMP analogs, the design of which will be based on the structures of those existing analogs shown in this laboratory to be potent in stimulating relaxation of the intact artery strip and in binding to the purified cGMP-dependent protein kinase and activating the enzyme. The approach will utilize two unique features of the kinase described below. These properties were recently discovered in this laboratory, and may allow enhancement of the specificity and potency of cGMP analogs.

Two isozymic forms of cGMP-dependent protein kinase are present in some mammalian tissues. One is the well-studied prototype referred to as type Iα, while the other is a novel isozyme, termed type Iβ. Since type Iβ appears to be prevalent in vascular smooth muscle, it could have an important role in mediating the effect of cGMP on relaxation of this tissue. We have previously carried out a study of cGMP analog selectivity of type Iα. Preliminary results indicate that although type Iβ has very similar physical properties to type Iα, it has a different cGMP analog selectivity. This potentially adds another level of specificity to the use of cGMP analogs if it is eventually shown that only one of the isozymes mediates the relaxation response.

In addition to the presence in smooth muscle of two different isozymes of cGMP-dependent protein kinase, each isozyme contains two different cGMP-binding sites that are positively cooperative for kinase activation. Since the two sites exhibit different cGMP analog specificities, a combination of two analogs which specifically target the separate sites will produce a synergistic effect in activating the kinase. Such a pair of analogs should be synergistically potent in producing the physiological responses due to cGMP-dependent protein kinase activation in intact tissues, including relaxation of smooth muscle. In addition to providing another level of specificity, the synergistic effect should allow the use of much lower total cGMP analog concentrations to elicit the response than was heretofore possible.

This proposed investigation is viewed as an opportunity to extrapolate findings from fundamental studies of purified enzymes and isolated tissues to drug design. Since cGMP-dependent protein kinase has a much more restricted tissue distribution than does cAMP-dependent protein kinase, this enzyme could be more amenable to specific pharmacological manipulation. The investigation should add new knowledge of the mechanisms of regulation of blood flow and pressure, and also lead to development of new treatments for angina pectoris, hypertension and other cardiovascular maladies.

4. RESEARCH PROPOSAL

BACKGROUND

Cyclic nucleotides have been implicated as mediators of smooth muscle relaxation in response to β-adrenergic agents, nitrovasodilators, atrial natriuretic peptide, adenosine, acetylcholine and specific phosphodiesterase inhibitors (1-4). Previously, the pharmaceutical industries have focussed on the design of cAMP analogs or of drugs that increase cAMP levels to relax smooth muscle. Although there is evidence that both cAMP and cGMP could play a role, our recent studies using cyclic nucleotide analogs to relax smooth muscle have indicated a definite role for cGMP, but not for cAMP (5).

Since comprehensive studies of cyclic nucleotide specificities for kinase activation and for binding to the purified cAMP- and cGMP-dependent protein kinases (cAMP and cGMP kinases) have been recently carried out in this laboratory (6,7), it has become possible to test these analogs in intact tissues with some degree of predictability of their rank order of potency if they act on either of the kinases. Although most tissues contain both protein kinases, smooth muscle tissues such as pig coronary artery possess atypically high levels of cGMP kinase, implying a potentially important role of this enzyme in smooth muscle function (5). Pig coronary artery and guinea pig trachea have been used to perform a systematic analysis of the relaxation induced by 24 cyclic nucleotide analogs, some of which exhibit high specificity for one or the other of the two protein kinases. For either tissue preparation, there is a good correlation between the K_a of cyclic nucleotide analogs for activation of cGMP kinase *in vitro* and their effectiveness in promoting relaxation (5, Table 1). In contrast, the correlation is poor for cAMP kinase. Despite the generally held belief that, due to poor cell penetration rates, cyclic nucleotides are not very potent for stimulation of intact tissues, the more effective compounds in Table 1 are much more potent than theophylline or caffeine, and as potent as the most effective methylxanthines (5,8). The most potent cGMP analog, 8-pCl-phenyl-S-cGMP, exhibits a very slow reversibility of its relaxant effects in the intact tissue, consistent with its strong resistance to hydrolysis by phosphodiesterase measured *in vitro* (5).

The results above suggest that in addition to modulating endogenous cyclic nucleotide levels as a means of treating cardiovascular diseases, another potential approach is the development of agents that act directly on cGMP kinase. As illustrated in Fig. 1, this proposal seeks to develop highly potent, specific, and reversible or persistent cGMP analogs that penetrate smooth muscle cells, activate cGMP kinase and cause relaxation. To conduct such an investigation, it is crucial to have an understanding of the properties of cyclic nucleotide-dependent protein kinases, and also of phosphodiesterases. Our laboratory has many years of experience in these specific areas; in addition, biochemical and molecular biological studies of these enzymes are ongoing. For instance, studies of site-specific mutagenesis of the cyclic nucleotide binding sites in these enzymes are in progress.

The cAMP and cGMP kinases are homologous proteins although there are several differences between them that presumably provide for specific functions (1). The regulatory component of each enzyme is inhibitory for the respective catalytic component, and cyclic nucleotide binding relieves the inhibition. The catalytic domains are not identical, which could allow for entirely different cellular substrate specificities. The native bovine lung cGMP kinase (type Iα), has been studied extensively, and is a dimer composed of two identical subunits of M_r = 76,331. The enzyme functions mechanistically as a monomer (9). Each subunit has a regulatory domain in the amino-terminal region which contains two cGMP binding sites, and a catalytic domain near the carboxy-terminus (10).

The two cGMP binding sites of type Iα, designated site 1 and site 2, differ in their cGMP binding kinetics. [^{3}H]cGMP dissociates relatively slowly from site 1, but much more rapidly from site 2. The binding activity of the kinase displays positive cooperativity since binding to either site stimulates cGMP binding to the other site (7,11). Both binding sites are specific for cGMP as compared with cAMP, although at very high concentrations cAMP can compete with cGMP for each binding site (7,10). The affinity for cAMP is increased by enzyme autophosphorylation (12), suggesting that this nucleotide could activate cGMP kinase under some cellular conditions. We have obtained or synthesized over 50 cGMP analogs and tested them for binding to and activation of cGMP kinase. In general, analogs with substitutions on the C-8 carbon are selective for site 1, whereas C-1-substituted analogs select site 2 (7,10). Both sites of the enzyme exhibit high specificity for the ribose cyclic phosphate moiety, and lower specificity for the guanine moiety. This latter feature has permitted the use of guanine-modified cGMP analogs to activate cGMP kinase and to stimulate physiological processes mediated by this enzyme in intact tissue preparations. Studies with analogs suggest that cGMP is bound at both sites by three hydrogen bonds at the 2'-OH, 3'-O, and 5'-O positions (7, Fig. 2). Other requirements include a negative charge in the cyclic phosphate and the presence of an amino group at C-2, a particularly important feature for site 1. The cGMP molecule is bound at both sites in the syn conformation.

Evidence suggests that sites 1 and 2 of both cAMP- and cGMP kinases are involved in enzyme activation (10,13,14). From results of kinetic approaches and a cGMP-induced "charge shift" of the enzyme on ion exchange columns, it has been suggested that only site 1 of cGMP kinase is occupied at low concentrations of cGMP, which causes a partial kinase activation (10,11). At higher concentrations of cGMP, site 2 occupation causes further kinase activation. The K_a for activation of the type Iα form of cGMP kinase by cGMP is 110 nM at 30° when using a synthetic peptide as substrate, and the Hill constant of 1.57 indicates positive cooperativity of cGMP action (10). Only a few cGMP analogs are more potent than cGMP in stimulating protein kinase activity (Table 1). The potencies of cGMP analogs as stimulators of kinase activity correlate better with the average affinity for both binding sites rather than with the affinity for either site alone (7). Two analogs added in combination are synergistic in kinase activation, particularly if one analog is selective for site 1 and the other for site 2 (7). Again, this is probably explained by the positive cooperativity between the two sites.

A novel isozymic form of cGMP kinase, termed type Iβ, has recently been discovered by this laboratory and has been shown to be prevalent in extracts of bovine aorta and porcine coronary artery (15). Although type Iβ is similar to type Iα in several respects, including the presence of two different positively cooperative binding sites for cGMP, this form has several properties quite different from those of the well-described type Iα of bovine lung and type II of intestinal brushborder (16). From a limited survey, type Iβ exhibits a different cGMP analog specificity from type Iα (Table 1).

The phosphodiesterases are a heterogeneous group of enzymes, and the role of each isozyme in degrading intracellular cGMP and cAMP is not completely understood. Since the potency and persistence of cGMP analogs in relaxing smooth muscle may depend in part on their resistance to degradation, a thorough knowledge of the phosphodiesterases is advantageous. We have investigated several forms of these enzymes in great detail, including studies of cGMP analog specificity (5). Extensive studies of phosphodiesterase inhibitors such as methylxanthines have also been carried out (8).

GLAXO.JDC/P0009/P. 10

DESCRIPTION OF RESEARCH

Objective:

The overall objective is to develop cGMP analogs, or combinations of analogs, that are potent and specific, and that exhibit an appropriate pattern of reversibility or persistence, in causing relaxation of vascular smooth muscle (Fig. 1). The proposal is based on the hypothesis that cGMP-dependent protein kinase (cGMP kinase) mediates the relaxant effects of various agents such as nitroglycerin and atrial natriuretic factor.

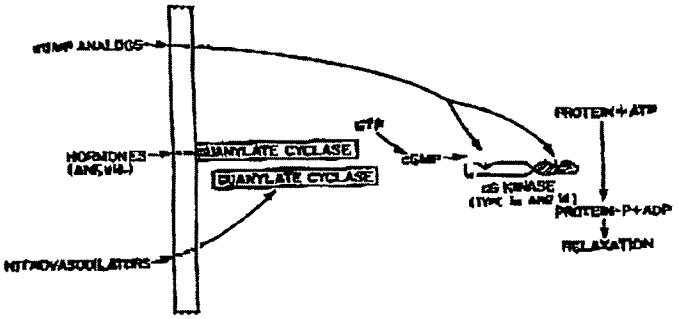


Figure 1. Mechanism of cGMP analog action in vascular smooth muscle.

Specific Aims:

1. Synthesize new cGMP analogs based on the structures of existing analogs that have been found to be effective in activating cGMP kinase and relaxing smooth muscle preparations.
2. Compare the new cGMP analogs with those that have previously been tested for potency and specificity in activating the purified preparations of smooth muscle type Iα and type Iβ isozymes of cGMP kinase, and in binding to the two different cGMP binding sites (site 1 and 2) of each isozyme. Test combinations of site 1- and site 2-specific cGMP analogs for synergistic effects in activating type Iα and type Iβ.
3. Using the studies described in Aim 2 for the initial screening, test single cGMP analogs for potency in relaxing pig coronary artery strips. Determine the rate of reversal of the relaxant effects after analog wash-out. Test combinations of site 1- and site 2-specific cGMP analogs for synergistic effects in relaxing artery strips.
4. Test combinations of cGMP analogs and specific cGMP phosphodiesterase inhibitors for synergistic effects in relaxing artery strips.

Timetable:

The synthesis of 14 new cGMP analogs (Aim 1) and the studies of potencies and specificities of these analogs for activating cGMP kinase, and for binding to the two sites (Aim 2), should require one year to complete. The effectiveness of the individual analogs, the rate of reversal of their effects, and synergistic effects of combinations of analogs in relaxing the artery preparation (Aim 3) will be determined during the second year. The studies of combinations of analogs and phosphodiesterase inhibitors (Aim 4) will be done in the third year. Concurrently with Aims 3 and 4, additional analogs will be synthesized based on effectiveness and structures of the first generation of new analogs.

Methodology:

1. Synthesis of new cGMP analogs - The procedures that will be used are modifications of either methods routinely used in this laboratory or published methods. Structures of the new analogs will be based on the structures of

GLAXO.JDC/P0009/P. 11

analogs that have been previously found by this laboratory to be potent in activating cGMP kinase (type Iα) in vitro and in promoting relaxation of pig coronary artery strips. It is theorized that the potency of those existing analogs is due to changes in the basic structure of the cGMP molecule caused by the appended group. These changes are likely to be in the electronic configuration of the guanine moiety, in allowing formation of an "extra" hydrophobic or other bond with the cGMP binding site, or in bulkiness. The design of analogs will also be based on our predicted model of the cGMP binding sites done in collaboration with Drs. Irene Weber and John Shabb. Modeling of the cGMP binding sites is done on a Vax 11/780 computer, using the Evans and Sutherland PS340 Graphic Display System. The binding site is modeled after the homologous cAMP-binding protein of bacteria termed CAP, the X-ray crystallographic structure of which is known. The conserved amino acids that are believed to make contact with the cGMP molecule in the binding pocket are indicated in Fig. 2. These interactions fit precisely with our cGMP analog studies. Site 2 is the one shown in Fig. 2, although site 1 is quite similar.

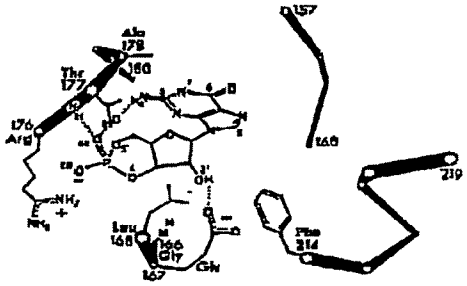


Fig. 2. Predicted cGMP-binding pocket of cGMP kinase. Predictions are based on homology with and crystallographic structure of the bacterial CAP.

Focus will be on four groups of analogs: (a) analogs that are selective for cGMP-binding site 1 of cGMP kinase; (b) analogs that are selective for site 2; (c) analogs that bind with high affinity to both sites; and (d) analogs that are persistent in causing relaxation of artery strips. Fig. 2 illustrates the cGMP molecule and the numbered positions where modifications will be made.

a. Site 1-selective cGMP analogs - The parent compounds of this group are 8-Br-cGMP and 7-deaza-cGMP.

8-Br-cGMP is 4.3-fold more potent than cGMP in activating purified type Iα cGMP kinase (Table 1) and is 7.9 times more selective for site 1 than site 2 (7). This compound is also one of the most potent in stimulating relaxation of the intact tissue. From an analysis of all C-8 modified analogs and from molecular modeling (Fig. 2), it is hypothesized that the potency of 8-Br-cGMP, especially for site 1 binding, is determined by bulk tolerance of large substituents at C-8, preference of the binding site for the syn conformation of cyclic nucleotides and the electron-withdrawing ability of the bromo group. Improvement will be sought by synthesizing other 8-halogenated compounds. It is noteworthy that 8-Cl-cAMP has been shown to be a very potent analog for stimulating cAMP kinase (17). The 8-Cl- (analog 1) and 8-I- (analog 2) cGMP analogs will be synthesized as described by Revankar and Robins for the halogenated cAMP analogs (18). 8-Br-cGMP will be the starting material. The chloro group would be less bulky than bromo but would have greater electron withdrawing power; whereas, iodo would have greater bulk but less electron withdrawing power.

Purification of all cGMP analogs will be by Sephadex G-25 (superfine) chromatography in 50 mM NH_4HCO_3 (pH 7.8) at 4°C, which is a method of high resolution developed in this laboratory (5). The fractions containing the analogs will be identified by absorbance at 260 nm.

7-Deaza-cGMP is slightly better than cGMP in activating type Iα cGMP kinase (Table 1) and is 23 times more selective for site 1 than site 2 (7). Because of the limited amounts available, it has not been tested on the intact artery strips. In addition to its other favorable features, this compound could be

quite resistant to cellular degradation by phosphodiesterases. In contrast to the protein kinases, most phosphodiesterases form a hydrogen bond at N-7 (19). 7-Deaza-cGMP (analog 3) will be synthesized as described by Ernaux et al. (19).

TABLE 1. Comparison of cyclic nucleotide analog potencies in relaxing pig coronary artery and activating the purified type Ia and Iβ isozymes of cGMP kinase

Compound	Potency in cGMP Kinase Activation K'_a		Potency in Artery Relaxation $1/EC_{50}$ (μM)
	Type Ia	Type Iβ	
8-Br-cGMP	4.3	0.76	0.021
β-Phenyl-1-N^2-etheno-cGMP	4.1	1.6	0.046
8-pCl-phenyl-β-cGMP	2.2	0.75	0.056
7-Deaza-cGMP	1.3	----	------
8-$H_2C_6H_4CS$-cGMP	1.1	0.65	0.017
8-HS-cGMP	1.3	0.15	0.013
cGMP	1.0	1.0	------
8-butyryl-cGMP	0.97	0.11	------
8-Methyl-cGMP	0.58	0.24	------
8-pCl-phenyl-S-cAMP	0.39	1.10	------
1-H_3C-cGMP	0.37	0.18	0.0003
8-H_2NS-cGMP	0.34	----	------
N^6-$nH_{11}C_6$-cGMP	0.22	----	------
8-$H_3C_2(O)C$-cGMP	0.22	0.019	------
1-H_2NS-cGMP	0.16	0.26	------
8-HO-cGMP	0.12	0.066	------
$8-H_2C_6H_4C(H)N$-cAMP	0.078	------	------
N^2-$nH_2C_2(O)C$-cGMP	0.053	------	0.0004
7-Deaza-cAMP	0.041	------	------
8-(2-HO-nH_7C_3)-cGMP	0.037	------	------
8-Thiomethyl-cAMP	0.032	------	<0.0008
8-Thiomethyl-cAMP	0.032	------	0.002
2-H_2N-cAMP	0.030	------	------
2-H_3C-cIMP	0.029	------	------
8-(6-aminohexylamino)-cAMP	0.028	0.11	<0.0017
8-$H_3C(O)C$-cGMP	0.026	------	------
8-Br-cAMP	0.019	------	0.0006
8-$(H_3C_7)_2N$-cGMP	0.018	------	------
2-F-cIMP	0.016	------	------
$2-nH_9C_4$-cIMP	0.016	------	------
N^2-[2,4-$(O_2N)_2$-H_3C_6]-cGMP	0.013	------	------
8-H_3-cAMP	0.013	------	------
cIMP	0.011	0.031	<0.001
N^6-Benzoyl-cAMP	0.0091	------	<0.0017
8-MS-cIMP	0.0091	------	------
$2-H_2N$-cIMP	0.0063	------	------
N^2,2'-d-[$nH_7C_3(O)C]_2$-cGMP	0.0059	------	<0.001
2'-Deoxy-cGMP	0.0051	------	------
$2-nH_9C_4$-cAMP	0.0038	------	------
cAMP	0.0037	0.024	------
N^6-$nH_7C_3(O)C$-cAMP	0.0032	------	------
2-H_3CS-cIMP	0.0021	------	------
8-NH-cAMP	0.0014	------	------

b. Site 2 selective analogs - Compounds of this group are based on the favorable properties of 1-CH_3-cGMP, 1-NH_2-cGMP, and β-phenyl-1-N^2-etheno-cGMP (Table 1). For 1-CH_3-cGMP and 1-NH_2-cGMP, the site 2 selectivity is explained by loss of site 1 binding affinity as compared with cGMP. This is not the case for β-phenyl-1-N^2-etheno-cGMP, which retains site 1 affinity and has a 10-fold greater site 2 affinity than does cGMP. This could be explained by formation of an additional "unnatural" bond between the phenyl group and an amino acid(s) near binding site 2, such as Cys-157 or Phe-214 (Fig. 2). Based on our present knowledge, efficacy of analogs should be improved by addition of an electron-rich, bulky hydrophobic constituent at the N-1 position. This will be done in two ways, the first of which will retain the intact 2-NH_2 group, which is believed to form a hydrogen bond between cGMP and a threonine residue in the binding site (Fig. 2). It will also contain the presumably favorable phenyl group of the parent compound, β-phenyl-1-N^2-ethano-cGMP.

Synthesis of 1-benzyl-cGMP (analog 4) will be by modification of the original method of Miller et al. (20) which we have used earlier for synthesis of 1-CH_3-cGMP (7). Benzyl bromide will substitute for methyl iodide as one of the reactants. In order to synthesize three additional analogs, the hydrophobicity of the phenyl ring will be changed by introduction of chloro

(analog 5) and hydroxy (analog 6) in the 3-position of the phenyl moiety and by replacement of phenyl by napthyl (analog 7).

Synthesis of derivatives of β-phenyl-1-N^2-etheno-cGMP will utilize a modification of the original method of Miller et al. (20) which has been used in our laboratory to synthesize the parent compound (7). α-Bromo-3-chloroacetophenone will be substituted for α-bromoacetophenone as one of the reactants. This procedure will produce the 3-Cl derivative (analog 8) of the phenyl group in the parent compound. The 3-OH derivative (analog 9) will be made by slight modification of the procedure, and the phenyl group will be substituted by napthyl (analog 10).

c. cGMP analogs that bind with high affinity to both sites - Since both sites 1 and 2 are involved in activating cGMP kinase, it might be expected that individual analogs having features of both sites 1- and 2-selective analogs might be quite potent in activating the enzyme. We will therefore combine the features of 8-Br-cGMP, the most potent site 1-selective analog, with the features of β-phenyl-1-N^2-etheno-cGMP, the most potent site 2-selective analog. This will be done by synthesizing 8-Br,β-phenyl-1-N^2-etheno-cGMP (analog 11) by the method described above for β-phenyl-1-N^2-etheno-cGMP with the exception of using 8-Br-cGMP instead of cGMP as the starting material. Pending initial success with this strategy, more potent analogs in this group may be designed after the site-1 and site 2-selective analogs of Sections a and b above have been characterized.

d. Persistent cGMP analogs - It is theorized from results of our previous intact tissue studies that persistence of analog effects is due to a high cGMP kinase affinity coupled with resistance to phosphodiesterase(s). The parent compound is 8-pCl-phenyl-S-cGMP. The high kinase affinity is apparently due primarily to the S atom, which changes the electronic configuration of the guanine moiety. The phosphodiesterase resistance may be due in part to the bulky 8-substituent. Our initial strategy will be to retain the S-linkage but to change the hydrophobicity and bulk of the 8-substituent.

The method is slightly modified from Miller et al. (21) by using m-chlorobenzenethiol instead of p-chlorobenzenethiol to react with 8-Br-cGMP. This procedure will produce 8-mCl-phenyl-S-cGMP (analog 12), which is more hydrophobic than the parent compound. By using m-hydroxybenzenethiol as the starting material, the less hydrophobic hydroxy derivative (analog 13) of the parent compound will be made. The more bulky 1-napthyl-S-cGMP (analog 14) will be made by using 1-naphthylenethiol as starting material.

2. Effects of cGMP analogs on cGMP kinase - Because of the ease of obtaining large quantities of pure preparations of both isozymes of cGMP kinase from bovine aorta, this tissue will be used as source of type Iα and type Iβ. We have not observed any differences in physical or kinetic properties between the enzymes from pig coronary artery and bovine aorta. The purification procedure is as described for preparation of the "cGMP-free" isozymes (15).

The activity of the cGMP kinase is determined (11) by measuring [^{32}P] incorporation from γ-[^{32}P]ATP into a heptapeptide substrate (RKRSRAE, Peninsula Labs). Hill plots will be constructed for determination of analog Ka values for type Iα and type Iβ. Analogs found to have Ka values > 1 μM will not be used for any of the studies described hereafter.

To determine the synergistic effect of pairs of analogs using the purified kinases, the concentration of each analog of the pair will be that which produces ~10% of the maximum kinase activation when used alone. The synergistic effect is the actual kinase activity produced by using the analog pair divided by the sum of the kinase activities produced by the two analogs used separately. Synergistic quotients of >10 have been observed using this method for cAMP kinase (13). It is expected that the most synergistic pairs of analogs will be those which contain individual analogs with the most extreme differences in specificity for cGMP binding sites 1 and 2.

GLAXO.JDC/P0009/P. 14

The standard [^{3}H]cGMP binding assay is the $(NH_4)_2SO_4$ method described earlier (7). The cGMP analog specificity for binding will be calculated as described by this laboratory (7). The presence of two different binding sites per subunit does not preclude the use of cGMP analogs for this study. As described, the two cGMP dissociation components can be utilized separately to obtain a K'_I of cGMP analog for each cGMP binding site (7).

3. Effects of cGMP analogs in relaxing pig coronary artery strips - The porcine coronary artery preparation described below is presently being used for another ongoing project, and we also have experience using guinea pig tracheal segments (5). The coronary arteries contain about equal amounts of type Iα and Iβ (15). Porcine hearts are obtained from a local slaughterhouse and packed in ice until dissection. A segment of the right coronary artery is dissected and placed in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 10 mM glucose and 1 mM pyruvate. The buffer is bubbled continuously with 95% O_2-5% CO_2. A small glass rod is inserted through each vessel and the vessel is carefully trimmed of all visible fat and fascia. Each artery is then cut into helical strips approximately 3 mm in width. The tissues are then stored at 0-4° overnight in the same buffer (with adjustment of the bicarbonate level to maintain pH 7.4 at this temperature) with aeration prior to use the following day. Eight small segments (1.5 cm in length, ~50 mg in weight) are cut from the spiral arterial strips and mounted by surgical threads in individual organ baths of 2.5 ml each at 37°. Two grams of resting tension are applied to the tissue for approximately 2 hours. Periodic adjustments of tension are made during this time until no further spontaneous relaxation is observed. Isometric tension is recorded by Statham strain gauge transducers connected to Gould-Brush 2400 recorders. After the initial 2 hours of equilibration and several buffer changes, the tissue segments are further conditioned by the sequential addition and removal of buffers containing 117 mM KCl and 5.9 mM KCl, respectively, to bring the tissues to a state of relatively constant responsiveness to the contractile stimulus. Contraction for the experimental studies is initiated by replacement of the buffer by Krebs-Ringer in which the KCl concentration is increased to 20 mM KCl and the NaCl level reduced to 128 mM. After tension is stabilized at an apparent maximum for those conditions, various concentrations of cGMP analogs are added. The tension generated by these strips in response to the 20 mM KCl challenge is typically 15-20% of the maximum tension generated by the 117 mM KCl induced contraction (12-25 g). At the end of the treatment, 100 μM 3-isobutyl-1-methylxanthine is added to achieve full relaxation. The difference between the stable tension achieved prior to addition of any relaxants and the tension achieved after 3-isobutyl-1-methylxanthine treatment is taken as the maximum relaxation and is used to calculate the percentage of analog effects on relaxation.

If cGMP analogs or other agents that act directly on cGMP kinase are ever to be used as pharmaceuticals, their rates of reversibility in relaxing vascular tissues should be scrutinized. There may be advantages of using either rapidly reversible or slowly reversible drugs depending on the circumstances. In order to determine rate of reversal of analog effects, the buffer in the bath will be exchanged ever 10 sec with the original buffer (5.9 mM KCl) to remove the analog and 3-isobutyl-1-methylxanthine. The strips will be tested for contraction to the extent of the original response at 30 min intervals by addition of buffer containing 20 mM KCl. If the full response is achieved at the first 30 min test, the experiment will be repeated with that particular cGMP analog using shorter time points. We have found that with 8-pCl-phenyl-S-cGMP, the effect persists for a very long time, 3 hr. with 15 buffer changes being required to reverse the effect (5).

Combinations of two analogs will be tested on the artery strips to assess synergism. We have used this approach successfully to stimulate physiological responses of cAMP kinase (22). In some cases ~50-fold lower total concentration of cAMP analog pairs produces the same response as either one of the individual

analogs. Because of the positive cooperativity of the two cGMP binding sites together with their different cGMP analog specificity, two analogs selective for the opposite sites added in combination should cause synergistic physiological responses. Analogs will be tested alone, in combination with an analog selective for the opposite site, and in combination with an analog selective for the same site as a control. The concentrations of analogs used for this test will be those which when used alone produce a very low response (~10% of maximum). We will rely on the in vitro studies described in Section 2 to determine the proper combinations to be used. The synergism quotient will also be determined as described in Section 2.

4. Synergistic effects of combinations of cGMP analogs and phosphodiesterase inhibitors - The synergistic effect of cGMP-elevating agents and phosphodiesterase inhibitors in relaxing smooth muscle has been demonstrated (23). Thus, it is expected that the potency of the cGMP analogs should be enhanced by the presence of 3-isobutyl-1-methylxanthine. We have also synthesized and characterized numerous other phosphodiesterase inhibitors, some of which are quite potent and specific for phosphodiesterases that prefer cGMP over cAMP (8), and that are available for testing if necessary. The experiments using artery strips will be conducted as described in Section 3 up to the stage of addition of cGMP analog. Before adding analog, a low concentration (1-5 μM) of 3-isobutyl-1-methylxanthine will be added for 15 min. This concentration is insufficient to produce a significant relaxation. The cGMP analog will then be added and the percent relaxation of the tissue determined as before. As is the case for the experiments testing synergistic effects of pairs of cGMP analogs described above, a disadvantage of this approach is that different tissue preparations will have to be used for comparing the effect of cGMP analog added alone with the effect of analog-phosphodiesterase inhibitor added in combination. For statistical evaluation, it will therefore be necessary to use at least four tissues for each treatment.

5. Potential Pitfalls and Concluding Remarks - The excellent correlation between potency of cGMP analogs in activating cGMP kinase and in relaxing smooth muscle suggests that the Ka value for cGMP kinase is by far the most important property of analogs to consider here. However, cell penetration rate and resistance to degradation could also play a role in relaxation potency, particularly in extreme cases. For instance, the impotency of cGMP itself and the persistence of 8-pCl-phenylthio-cGMP might be explained by high and low degradation rates, respectively. In the course of the studies of cGMP analog specificity for relaxing the artery strips, it could become apparent that only one of the two cGMP kinase isozymes mediates the response. If so, then subsequent studies would focus on design of analogs specific for that particular isozyme. Another possible finding that could alter the strategy would be that type Iβ, in contrast to type Iα, does not exhibit differences in site 1 and site 2 specificity for cGMP analogs. Although from preliminary results this does not appear likely, this finding would render searches for synergistic analogs for type Iβ less attractive. After the first generation of new cGMP analogs have been synthesized and tested, the most effective analogs will be carefully scrutinized for bulkiness, electron-withdrawing power, and hydrophobicity of the appended group. A second generation of new analogs will then be synthesized and their effectiveness determined. Finally, since our fundamental studies of regulation of the two isozymes will be done in parallel using another funding source, new findings of enzyme mechanisms could alter the research plan. If early successes occur during this investigation, it would be possible to proceed to the next stage of the project, which is the testing of analogs in a preparation in which blood pressure can be conveniently measured. Dr. Ed Jackson of the Department of Pharmacology has indicated his willingness to collaborate using a blood-perfused rat mesenteric preparation, which would require only small amounts of analogs for the study. The long-range goal would be, of course, to test cGMP analogs in the intact organism.

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Facilities Available:

The laboratory of the principal investigator is presently situated on the Howard Hughes Medical Institute floor of Light Hall in the Vanderbilt Medical complex. This laboratory will move to ~2200 sq. ft. of space in the new Medical Research Building in the Spring of 1989. An advantage of this move will be the close proximity of the laboratory of the collaborator, Dr. Jack Wells, who will also be moving to the new building. In addition to Dr. Sharron Francis, the Corbin lab consists of three graduate students, three technicians and four postdoctorals. The Howard Hughes Medical Institute support will terminate in August 1989, which should allow more effort to be devoted to this project if it were funded.

No new equipment is being requested since the major items of necessary equipment are in place. These are:

2 preparative centrifuges, 1 ultracentrifuge, 2 scintillation counters (beta)

GLAXO.JDC/P0009/P. 17

1 spectrophotometer (UV and visible, recording), 2 HPLC systems, and 1 cold room. A Gould 3400 Physiographic System (8-channel) is installed, and a similar system is in use in Dr. Wells' laboratory. Centrifuges, cold rooms, spectrophotometers, and scintillation counters are also available in the departmental core facilities. A Hewlett Packard 3350 Lab Automation System with lab terminal, A VAX 11/780 and VAX II are in place. The Evans and Sutherland PS340 Graphic Display system which will be used to model the cGMP binding sites is a departmental core instrument. Software in use includes the Mogli Display Program (Evans and Sutherland, Inc.) and Insight Graphics Program (Biosym. Tech., Inc.). Molecular dynamics and mechanisms are examined using the Discover Simulation Program (Biosym. Tech., Inc.). The VAX II computer system with Mass 11 word processing software is available for use by secretarial staff and individual investigators via direct connections. Duplicating and mailing services are available.

There is a University machine shop, electronics shop and illustrations department which provide services requiring fees.

EXHIBIT B
SCHEDULE OF PAYMENTS

University: Vanderbilt University
Principal Investigator: Jackie Corbin

YEAR 1: $168,023
Payment 1; on or about July 1, 1989: $84,012

Payment 2; on or about January 1, 1990: $84,011

YEAR 2*: $181,465
Payment 3; on or about July 1, 1990: $90,733

Payment 4; on or about January 1, 1991: $90,732

YEAR 3*: $195,982
Payment 5; on or about July 1, 1991: $97,991

Payment 6; on or about January 1, 1992: $97,991

GRANT TOTAL: $545,470

* Payment subject to Glaxo's receipt of annual reports demonstrating progress of the Discovery Research Project as provided for in Paragraph VII of the agreement.

5. Budget Proposal

		1989-1990	1990-1991	1991-1992
Salary and Fringe Benefits				
Jackie Corbin, Professor	25%	$25,484	$27,523	$29,725
Jack Wells, Professor	10%	8,326	8,992	9,711
Sharon Francis, Asst Prof.	25%	14,022	15,144	16,356
Research Associate, TBA	100%	32,556	35,160	37,973
Research Assistant, TBN	100%	26,474	28,592	30,879
Total		$106,862	$115,411	$124,644
Supplies		30,000	32,400	34,992
Travel		2,000	2,160	2,333
		32,000	34,560	37,325
Total Direct		$138,862	$149,971	$161,969
Indirect Costs @ 21%		29,161	31,494	34,013
Total		$168,023	$181,465	$195,982

The percent effort for each investigator is the approximate effort that will be devoted to this project if it is funded. It is preferable that the research associate recruited for the position listed will have experience in organic chemical syntheses. This person will mainly be involved in cGMP analog synthesis in collaboration with Wells. The research assistant will carry out the experiments using intact coronary artery slices and will do the kinase and binding assays. Corbin and Francis will coordinate these aspects of the project. It is expected that the three scientific members will be in daily consultation concerning results of the experiments. The Supplies request seems modest in view of the chemicals and materials needed for this project. The Travel request is for one meeting per investigator. The annual increases in each category are eight percent.