The design and biological properties of potent and selective inhibitors of protein kinase C

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Introduction: the PKC isoenzyme family
The isoenzyme family protein kinase C (PKC) so far consists of nine members (α, βI, βII, γ, δ, ε, η, θ, ι) which phosphorylate their target proteins on serine and threonine residues [1]. The members of this family are single polypeptide chains, with molecular masses around 80 KDa, containing two functional domains. The catalytic C-terminal domain contains binding sites for the protein substrate and ATP. All PKC isoenzymes contain within their N-terminal regulatory domain sequences which resemble their substrate phosphorylation sites [2]. These pseudosubstrate sites probably interact with the enzyme’s active site and thus prevent substrate access. Additionally the regulatory domain contains binding sites for Ca²⁺, phospholipid and the physiological activator diacylglycerol (DAG). These cofactors, when bound, presumably induce a conformational change which exposes the catalytic site and renders the enzyme active. Phorbol esters such as tetradecanoil phorbol acetate (TPA) are potent activators of PKC [3] and probably bind to the same site as DAG.

Isoenzyme forms of PKC differ in their activation requirements [4] and in their recognition of the flanking sequences around the phosphorylation sites in their target proteins. For instance, the regulatory domains of PKC α, β and γ all contain a Ca²⁺-binding region that is absent in the δ and ε isoforms [5, 6]. There is increasing evidence that certain isoforms are preferentially activated by different phorbol esters [6]. The tissue and subcellular distribution of PKC sub-species also varies. This information, taken together with the differences in regulation and target phosphorylation sites, suggests distinct roles for the different isoenzyme forms of PKC.

Role of PKC in cell signalling
Activation of PKC is an early event in a wide range of signal transduction processes. Receptor binding by a variety of hormones, neurotransmitters and growth factors stimulates phospholipase C-mediated hydrolysis of phosphoinositides. This leads to generation of two intracellular messengers: inositol triphosphate (InsP₃) which stimulates Ca²⁺ release from intracellular stores and DAG which triggers PKC [7, 8]. An alternative pathway involving PKC activation operates through receptor-linked phospholipase D and leads to DAG generation without InsP₃ formation [9]. The events which link PKC activation to the end cellular response are, in most instances, poorly understood and the degree of redundancy involved in these systems (i.e. the degree to which PKC can be bypassed) is not known. However, several lines of evidence (use of phorbol esters; detection of increased DAG; translocation of PKC) have implicated PKC in a variety of cellular responses such as cell proliferation, secretion and gene expression. The use of potent, cell-permeable and selective inhibitors of PKC may clarify the precise role this enzyme plays in regulating cell function. Furthermore such agents may provide therapies for diseases such as asthma, cancer, dermatological disorders, rheumatoid arthritis and AIDS.

Targets for inhibitor design
Both regulatory (phospholipid- and phorbol ester-binding sites) and catalytic domains (peptide- and ATP-binding sites) on PKC offer targets for inhibitor design. Examples of inhibitors directed against the regulatory site include sphingosine [10] and calphostin C [11]. The latter is a perylenquinone which is competitive with phorbol ester and is three orders of magnitude more potent against PKC (IC₅₀ = 50 nM) than against any other protein kinase. However, the mechanism of action of this compound appears to involve photo-activation to yield a short-lived species which reacts with PKC resulting in permanent inactivation of phorbol ester binding [12]. Peptide sequences directed against the substrate binding site, for instance the pseudosubstrate sequence PKC19–36 (IC₅₀ = 300 nM [13]), are potent and selective inhibitors but do not penetrate cells. By far the greatest progress has been made with the design of ATP-competitive inhibitors of PKC.

Abbreviations used: PKC, protein kinase C; DAG, diacylglycerol; TPA, tetradecanoil phorbol acetate; PKA, cyclic AMP-dependent protein kinase; IL-2, interleukin 2; PDBu, phorbol dibutyrate.
ATP-competitive inhibitors

1. Isoquinoline sulphonamides

A series of isoquinoline sulphonamides exemplified by N-[2-(methylamino)ethyl]-5-isooquinoline sulphonamide (H18, Fig. 1) and 1-(5-isooquinolinyl-sulphonyl)-2-methylpiperazine (H17, Fig. 1) gave $K_i$ values in the micromolar range against a selection of protein kinases [14]. H18 showed some selectivity towards cyclic nucleotide-dependent protein kinases whereas H17 was an inhibitor of cyclic AMP.

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**Fig. 1**

ATP-competitive inhibitors of PKC

(a) Isoquinolinesulphonamides

(b) Indolocarbazoles

| Stauroporine        | H | H | H | 9 | 21 |
| CGP41295           | H | H | Benzoyl | 50 | 20 |
| UCN-01             | H | OH | H | 4 | 19 |

(c) Bisindolylmaleimides

| Ro31-6045     | CH$_3$ | H | H | >100000 | 21 |
| Ro31-6233     | H | H | H | 600 | 21 |
| Ro31-7208     | H | CH$_3$ | CH$_3$ | 300 | 21 |
| Ro31-7549     | H | (CH$_3$)$_2$NH$_2$ | CH$_3$ | 70 | 21 |
| Ro31-8220     | H | (CH$_3$)$_2$S(-NH)NH$_2$ | CH$_3$ | 10 | 21 |

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cyclic GMP-dependent protein kinases and PKC (K = 6 μM). H7 has been used as a tool to probe the involvement of PKC in a variety of cellular processes. This use is inappropriate for two reasons [15]. First, H7 is not particularly selective for PKC over other protein kinases. Secondly, ATP concentrations in cells are sufficiently high to prevent low micromolar concentrations of H7 from inhibiting PKC. Therefore, it is not surprising that 100 μM H7 does not inhibit phorbol ester-induced cellular events.

2. Indolocarbazoles
An alternative approach to the design of selective ATP-competitive inhibitors was provided by the identification of the microbial metabolite staurosporine (Fig. 1) as a potent, but non-selective, protein kinase inhibitor [16, 17] (IC50 versus PKC = 9 nM). Replacement of the methylamino substituent on the pyranose ring in this molecule with –OH resulted in a considerable loss in potency against PKC [18], suggesting the presence of a cation-binding site on the enzyme. Modification of the lactam and substitution of the amine group in the sugar moiety of staurosporine has yielded, in UCN-01 [19] and CGP41 251 [20], compounds which show a significant improvement in selectivity for PKC over cyclic AMP-dependent protein kinase (PKA). However, the improvement in selectivity was only modest (10-fold) for UCN-01 and was offset by a loss in potency against PKC for CGP41 251. Furthermore, CGP41 251 was also a potent inhibitor of phosphorylase kinase. Nevertheless, given the close sequence similarity between ATP binding sites of protein kinases, the indentification of PKC-selective indolocarbazoles is encouraging.

3. Bisindolylmaleimides
Removal of the 12a–12b bond in staurosporine aglycone and introduction of a second carbonyl function into the lactam ring resulted in a bisindolylmaleimide. In this molecule neither indole group could attain coplanarity with the maleimide ring system in a low-energy conformation. Simple bisindolylmaleimides such as Ro31-6233 and Ro31-7208 (Fig. 1) were ATP-competitive but showed reduced activity against PKC compared with staurosporine [21]. However, these bisindolylmaleimides were much more selective for PKC over PKA than staurosporine.

A molecular graphics approach was adopted to place substituents on the bisindolylmaleimide which would dock to the putative cation-binding site on the enzyme. Assuming a common binding mode for staurosporine and Ro31-6233, the imide moiety of the bisindolylmaleimide was matched to the lactam of staurosporine. The indoles were allowed to approach the plane of the indolocarbazole as far as possible without energy penalty and a side-chain containing a cationic substituent was introduced on one indole nitrogen, the only position in the molecule known to tolerate any significant substitution. The graphics model predicted that a variety of cation-containing side-chains, substituted on the indolyl nitrogen, should address the putative binding site for staurosporine. Indeed, such compounds, exemplified by the n-propylamine derivative Ro31-7549 (IC50 versus PKC = 70 nM; Fig. 1), were significantly more potent PKC inhibitors than the parent bisindolylmaleimide [21].

A further 10-fold potency improvement was obtained by conformational restriction of the primary amine at specific spatial positions relative to the bisindolylmaleimide. The most potent of these inhibitors Ro31-8425 (IC50 versus PKC = 8 nM; Fig. 1) was equipotent with staurosporine against PKC, but showed a markedly different selectivity profile (Fig. 2) [22]. Staurosporine showed little evidence of selectivity for PKC over a range of closely related protein kinases. In contrast, Ro31-8425 was highly selective for PKC (350-fold selectivity over PKA; 2400-fold selectivity over Ca2+/calmodulin-dependent protein kinase and 160-fold selectivity for PKC over phosphorylase kinase).

Demonstration of PKC inhibitory activity in cells
Phorbol esters which are direct, potent and selective activators of PKC were used to drive phosphorylation of 47 KDa protein in intact platelets [23]. A range of bisindolylmaleimide PKC inhibitors was able to antagonize TPA-induced p47 phosphorylation in platelets with structure–activity relationships which paralleled those obtained against the isolated enzyme (Table 1). However, absolute potencies were 50–100-fold less than those obtained against isolated PKC [21]. The same pattern of similar structure–activity relationships but reduced potencies was repeated with other phorbol ester-induced events in cells [e.g. phorbol dibutyrate (PDBu) induced down-regulation of CD3 from T cells; TPA induced degranulation of human neutrophils]. Cellular concentrations of ATP (1–10 μM) are much higher than those used in the enzyme assay (10 μM), and this probably contributes to the reduced potency of these ATP-competitive inhibitors in cells.
**Fig. 2**

Selectivity profile for Ro 31-8425

Abbreviations used: PKC<sup>a</sup>, Rat brain PKC; PKC<sup>h</sup>, Human neutrophil PKC; PKA, Bovine Heart PKA; PhK, Rabbit muscle phosphorylase kinase; CCD-PK, Ca<sup>2+</sup>/calmodulin-dependent protein kinase. Assay procedures detailed in [17, 21].

<table>
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<tr>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>PKC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PKC&lt;sup&gt;h&lt;/sup&gt;</th>
<th>PKA</th>
<th>PhK</th>
<th>CCD-PK</th>
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<td>5</td>
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<td>1300</td>
<td>19000</td>
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</table>

![Chemical structure of Ro31-8425 and Staurosporine]

**Table 1**

Inhibition of TPA-induced P47 phosphorylation in platelets and PDBu-induced CD3 down-regulation in T cells

Assay procedures detailed in [21]

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Rat brain PKC (in vitro)</th>
<th>P47 phosphorylation platelet</th>
<th>CD3 Down-regulation</th>
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<td>Ro31-6045</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Ro31-6233</td>
<td>0.6</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Ro31-7549</td>
<td>0.07</td>
<td>4.4</td>
<td>3.1</td>
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<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Ro31-8425</td>
<td>0.008</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>H-7</td>
<td>18</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>0.009</td>
<td>0.6</td>
<td>0.2</td>
</tr>
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</table>

**Effects of bisindolylmaleimides on proinflammatory cellular responses**

The effects of these bisindolylmaleimide inhibitors have been most extensively studied on cellular responses associated with neutrophil activation and with immunoregulation. Superoxide generation by phagocytic cells such as neutrophils occurs in response to a wide range of stimuli and is thought to be an important factor in tissue damage. Ro31-8425 inhibits the neutrophil respiratory burst induced by a variety of soluble and particle agonists implying the involvement of PKC in the signal transduction mechanisms stimulated by these agents. The burst triggered by dioctanoylglycerol, a direct PKC activator, was more sensitive to inhibition by Ro31-8425 than were the responses induced by formyl Met-Leu-Phe, IgG, heat-aggregated IgG and opsonized zymosan [22]. This suggests that PKC is involved in propagating the signal invoked by this second group of agonists, but is not a rate limiting step in these pathways.

Presentation of specific antigen to T lympho-
cytes results in the clonal expansion of particular T-cell populations bearing T-cell receptors which recognize that antigen. These expanded populations are then able to modulate the activities of a variety of other cell types involved in mounting an immune response to that antigen. Antigen-driven T-cell proliferation may be regarded as a two-stage process [24, 25]. In the first stage, the antigenic signal delivered via the T-cell receptor CD3 complex results in increased surface expression of the interleukin 2 (IL2) receptor and in the production of IL2. In the second stage, binding of IL2 to its receptor triggers cell division. Inhibition of the allogenic mixed lymphocyte reaction by Ro31-8830 suggests a role for PKC in antigen-driven proliferation of T cells [22]. Ro31-8830 had little effect on IL2-

Fig. 3
Oral, anti-inflammatory activity of Ro31-8830

(a) Effect of Ro31-8830 on PDBu-induced paw inflammation in the mouse. Male C57BL/6 mice were randomized into groups of eight animals and given standard laboratory diet and water ad libitum. Animals were injected into one hind footpaw with 20 μl of a 10⁻¹ M solution of phorbol 12, 13-dibutyrate in saline. The contralateral paw received vehicle alone. Paw swelling was monitored using water displacement plethysmography. Results are expressed as the mean differences between PDBu-injected and saline-injected paws at each time point, with statistical significance being calculated using non-paired Student's t-test. Key to symbols: ---, Control; ---, 15 mg/kg; ---, 30 mg/kg; ---, 50 mg/kg. (b) Effect of Ro31-8830 on paw inflammation in a model of developing adjuvant arthritis in the rat. Two groups of five A/J-HR female rats were injected in the right hind paw with a suspension of M. tuberculosis in liquid paraffin. One group of rats was dosed with Ro31-8830 (200 mg/kg, daily), the first dose being given one hour before the injection of M. tuberculosis. The second group received the dosing vehicle, 10% succinylated gelatin (10 ml/kg). Paw volumes were measured by water displacement plethysmography on the days indicated. Key to symbols: ---, Control (non-injected paw); ---, control (injected paw); ---, 31-8830 treated (non-injected paw); ---, 31-8830 treated (injected paw). (c) Dose-response for Ro31-8830 in adjuvant arthritis. The procedure described for Fig. 3(b) was followed. Ro31-8830 was given once a day at the doses indicated. Paw swelling and plantar lymph node weights were measured on day 13 following injection of M. tuberculosis. Ro31-8830 dose-dependently inhibited inflammation in the non-injected paws. Ro31-8830 also inhibited the increase in lymph node weight which occurs in adjuvant-treated animals. Key to symbols: ---, Paw swelling; ---, lesion score; ---, joint mobility.
induced T-cell proliferation, implying that involvement of PKC in antigen-driven proliferation occurs before this second stage [22].

Demonstration of PKC inhibitory activity of bisindolylmaleimides in vivo
Phorbol esters induce inflammatory responses when administered to rodents, and antagonism of this response can be used to demonstrate inhibition of PKC in vivo. Intraplantar injection of the phorbol ester PDBu into the hind paw of a mouse produces an oedema which develops rapidly and is maintained for over six hours following a single injection. Although Ro31-8425, dosed orally at 30 mg/kg, was ineffective in this model, a tertiary amine derivative Ro31-8830 (Fig. 1), dosed orally one hour before the PDBu injection, inhibited the oedema with an ED50 of 20 mg/kg (Fig. 3).

Profile, in vivo, of Ro31-8830 in T-cell-mediated models of inflammation
Ro31-8830, dosed orally at 100 mg/kg, showed little or no effect in a number of acute models of inflammation (zymosan or carrageenin induced paw oedema in the mouse, carrageenin induced pleurisy in the rat). This distinguishes the profile of Ro31-8830 in vivo from that of the classical non-steroidal anti-inflammatory drugs such as indomethacin which are active in these models.

Since bisindolylmaleimide PKC inhibitors are potent inhibitors of T-cell proliferation in vitro, the effect of Ro31-8830 on T-cell-mediated inflammation in vivo was examined. An arthritis was induced by intraplantar injection of Mycobacterium tuberculosis in mouse into the hind paw of a rat. Ro31-8830 was administered orally once daily and its effects on various parameters of the arthritic response were measured. In this model of arthritis, the paw inflammation proceeds in two phases. A primary response which affects only the injected paw is followed by a secondary response which affects both injected and non-injected hind paws. This secondary response is T-cell mediated. Ro31-8830, at doses up to 200 mg/kg given once daily, had no significant effect on the primary inflammation, but dose-dependently inhibited the secondary inflammatory response with an ED50 of 12.5 mg/kg given once daily (Figs. 3b and 3c). Lesion scores (a visual assessment of swelling in ears, fore paws, hind paws and tail) and lymph node weights, which increase in arthritic animals, were reduced in Ro31-8830-treated animals.

Conclusions
The structural lead provided by staurosporine, a non-selective protein kinase inhibitor, was used to derive a series of potent and highly selective bisindolylmaleimide PKC inhibitors. These agents are useful tools for clarifying the role of PKC in mediating functional responses in cells. The inhibition of T-cell proliferation in vitro and in vivo by certain of these bisindolylmaleimides, suggests that PKC inhibitors may have therapeutic potential in the treatment of autoimmune diseases such as rheumatoid arthritis.

Suppression of malignancy targeting cyclic AMP signal transducing proteins
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Introduction
Cyclic AMP as a transducer of hormonal signals [1] acts through binding to its receptor proteins, the cyclic AMP-dependent protein kinases [2, 3]. There are two different classes of cyclic AMP-dependent protein kinase, type I and type II, and these two isoforms of cyclic AMP receptor proteins are inversely expressed during ontogeny and differentiation of normal cells, and the disruption in normal patterns of cyclic AMP-receptor proteins correlates with malignant transformation [4, 5]. Thus malignancy correlates with an abnormal signal transduction of cyclic AMP.

Elevation of intracellular levels of cyclic AMP or a cyclic AMP derivative exogenously supplied causes, in some cases, inhibition of cell proliferation in normal as well as transformed cells along with morphological changes and induction of differentiation [6-8]. In contrast, in other instances, especially in the primary cultures of normal cells, elevation of cellular cyclic AMP levels often results in stimulation rather than inhibition of cell growth [9]. These results, rather than representing conflicting data, may instead demonstrate that cyclic AMP, dependent on the particular physiological status of the cell, exerts dual controls, either positive or negative, over cell proliferation.

I will show that cancer cells can be made to differentiate and acquire growth control when the functional balance of these signal transducers of cyclic AMP is restored by the use of site-selective cyclic AMP analogues, antisense strategy, or retroviral vector-mediated gene transfer, suggesting new approaches to cancer therapy. In addition, the promising results obtained from the first clinical application of 8-Cl-cyclic AMP to a terminal cancer will be presented.

Two isoforms of cyclic AMP receptor proteins: the positive and negative regulators of cell proliferation
Cyclic AMP-dependent protein kinase is composed of two genetically distinct catalytic (C) and regulatory (R) subunits. The activating ligand, cyclic AMP, which binds to the R subunit, induces conformational changes and dissociates holoenzyme R,C, into an R (cyclic AMP), dimer and two free C subunits that are catalytically active [2, 10, 11]. There are two different classes of cyclic AMP-dependent protein kinase designated as type I and type II, which contain distinct R subunits, RI and RII, respectively, but share a common C subunit [2]. Four different regulatory subunits (RIα [12], RIβ [13], RIIα [14], and RIIβ [15]) have been identified at the gene/mRNA level. Three isoforms of the C subunit (Cα [16], Cβ [17, 18], and Cγ [19]) have also been discovered. However, preferential coexpression of any of these C subunits with either the type I or type II protein kinase R subunit has not been found [18, 19].

The two general classes of R subunits, RI and RII, contain two tandem cyclic AMP-binding domains at the C-terminus of which amino acid sequences are highly conserved. The RI and RII differ significantly in the N-terminus at a proteolytically sensitive hinge region that occupies the peptide substrate binding site of the C subunit in the holoenzyme complex [20]. In this segment, RII contains the sequence (54Arg-Arg-Val-Ser(?)