Photodynamic action of aluminium phthalocyanine tetrasulphonate (AlPcS₄) on smooth muscle: effects of thiols and a cyclic GMP analogue

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1 The smooth muscle system of the guinea-pig taenia caeci has been used *in vitro* to characterize the photodynamic action of aluminium phthalocyanine tetrasulphonate (AlPcS₄) in the presence or absence of the thiol reductants L-cysteine (Cys), N-acetyl-L-cysteine (NAC), DL-dithiothreitol (DTT) or reduced glutathione (GSH).

2 In all photodynamic experiments the muscle was exposed to AlPcS₄ (10⁻⁵ M) for 30 min, followed by a 30 min washout period before photon irradiation at 32,000 lux ($\lambda > 570$ nm) for 30 min. Photodynamic contractions were measured relative to the contractile response to carbachol (5 × 10⁻⁵ M) and relaxation responses were determined in muscle precontracted with either carbachol 5 × 10⁻⁵ M or KCl 23.5 mM.

3 Photon-activation of AlPcS₄-sensitized smooth muscle evoked a triphasic response: an initial transient contraction and subsequent relaxation followed by a secondary sustained contraction. Cys 10 mM, NAC 10 mM and DTT 5 mM had no effect on the initial photodynamic contraction but significantly decreased the magnitude of the sustained contraction from mean values of 98% to 18%, 95% to 72% and 93% to 6% of the standard carbachol contraction (5 × 10⁻⁵ M), respectively; GSH 10 mM was without significant effect on either the initial or sustained contraction.

4 In the absence of extracellular calcium the AlPcS₄-sensitized smooth muscle did not respond to photon activation but re-introduction of calcium after cessation of illumination produced a sustained contraction which was markedly inhibited by Cys 10 mM.

5 In precontracted AlPcS₄-treated muscle preparations photon activation produced a triphasic relaxation response, i.e. a rapid relaxation followed by a transient contraction and a secondary more sustained relaxation. The sustained phase of photodynamic relaxation was potentiated significantly by Cys 10 mM, NAC 10 mM, DTT 5 mM and GSH 10 mM, the relaxation being approximately doubled in magnitude from mean values of 34% to 68%, 30% to 73%, 34% to 68%, and 48% to 77%, respectively, relative to the standard carbachol (5 × 10⁻⁵ M) response.

6 The cyclic GMP analogue, 8-(4-chlorophenylthio)-guanosine-3':5'-cyclic monophosphate (8-PCPT-cGMP) (2 × 10⁻⁴ M) alone caused a triphasic relaxation response similar to that produced by photon activation of an AlPcS₄-sensitized precontracted preparation in the presence of thiol reductants. The pattern of 8-PCPT-cGMP-induced relaxation was similar in muscle precontracted with carbachol 5 × 10⁻⁵ M or KCl 23.5 mM.

7 It is concluded that the rapid generation of reactive intermediates by photon-activation of bound AlPcS₄ leads to membrane permeabilization, calcium entry and muscle contraction. These effects may be opposed by a direct stimulatory action of singlet oxygen on guanylate cyclase which is enhanced by the action of thiol reagents and mimicked by the cyclic GMP analogue, 8-PCPT-cGMP.

Keywords: Photodynamic drug action; singlet oxygen; smooth muscle; guinea-pig taenia caeci (coli); aluminium phthalocyanine tetrasulphonate; thiols; guanylate cyclase; cyclic GMP analogue

Introduction

Photodynamic drugs when photon-activated are believed to exert their effects primarily via the local generation of highly-reactive singlet oxygen (Matthews & Cui, 1990a,b). It is, however, often difficult to assess the nature and extent of the biological effects produced by photodynamic agents. As a model system the guinea-pig taenia caeci (coli) has many advantages for analysing the mechanisms of photodynamic drug action since it is composed of a relatively transparent syncytium of smooth muscle cells which readily take up the photosensitizer, respond rapidly to receptor activation or changes in membrane permeability, and produce a well-defined calcium-dependent photodynamic contraction (Matthews & Mesler, 1984b).

In this study the new and highly-effective photosensitizer aluminium phthalocyanine tetrasulphonate (AlPcS₄) has been used to investigate the molecular mechanisms of photo-

dynamic drug action on the smooth muscle cells of the taenia caeci and the way in which these actions can be enhanced or inhibited. Photon absorption by a sensitizer molecule converts it from the ground state (S) to the lowest excited singlet state (¹S). Intersystem crossing with spin inversion yields the less energetic but longer-lived triplet excited state (³S). In the presence of molecular oxygen, energy transfer from ³S to ground state oxygen (O₂) leads to the formation of highly reactive single oxygen (¹O₂), (Girotti, 1990; Foote, 1991). With an absorbance maximum of 675 nm to ensure effective tissue penetration, a good quantum yield of the triplet state, and extended triplet lifetime, AlPcS₄ is a potent photodynamic drug the activation and effects of which can be controlled precisely by the wavelength, intensity, and duration of the incident photon irradiation. The free radicals and singlet oxygen generated may react at a number of membrane and subcellular sites although the more immediate effects of AlPcS₄ activation are likely to be exerted in the proximity of the cell membrane to which much of the AlPcS₄

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is confined by its high net negative charge (Rosenthal, 1991). Membrane lipid peroxidation, membrane permeabilization and the activation of membrane receptor-transduction pathways may all contribute to photodynamic drug action (Matthews & Cui, 1990a,b); guanylate cyclase is also known to be readily activated by oxidation (Waldman & Murad, 1987). Some of these effects may be counteracted by thiol containing reductants. Different thiol reagents have therefore been employed to investigate the involvement of thiol - disulphide oxidation and reduction reactions in photodynamic drug action. In order to assess the extent to which guanylate cyclase and guanosine 3':5'-cyclic monophosphate (cyclic GMP) production may participate in the photodynamic process, the cyclic GMP analogue, 8-(4-chlorophenylthio)-guanosine-3':5'-cyclic monophosphate (8-PCT-cGMP) was used as a potent selective activator of cyclic GMP-dependent protein kinase. In this membrane permeant analogue the lipophilicity of the molecule has been markedly enhanced by replacing the hydrogen in position 8 of the heterocyclic base with the *para*-chlorothio moiety (Francis *et al.*, 1988), so promoting cellular uptake. The results of some of this work were com-

municated to the Cambridge meeting of the British Pharmacological Society in January, 1993 (Matthews *et al.*, 1993).

Methods

Superfusion of taenia caeci

Male guinea-pigs (200–400 g) were killed by cranial percussion and exsanguination. Strips of taenia muscle were dissected from the caecum, divided into four lengths of approximately 1 cm and superfused at 32°C in a system described in detail by Matthews & Mesler (1984b). Isotonic recordings of muscle contraction were made via a Harvard transducer – amplifier system and Kipp and Zohnen BD 40 chart recorders. A modified Krebs-Henseleit solution was employed of the following composition (mM): NaCl 118, CaCl₂ 2.56, KCl 4.7, MgCl₂ 1.13, NaH₂PO₄ 1.15, NaHCO₃ 25, and D-glucose 11.2. The solution was bubbled with 95% O₂ and 5% CO₂ to give a final pH of 7.4. When, in some experiments, the KCl concentration was increased to 23.5 mM the NaCl concentra-

Figure 1 Photon-activation of AlPcS₄-sensitized taenia coli. Photodynamic contractile responses (a) in the absence, and (b) in the presence of DL-dithiothreitol (DTT) 5 mM. Contractions to carbachol, 5×10^{-5} M for 10 s (●) and exposure to AlPcS₄, 10^{-5} M for 30 min (between the arrows). Illumination at 32,000 lux for 30 min (open rectangles). In (b) DTT, 5 mM was present from the withdrawal of AlPcS₄ until the end of the experiment.

Figure 2 Photon-activation of AlPcS₄-sensitized taenia coli. Photodynamic contractile responses (a) in the absence, and (b) in the presence of L-cysteine, 10 mM. Contractions to carbachol, 5×10^{-5} M for 10 s (●) and exposure to AlPcS₄, 10^{-5} M for 30 min (between the arrows). Illumination at 32,000 lux for 30 min (open rectangles). In (b) L-cysteine, 10 mM, was present from the withdrawal of AlPcS₄ until the end of the experiment.

tion was decreased correspondingly to 99.2 mM to maintain isotonicity. In other experiments, when CaCl_2 was omitted ETGA 1 mM was added.

Light intensity

All experiments were carried out in a light-sealed cabinet. Photon irradiation of the tissue was via a fibre-optic probe from a Schott KL150 quartz-halogen light source equipped with a KG1 heat filter. An additional Schott OG-570 sharp-cut glass filter with transmission > 570 nm was also used to restrict the spectral output to the range required for activation of AlPcS_4 (peak absorbance of 675 nm). Light intensities were measured with a Minolta Illuminance meter T-IH.

Data analysis

Results are expressed as the mean $\pm$ s.e.mean and statistical significance was evaluated by Student's *t* test. Photodynamic contraction or relaxation responses are expressed as a percentage of the sustained response to carbachol 5×10^{-5} M. This use of a standardized response to carbachol as a reference allowed for any variation in the magnitude of muscle contraction due to differences in tissue length for each preparation.

Drugs used

Carbachol, L-cysteine, N-acetyl-L-cysteine and glutathione (reduced form) were from Sigma U.K. Chloroaluminium phthalocyanine tetrasulphonate was obtained from Porphyrin Products, Logan, Utah, U.S.A.

Results

AlPcS₄-sensitized photodynamic contraction of taenia caeci: effect of thiol reductants

In order to photosensitize the superfused muscle it was first exposed to AlPcS_4 (10^{-5} M) for 30 min followed by washout of the drug for a further 30 min. It is clear that the muscle had taken up and retained the AlPcS_4 because when illuminated at 32,000 lux ($\lambda > 570$ nm) for 30 min the muscle showed a light-induced contraction (Figure 1a). This effect was not seen in control preparations which had not been exposed to AlPcS_4 . Thus neither illumination nor AlPcS_4 treatment alone had any effect on muscle tone, nor were the contractile responses to repetitive doses of carbachol (5×10^{-5} M) affected by AlPcS_4 in the absence of light (Figure 1).

The response of the phthalocyanine-sensitized muscle to light activation was triphasic, i.e. (i) an initial brief contrac-

Figure 3 Effects of thiols on (a) the initial, and (b) the sustained photodynamic contraction of AlPcS_4 -sensitized taenia coli. Experimental protocol as in Figures 1 and 2: Cyst, L-cysteine 10 mM; NAC, N-acetyl-L-cysteine 10 mM; GSH, glutathione 10 mM; DTT, dithiothreitol 5 mM. Responses in the absence (solid columns) and presence (hatched columns) of thiol. * $P < 0.05$; *** $P < 0.001$; $n = 4$ or 5.

Figure 4 Calcium-dependence of the photodynamic contraction in AlPcS_4 -sensitized smooth muscle. Contractions to carbachol, 5×10^{-5} M, for 10 s (●) and exposure to AlPcS_4 , 10^{-5} M, for 30 min (between the arrows). Illumination at 32,000 lux for 30 min (open rectangles). In (a) and (b) extracellular calcium was removed and EGTA, 1 mM, added from the time of AlPcS_4 withdrawal until normal $[\text{Ca}^{2+}]_o$ was restored at Δ . In (b) L-cysteine, 10 mM, was present from the time of AlPcS_4 withdrawal until the end of the experiment.

Figure 5 Photodynamic relaxation response produced in muscle precontracted with carbachol. Contractions to carbachol, 5×10^{-5} M for 10 s (●) and exposure to AlPcS₄, 10^{-5} M, for 30 min (between the arrows). The smooth muscle cells were exposed continuously to carbachol, 5×10^{-5} M, from the withdrawal of AlPcS₄ until the end of the experiment. Illumination at 32,000 lux for 30 min (open rectangle).

Figure 6 Photodynamic relaxation of smooth muscle in (a) the absence, and (b) the presence of L-cysteine, 10 mM. Contractions to carbachol, 5×10^{-5} M for 10 s (●) and exposure to AlPcS₄, 10^{-5} M, for 30 min (between the arrows). Other details as for Figure 3. In (b) the smooth muscle cells were exposed to L-cysteine, 10 mM, in addition to carbachol, 5×10^{-5} M, from the withdrawal of AlPcS₄ until the end of the experiment.

Figure 7 Effects of thiols on the photodynamic relaxation of AlPcS₄-sensitized taenia coli. Experimental protocol as in Figures 5 and 6: Cyst, L-cysteine, 10 mM; NAC, N-acetyl-L-cysteine, 10 mM; GSH, glutathione, 10 mM; DTT, dithiothreitol, 5 mM. Responses in the absence (solid columns) and presence (hatched columns) of thiol. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$; $n = 4$ or 5.

tion, (ii) a subsequent relaxation, followed by (iii) a secondary sustained contraction (Figure 1a). The third phase of the response, i.e. the sustained contraction, was found to be blocked selectively by DL-dithiothreitol (5 mM) (Figure 1b), by L-cysteine (10 mM), and by other thiols, leaving the initial contractile response unaffected (Figures 1 and 2). Thus in a series of experiments neither L-cysteine (10 mM), N-acetyl-L-cysteine (10 mM), GSH (10 mM), nor dithiothreitol (5 mM) affected the initial contraction induced by photo-activation of AlPcS₄ (Figure 3a). In contrast, when compared to the standard carbachol contraction (5×10^{-5} M), there was a major inhibition of the sustained light-induced contraction by L-cysteine and by DTT, and a smaller but significant inhibition with N-acetyl-L-cysteine. (Figure 3b). Interestingly, GSH (10 mM) was without effect (Figure 3b). None of the thiols had any effect on the standard contraction to carbachol (5×10^{-5} M); see Figures 1 and 2.

In a previous investigation (Matthews & Mesler, 1984b) it was suggested that one action of photodynamic drugs may be to increase membrane permeability to calcium and other ions. It is evident from the present study that the photon-induced contraction of the taenia sensitized with AlPcS₄ is calcium-dependent since it was abolished when extracellular calcium was removed (Figure 4a). However, as soon as extracellular calcium was re-introduced the muscle contracted, indicating that a photodynamic effect, possibly on membrane permeability, had been produced but could not be expressed until extracellular calcium was restored. It is also of interest to note that the contraction seen on the re-introduction of calcium was sustained and monophasic. Furthermore this calcium-dependent contraction was markedly inhibited by the presence of L-cysteine (Figure 4b). In some experiments (Figures 2b and 4b) a small transient contraction of variable magnitude was seen on the addition of cysteine and this may be attributable to a brief opening of calcium release channels in the sarcoplasmic reticulum.

AlPcS₄-sensitized photodynamic relaxation of taenia caeci: effect of thiol reductants

An additional photodynamic effect was seen in response to light if, after exposure to the phthalocyanine compound, the smooth muscle of the taenia was maintained in a contracted state by continuous exposure to carbachol (5×10^{-5} M). When under these conditions, the muscle was illuminated (32,000 lux, $\lambda > 570$ nm), a triphasic relaxation response was

seen, i.e. (i) a rapid relaxation, followed by (ii) a transient contraction, preceding (iii) a secondary more sustained relaxation. The time course of this effect is clearly demonstrated in Figure 5. In comparative experiments the triphasic relaxation response was enhanced in the presence of L-cysteine (Figure 6) and by other thiols. L-Cysteine, N-acetyl-L-cysteine, GSH, and DTT, all significantly increased the magnitude of the photodynamic relaxation relative to the sustained carbachol response, approximately doubling it, although GSH was again the least effective agent (Figure 7). The duration of the photodynamic relaxation was also extended considerably in the presence of L-cysteine, N-acetyl-L-cysteine, and DTT, i.e. from 20 ± 4 min, 15 ± 1 min, and 16 ± 2 min, respectively, to $>> 30$ min; $n = 3$ to 5.

Effects of 8-PCPT-cGMP on smooth muscle contraction

Underlying the relaxation response in many smooth muscle cells, including the taenia coli (Katsuki *et al.*, 1977) is the activation of guanylate cyclase and the formation of cyclic GMP. It is also known that guanylate cyclase can be activated by oxidation (Waldman & Murad, 1987). The potent membrane-permeant cyclic GMP analogue, 8-PCPT-cGMP which has recently become available was therefore used to mimic the effect of cyclic GMP generation in muscle pre-contracted with carbachol. There was a reversible and

dose-dependent response to the cyclic GMP analogue. Relatively little action was seen with $20 \mu\text{M}$ but a 10 fold increase to $200 \mu\text{M}$ for 30 min not only induced a marked relaxation (Figure 8) but one reproducing almost exactly that evoked by photodynamic drug action (see Figures 5 and 6). Furthermore, the effect of the cyclic GMP analogue was still seen whether the muscle was pre-contracted by carbachol acting via a receptor-operated pathway or more directly by depolarization with a five fold increase in $[\text{K}^+]_o$ from 4.7 mM to 23.5 mM (Figure 9).

The ability of 8-PCPT-cGMP ($200 \mu\text{M}$) to affect the photodynamic contractile response was also investigated. Illumination (32,000 lux, $\lambda > 570$ nm), in the presence of the cyclic GMP analogue produced no change in the magnitude of the initial contractile response of AlPcS_4 -sensitized muscle but both the rate of rise and final plateau level of the sustained contraction was markedly reduced when compared to the preparation not treated with 8-PCPT-cGMP (Figure 10).

Discussion

The characteristic features of photodynamic drug action on smooth muscle have been defined here in an attempt to identify the molecular mechanisms by which these drugs exert their effects at the cellular and subcellular level. The major factor underlying photodynamic drug action is likely to be the generation of singlet oxygen which is highly reactive chemically but has an extremely short half-life of micro-seconds. The results establish that on an AlPcS_4 -sensitized photodynamic contraction in the taenia caeci the thiol reductants L-cysteine, N-acetyl-L-cysteine, DTT and GSH have a similar action with GSH being the least potent agent. Furthermore there is a common potentiating effect of the thiols on the photodynamic relaxation response in the precon-

Figure 8 Effects of 8-PCPT-cGMP on smooth muscle precontracted with carbachol. Contractions were evoked with carbachol, 5×10^{-5} M, for 10 s (●) or by continuous exposure (▲). 8-PCPT-cGMP was present for 30 min (open rectangle) at the concentrations shown.

Figure 9 Effects of 8-PCPT-cGMP on smooth muscle contracted with potassium chloride. Contractions were evoked in response to KCl, 23.5 mM for 30 s (○) or continuously (▲). 8-PCPT-cGMP was present for 30 min (open rectangle).

Figure 10 Photodynamic contractile responses (a) in the absence, and (b) in the presence of 8-PCPT-cGMP, 200 μ M. Contractions to carbachol, 5×10^{-5} M, for 10 s (●). In both (a) and (b) the smooth muscle cells had been exposed previously to AlPcS₄, 10^{-5} M, for 30 min followed by washout period of 30 min before illumination at 32,000 lux for 30 min (open rectangle). In (b) the muscle was also exposed to 8-PCPT-cGMP immediately following the carbachol response until the end of the experiment.

tracted photosensitized muscle. In addition cyclic GMP analogues cause a relaxation of the smooth muscle cells which mimics the photodynamic relaxation response.

In the absence of thiols, light activation of AlPcS₄-sensitized muscle produced a typical triphasic response, an initial transient contraction and subsequent relaxation followed by a sustained contraction. Both contractile responses were blocked by the removal of extracellular calcium but the thiol reductants blocked only the sustained secondary contraction. The initial transient contraction was seen within a few seconds of illumination and, as in other cells (Matthews & Ciu, 1990a; Al-Laith *et al.*, 1993a,b), may be due to a rapid, direct stimulatory effect of photon-activated AlPcS₄ or singlet oxygen upon the membrane transduction-intracellular signalling system involving the G-protein-IP₃ dependent release of calcium from intracellular stores and subsequent contraction. The intracellular calcium stores are labile and dependent upon extracellular calcium, becoming depleted after the smooth muscle cells have been in a calcium-free EGTA solution for 5 or 6 min (Brading & Sneddon, 1980).

The inhibition of the secondary contractile photodynamic response can be attributed to two effects exerted by the thiols i.e. (i) a potentiation of the relaxation observed after the initial contraction, since all four thiols were able to markedly increase the photorelaxation effect in the precontracted taenia, or (ii) inhibition of the singlet oxygen-dependent membrane permeabilization and subsequent massive influx of extracellular calcium thought to be responsible for a sustained photodynamic contraction (Matthews & Mesler, 1984a,b). The differing abilities of the reductants to potentiate the magnitude and duration of the relaxation or to inhibit singlet oxygen generation or action sufficiently to diminish the secondary sustained contraction are probably due to differences in membrane uptake or reductant potency. L-Cysteine is a natural amino acid, neutral at physiological pH, and shown to be actively transported into cells (Bannai, 1984). Although N-acetyl-L-cysteine may have some reducing activity itself (Tang *et al.*, 1991) it possesses a bulky acetyl substituent which may prevent or hinder its uptake on the L-cysteine transporter; alternatively, the smooth muscle cells may lack the deacetylation enzyme required to convert the molecule to L-cysteine and so account for its lesser action, DTT has a low redox potential and is a potent thiol reductant. It was

Figure 11 Diagram summarizing the photodynamic effects of AlPcS₄ on the smooth muscle cells of the taenia caeci.

therefore, not surprisingly, the most effective of the thiols tested. In contrast, the relative lack of action of GSH may be due to the fact that little exogenous GSH crosses cell membranes as the intact tripeptide (Butterworth *et al.*, 1992) or it is possible that the reduced glutathione is oxidized by oxygenation of superfusion solutions so decreasing its activity. Oxidation may also decrease the activity of L-cysteine (Bannai, 1984), but not DTT, and hence account for the greater effect of the DTT. All thiol reductants can diminish local singlet oxygen concentrations in proportion to their free radical scavenging capacity; they may also delay the process of membrane permeabilization by recycling some of the membrane lipid oxidized by singlet oxygen (Buettner & Hall, 1987). Together these various mechanisms are clearly effective in minimizing the photodynamic process of membrane permeabilization because the restoration of calcium to a previously calcium-free but cysteine-treated muscle produced a subsequent contraction of much slower onset and smaller magnitude than that in an L-cysteine-free preparation (see Figure 4).

To investigate the photodynamic relaxation response, AIPcS₄-sensitized muscle was pre-contracted either with carbachol or KCl; in both experimental conditions a photodynamic relaxation was seen which was enhanced by L-cysteine. This makes it unlikely that the relaxation is due simply to permeabilization of nerve endings in the muscle and the release of inhibitory transmitters because the high K⁺ concentration will stimulate and persistently depolarize any neurones in the muscle, as well as the muscle itself. A major nerve-mediated component of the relaxation response can therefore be largely discounted. It now seems more probable that an effect of singlet oxygen on guanylate cyclase increases cyclic GMP production. Oxidizing agents have been shown to activate guanylate cyclase directly and dithiothreitol and other reducing agents potentiate activation of the enzyme (Waldman & Murad, 1987). Thiol-disulphide oxidation and reduction thus forms an important mechanism for regulating guanylate cyclase and modulating singlet oxygen action. This conclusion is substantiated by the responses of

the precontracted muscle to the membrane permeant cyclic GMP analogue, 8-PCPT-cGMP which closely mimicked those evoked by photodynamic activation of AIPcS₄. Specific protein kinases activated by cyclic GMP may not only cause sequentially, phosphorylation and inactivation of myosin light chain kinase, dephosphorylation of the myosin light chain, and smooth muscle relaxation (Ignarro, 1991) but also decrease [Ca²⁺]_i by (i) reducing Ca²⁺ influx, (ii) increasing Ca²⁺ efflux, and (iii) promoting subcellular sequestration of Ca²⁺ (Francis *et al.*, 1988). All these effects will contribute to the photodynamic relaxation response and can be enhanced in the presence of thiol reductants. Such effects would be expected also to diminish or delay the onset of the sustained photodynamic contraction as does the cyclic GMP analogue itself.

In conclusion, photo-activation of cell-bound AIPcS₄ raises it to the higher energy triplet state which in turn generates highly-reactive but short-lived singlet oxygen (Figure 11). This, in the smooth muscle cell of the taenia caeci produces an initial contraction not directly affected by thiol reagents. The initial contraction is followed by a relaxation due to oxidative activation of guanylate cyclase and the production of cyclic GMP and is the major effect seen in precontracted muscle; it is enhanced by thiol reagents. Finally, permeabilization of the cell membrane and calcium influx leads to a sustained contraction and this may be diminished by thiol reductants either by directly opposing the photo-oxidation of membrane proteolipid structure, or by enhancing the generation of cyclic GMP, and some of these effects can be mimicked by cyclic GMP analogues. More experiments are therefore now clearly required to assess fully the photodynamic action of AIPcS₄ on purified guanylate cyclase *in vitro*.

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