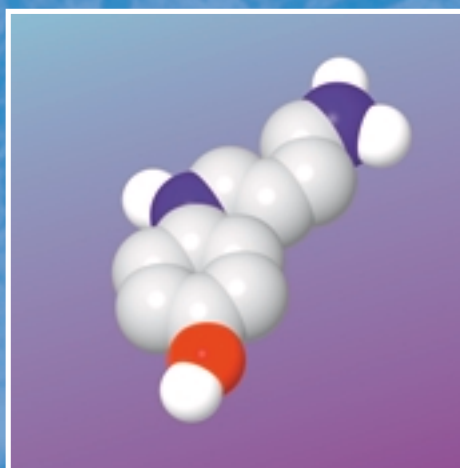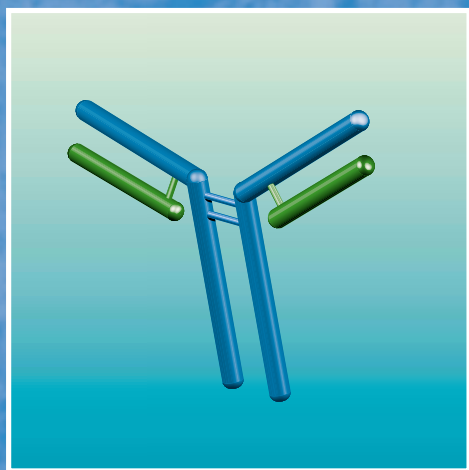# Celltransmissions

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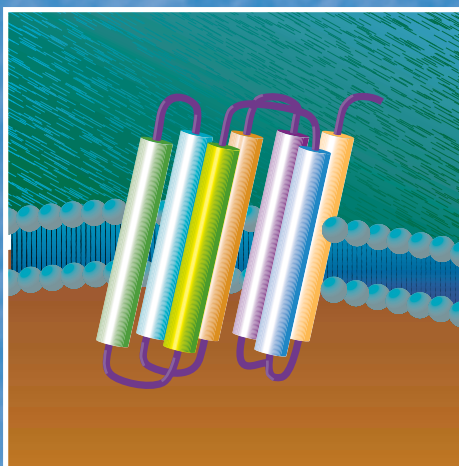
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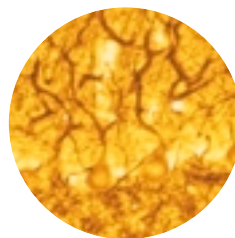
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Past Winners of Photo Contests

First Place, Dr. Giuseppina Di Stefano, Neurobiology of Aging Lab, INRCA Res. Dept., Ancona, ITALY **Description**: Rat cerebellar cortex immunostained with monoclonal Anti-MAP-2 (Clone HM2, Sigma Product No. M 4403). Purkinje cell bodies and proximal dendrites are faintly stained, while distal Purkinje cell dendrites and glomeruli are intensely labeled. No staining can be observed in the white matter.
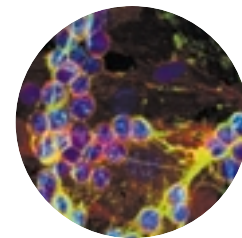
Second Place, Carol de la Motte and Judy Dragba, Cell Biologists, Cleveland Clinic Foundation, Cleveland, Ohio, USA **Description**: HeLa cells in culture. The cells were labeled for microtubules with an antibody to tubulin (green-FITC - Sigma Product No. T 3526) and stained with propidium iodide to label DNA and RNA (red- Sigma Product No. P 4170) and observed in a Leica confocal micro-scope. The image is from an optical section of 0.7 micrometers in thickness. The figures show interphase cells, and cells in mitosis (anaphase, metaphase, and cytokinesis).

Matrix Metalloproteinases and their Inhibitors – Current Status and Future Challenges

Mark Whittaker and Andrew Ayscough

Matrix metalloproteinases are a family of structurally-related, zinc-containing enzymes that have the ability to breakdown connective tissue. Their expression is known to increase in various inflammatory, malignant and degenerative diseases thereby raising the possibility that inhibitors of these enzymes may possess therapeutic potential [1-3]. Over the past decade, there have been significant advances in matrix metalloproteinase (MMP) research. These have included a better under-standing of the biochemistry of these zinc-dependent enzymes in terms of their activation, regulation and substrate specificity, the determination of their structure by X-ray crystallography and NMR, and the design of orally-available inhibitors [4-7]. A disappoint-ment to researchers in this field has been the lack of success to date in the clinic for such compounds in spite of the encouraging preclinical efficacy in pharmacological models. The new challenge in MMP research is to better understand the complex role these enzymes play in human disease, and to design inhibitors that are successful in the clinic. The purpose of this review is therefore not only to provide an introduction to MMPs and their inhibitors and give an overview of what has been achieved to date, but also to review some of the current challenges for this area of life science research.

Ten years ago there were around 7 known members of the mammalian MMP or matrixin family [8]. This family continues to grow with the most recent member identified as MMP-26 [9] (see Table 1). This group of enzymes was initially classified into collagenases, gelatinases and stromelysins on the basis of substrate specificity. A further distinct class of enzymes within the matrixin family are the "membrane type" MMPs which are characterized by a C-terminal transmembrane domain that allows them to be anchored in the cell membrane [10,11]. There are also a number of MMPs that do not readily fit into any of the above four classes. Thus, matrilysin (MMP-7) is a short "truncated" proteinase that can degrade non-fibrillar collagen, fibronectin and laminin [12]. Metalloelastase (MMP-12) is a relatively non-specific enzyme capable of degrading many substrates including elastin [13]. Newer members to the MMP family are MMP-19 (also known as RASI-1), an enzyme with some homology to the stromelysins [14,15], enamelysin (MMP-20) [16], two genomic sequences on chromosome 1 (MMP-21 and MMP-22) [17] and a short MMP cloned from an ovary cDNA library (MMP-23) [18]. The most recent discovery, MMP-26, is an endometrial tumor-derived metalloproteinase [9]. While not covered in this review, there is a related series of metalloenzymes known as the ADAMs (A Disintegrin And Metalloproteinase) family [19]. Members of this group of metallo-proteinase disintegrins have been shown to be involved in cytokine processing. An example is ADAM-17 (TNF convertase or TACE) which cleaves the membrane-bound form of tumor necrosis factor-α [20]. Similar activity has been shown for members of the MMP family and the relative contributions of MMPs and disinte-grin metalloproteinases to the shedding of cell surface molecules, such as soluble CD23 [21] and transforming growth factor alpha (TGF-α), requires clarification [22].

About the Authors

Mark Whittaker received his D.Phil. in chemistry from the University of York in the UK in 1982, and then undertook postdoctoral studies, firstly in the laboratory of Clifford Leznoff at York University (Toronto, Canada) and secondly from 1985 in the laboratory of Steve Davies at the Dyson Perrins Laboratory (Oxford, UK). In 1987, he joined British Biotech and initially worked on a platelet-activating factor antagonist medicinal chem-istry program. He is currently Director of Chemistry and his research focus is on the medicinal and combinat-orial chemistry of metalloenzyme inhibition.

Andrew Ayscough received his D.Phil. in chemistry from the University of Oxford in 1988, where he worked in the laboratory of Steve Davies. In 1988, he joined British Biotech, where he has worked primarily on the design and synthesis of metalloenzyme inhibitors, and is currently Head of Medicinal Chemistry.

Table 1. Classification of Matrix Metalloproteinases

MMP	Alternative Names	EC Number	Chromosome	Substrates
MMP-1	Collagenase (Type I, interstitial)	EC3.4.24.7	11q22-q23	Collagens (I, II, III, VII, VIII and X); gelatin; aggrecan; L-selectin; IL-1β; proteoglycans; entactin; ovostatin; MMP-2; MMP-9
MMP-2	Gelatinase A 72kDa Gelatinase Type IV Collagenase	EC3.4.24.24	16q13	Collagens (I, IV, V, VII, X, XI and XIV); gelatin; elastin; fibronectin; aggrecan; MBP; osteonectin; laminin-1; MMP-1; MMP-9; MMP-13
MMP-3	Stomelysin-1 Proteoglycanase	EC3.4.24.17	11q23	Collagens (III, IV, V and IX); gelatin; aggrecan; perlecan; decorin; laminin; elastin; casein; osteonectin; ovostatin; entactin; plasminogen; MBP; IL-1β; MMP-2/TIMP-2; MMP-7; MMP-8; MMP-9; MMP-13
MMP-7	Matrilysin, PUMP	EC3.4.24.23	11q21-q22	Collagens (IV and X); gelatin; aggrecan; decorin; fibronectin; laminin; entactin; elastin; casein; transferrin; plasminogen; MBP; β4-integrin; MMP-1; MMP-2; MMP-9; MMP-9/TIMP-1
MMP-8	Neutrophil collagenase	EC3.4.24.34	11q21-q22	Collagens (I, II, III, V, VII, VIII and X); gelatin; aggrecan; fibronectin
MMP-9	Gelatinase B	EC3.4.24.35	20q11.2-q13.1	Collagens (IV, V, VII, X and XIV); gelatin; entactin; aggrecan; elastin; fibronectin; osteonectin; plasminogen; MBP; IL-1β
MMP-10	Stromelysin-2	EC3.4.2.22	11q22.3-q23	Collagens (III-V); gelatin; casein; aggrecan; elastin; MMP-1; MMP-8
MMP-11	Stromelysin-3	*	22q11.2	Unknown (Casein)
MMP-12	Macrophage metalloelastase	EC3.4.24.65	11q22.2-q22.3	Collagen IV; gelatin; elastin; casein; fibronectin; vitronectin; laminin; entactin; MBP; fibrinogen; fibrin; plasminogen
MMP-13	Collagenase-3	*	11q22.3	Collagens (I, II, III, IV, IX, X and XIV); gelatin; plasminogen; aggrecan; perlecan; fibronectin; osteonecting; MMP-9
MMP-14	MTI-MMP	*	14q11-q12	Collagens (I-III); gelatin; casein; fibronectin; laminin; vitronectin; entactin; proteo-glycans; MMP-2; MMP-13
MMP-15	MT2-MMP	*	16q12.2-q21	Fibronectin; entactin; laminin; perlecan; MMP-2
MMP-16	MT3-MMP	*	8q21	Collagen III; gelatin; casein; fibronectin; MMP-2
MMP-17	MT4-MMP	*	12q24	Unknown
MMP-19	RASI-1	*	12q14	Gelatin; aggrecan; fibronectin
MMP-20	Enamelysin	*	Unknown	Amelogrenein; aggrecan
MMP-21		*	1p36.3	Unknown
MMP-22		*	1p36.3	Unknown
MMP-23		*	Unknown	Unknown
MMP-24	MT5-MMP	*	20q11.2	Unknown
MMP-25	Leucolysin / MT6-MMP	*	16p/3.3	Pro-gelatinase A
MMP-26	Endometase	*	Unknown	Gelatin Iα, P1

* EC number not assigned.

Abbreviations:
PUMP: Putative metalloproteinase
RASI-1: Name of gene encoding MMP19
IL-1β: Interleukin-1β
PI: Protein inhibitor
MBP: Myelin basic protein

The activity of MMPs is controlled both through activation of pro-enzymes and inhibition by endogenous inhibitors, such as the TIMPS (Tissue Inhibitors of Metallo-Proteinases). There are currently four known TIMPs in humans which all bind tightly to most MMPs. Interestingly, only TIMP-3 is a good inhibitor of TACE.
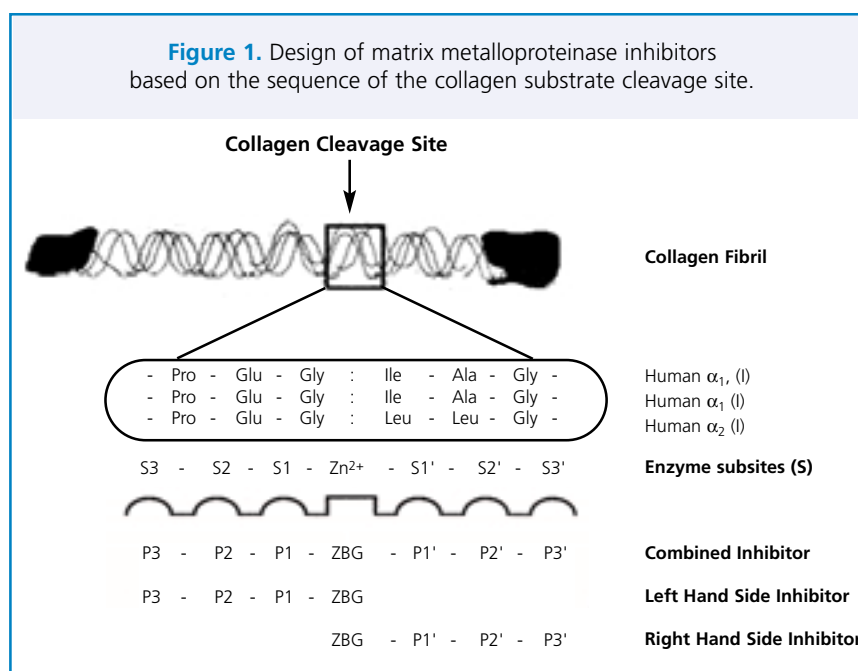
MMPs in Disease

Inappropriate expression of MMP activity constitutes part of the pathogenic mechanism associated with a wide range of diseases. These include; the destruction of cartilage and bone in rheumatoid and osteoarthritis [1,23], tissue breakdown and remodelling during invasive tumor growth and tumor angio-genesis [24], degradation of myelin-basic protein in neuroinflammatory diseases [25], opening of the blood-brain barrier following brain injury [26], increased matrix turnover in restenotic lesions [27], loss of aortic wall strength in aneurysms [28], tissue degradation in gastric ulceration [29], liver fibrosis [30], breakdown of connective tissue in periodontal disease [31], acute lung injury and acute respiratory distress syndrome [32]. As the role of MMPs in disease has become better understood, interest in the control of their activity has increased. However, which MMPs are involved in which diseases remains a key question? The issue to date is that disease association studies have tended to be limited by the lack of appropriate research tools (for example, anti-bodies) and the fact that not all MMPs have been investi-gated. However, what is clear is that MMPs are often over expressed in "gangs" in diseases such as cancer. In time, MMP disease association will presumably be effectively addressed by the new high-throughput biology technologies of expression profiling and proteomics [23,24]. A clear disease association has been established for MMP-12 in the development of emphysema that results from chronic inhalation of cigarette smoke [33].

In vivo, the degradative actions of the MMPs are limited by the TIMP family of natural macromolecular inhibitors [34]. Therefore, in disease association studies, it is also necessary to determine the relative levels and inhibitory effects of the four TIMPs (numbered TIMP-1 through TIMP-4).

Matrix Metalloproteinase Inhibitors

The most important structural requirement of an MMP inhibitor is a zinc binding group (ZBG) capable of chelating the active-site zinc (II) ion. The discovery of early MMP inhibitors followed a substrate-based approach to inhibitor design from a knowledge of the amino acid sequence of human triple helical collagen at the site of cleavage by MMP-1 [4] (see Figure 1).

Figure 1. Design of matrix metalloproteinase inhibitors based on the sequence of the collagen substrate cleavage site.

Collagen Cleavage Site

- Pro -	Glu -	Gly	:	Ile	- Ala -	Gly -	Human α_1, (I)
- Pro -	Glu -	Gly	:	Ile	- Ala -	Gly -	Human α_1 (I)
- Pro -	Glu -	Gly	:	Leu	- Leu -	Gly -	Human α_2 (I)

S3 - S2 - S1 - Zn^{2+} - S1' - S2' - S3'   **Enzyme subsites (S)**

P3 - P2 - P1 - ZBG - P1' - P2' - P3'   **Combined Inhibitor**

P3 - P2 - P1 - ZBG   **Left Hand Side Inhibitor**

ZBG - P1' - P2' - P3'   **Right Hand Side Inhibitor**

Collagen Fibril

Consideration of the amino acid sequence to the right of the cleavage site led to the discovery of potent inhibitors such as BB-94 (**1**; see Figure 2). In general, much weaker inhibitors (for example **2**) have been identified through design strategies based on the amino acid sequence to the left of the cleavage site [4]. Related compounds may be used as affinity ligands for the purification of MMPs, e.g. Pro-Leu-Gly-Hydroxamate (Prod. No. **P 5298**).

In a study to select the best ZBG for inhibition of MMP-1, Castelhano and co-workers compared different ZBGs while keeping the rest of the inhibitor structure constant. Using this approach they arrived at the following preference: hydroxamate>>formylhydroxy-lamine>sulfhydryl>phosphinate>amino-carboxylate>carboxylate [35]. The hydrox-amate acts as a bidentate ligand with each oxygen an optimal distance (1.9-2.3 Å) from the active-site zinc (II) ion and the position of the hydroxamate nitrogen suggests that it is protonated and forms a hydrogen bond with a carbonyl oxygen of the enzyme backbone.

Much of the early medicinal chemistry in this area focused on a series of succinyl hydroxamates [4]. Modification of sidechains from the early leads led to compounds suitable for *in vivo* evaluation, and later for clinical studies. Our own research program led to the discovery of BB-94 (**1**), BB-1101 (**3**) and later BB-2516 (**4**) (see Figure 2) [36]. BB-94 possesses a thienylthiomethylene α-substituent and BB-1101 features a smaller allyl α-substituent while the α-substituent for BB-2516 is a hydroxyl group [36]. All three compounds are broad spectrum inhibitors that have displayed efficacy in animal models of human disease (see below). From analysis of the X-ray crystal structure of succinate based hydroxamates, the possibility of joining the P1 and P2' side chains together to form cyclic inhibitors has been investigated [37]. These studies resulted in the identification of compounds such as SE205 (**5** see Figure 2), which possess similar potency to the uncyclised analogues [37]. X-ray crystallographic analysis indicates that the S1' pocket is short for MMP-1 and MMP-7, but is longer for MMP-2, MMP-3,

MMP-8 and MMP-13 [6]. Thus, small P1' groups generally give broad spectrum activity while longer P1' groups tend to provide selectivity for the deep pocket enzymes over the short pocket enzymes. Truncation of the P2'-P3' group of pseudo-peptide succinyl hydroxamic acid derivatives with small P1' groups leads to MMP inhibitors selective for the collagenases, e.g. Ro 32-3555 (**7**, see Figure 2) [38].

N-Sulfonyl amino acid hydroxamates have been identified as inhibitors of MMPs and the first such compound to enter development was the orally-available, broad-spectrum inhibitor, CGS 27023A (**8**, see Figure 2) [39]. AG3340 (**9**, see Figure 2), is a derivative of CGS 27023A, that was selected for develop-ment based on efficacy in a murine model of cancer growth and metastasis [40]. The preparation of analogs of AG3340 has been a very fertile area for inhibitor design with different strategies for cyclization between the position alpha to the hydroxamic acid, and the sulfonamide nitrogen being explored [5]. A new class of recently identified MMP inhibitor is aryl hydroxamates such as **10** (see Figure 2) and the corresponding heterocyclic analogs [7]. It will be interesting to see if these compounds progress through the clinic given the known mutagenic properties of aryl hydroxamic acids.

Due to the intense competition in the area of hydroxamic acid MMP inhibitors, there has been considerable interest in compounds with alternative zinc binding groups (ZBGs). The most successful to date are carboxylic acid and thiol ZBGs. Although the carboxylate group is a less effective binding group towards zinc, many carboxylates are effective MMP inhibitors and some appear to have promise as clinical candidates. A substituted derivative of the anti-inflammatory drug Fenbufen was identified using high-throughput screening and provided the starting point for a series of carboxylic acid MMP inhibitors such as the biphenyl derivative BAY 12-9566, **11** (see Figure 2) [41].

Antibodies to Matrix/Tissue Inhibitors of Metalloproteinase

PROD. # ANTIBODY

Monoclonal Antibodies

M 6427 Anti-MMP-1, Mab (Mouse), Clone 3A9.3
M 6552 Anti-MMP-3, Mab (Mouse), Clone 4B7.3
T 8687 Anti-TIMP-1, Mab (Mouse), Clone 2E7.1

Polyclonal Antibodies*

M 4302 Anti-MMP-1, C-Terminal
M 4053 Anti-MMP-1, Hemopexin Domain
M 4177 Anti-MMP-1, Hinge Region
M 4427 Anti-MMP-1, N-Terminal
M 3178 Anti-MMP-1, Propeptide Region
M 4428 Anti-MMP-2, C-Terminal
M 4552 Anti-MMP-2, Hemopexin Domain
M 4677 Anti-MMP-2, Hinge Domain
M 6302 Anti-MMP-2, N-Terminal
M 5928 Anti-MMP-2, Propeptide Region
M 4927 Anti-MMP-3, C-Terminal
M 4802 Anti-MMP-3, Hinge Region
M 5052 Anti-MMP-3, N-Terminal
M 6053 Anti-MMP-3, Propeptide Region
M 8308 Anti-MMP-7, Middle of Active Region
M 8808 Anti-MMP-7, N-terminal of Active Enzyme
M 8683 Anti-MMP-7, N-terminal of Catalytic Zinc Site
M 7429 Anti-MMP-7, Propeptide Region
M 8305 Anti-MMP-8, C-Terminal
M 5552 Anti-MMP-8, Hinge Region
M 8933 Anti-MMP-8, N-Terminal
M 8430 Anti-MMP-8, Propeptide Region
M 9555 Anti-MMP-9
M 5177 Anti-MMP-9, C-Terminal
M 5427 Anti-MMP-9, Hinge Region
M 5302 Anti-MMP-9, N-Terminal
M 0306 Anti-MMP-9, Propeptide Region
M 0431 Anti-MMP-11, C-Terminal
M 9058 Anti-MMP-11, Hinge Region
M 0806 Anti-MMP-11, N-Terminal
M 2556 Anti-MMP-11, Propeptide Region
M 0807 Anti-MMP-12, C-Terminal
M 6056 Anti-MMP-12, Hinge Region
M 4057 Anti-MMP-12, N-Terminal
M 6057 Anti-MMP-12, Propeptide Region
M 6307 Anti-MMP-13, C-Terminal
M 4052 Anti-MMP-13, Hinge Region
M 4683 Anti-MMP-13, N-Terminal
M 4933 Anti-MMP-13, Propeptide Region
M 3927 Anti-MMP-14, Hinge Region
M 5808 Anti-MMP-14, N-Terminal
M 9933 Anti-MMP-14, Propeptide Region

PROD. # ANTIBODY

M 8183 Anti-MMP-16, Hinge Region
M 3684 Anti-MMP-17, N-Terminal
M 4934 Anti-MMP-18, C-Terminal
M 5059 Anti-MMP-18, Hinge Region
M 5184 Anti-MMP-18, N-Terminal
M 5309 Anti-MMP-19, C-Terminal
M 5434 Anti-MMP-19, Hinge Region
M 5559 Anti-MMP-19, N-Terminal
M 5684 Anti-MMP-19, Propeptide Region
M 5809 Anti-MMP-20, C-Terminal
M 9183 Anti-MMP-20, Hinge Region
M 5934 Anti-MMP-20, N-Terminal
M 6059 Anti-MMP-20, Propeptide Region
M 6184 Anti-MMP-23, C-Terminal
M 6309 Anti-MMP-23, Hinge Region
M 6434 Anti-MMP-23, N-Terminal
M 6559 Anti-MMP-24, Cytoplasmic Domain
M 6684 Anti-MMP-24, Hinge Region
M 6809 Anti-MMP-24, N-Terminal
M 6934 Anti-MMP-24, Propeptide Region
T 8187 Anti-TIMP-1, C-Terminal
T 7562 Anti-TIMP-1, Third Loop
T 4918 Anti-TIMP-1, First and Second Loop
T 7937 Anti-TIMP-2, C-Terminal
T 8062 Anti-TIMP-2, First Loop
T 5160 Anti-TIMP-2, Third Loop
T 7812 Anti-TIMP-3, C-Terminal
T 7687 Anti-TIMP-3, First Loop
T 5285 Anti-TIMP-3, Third Loop
T 8312 Anti-TIMP-4, C-Terminal
T 8537 Anti-TIMP-4, First Loop
T 0913 Anti-TIMP-4, Second Loop
T 9289 Anti-TIMP-4, Third Loop

Proteins

M 2928 MMP Control-1
M 3303 MMP Control-2
M 1802 MMP-1 (Human)
M 1927 MMP-2 (Human)
M 1677 MMP-3 (Human)
M 4809 MMP-9 (Human)
M 1552 MMP-9 (Mouse)

Other Products

A 6671 Actinonin
D 9891 Doxycycline
M 9511 Minocycline
P 5298 Pro-Leu-Gly-Hydroxamate

* Host: Rabbit

Thiols are an attractive ZBG for incorporation in MMP inhibitors since, although the intrinsic affinity of a monodentate thiol ZBG is less than that of the bidentate groups, such as carboxylate or hydroxamate, lower dissolution costs and easier ionization tend to make such inhibitors only slightly less potent than the hydroxamate class of MMP inhibitors. Baxter et al. have identified a series of inhibitors incorporating a mercaptoacyl ZBG [42], including D2163 (**12**) and D1927 (**13**, see Figure 2) both of which have entered clinical development. While the above ZBGs have led to the discovery of potent inhibitors of MMPs, there is a clear challenge to identify alternatives which may provide compounds with improved pharmacokinetic and toxicological profiles and avoid an increasingly crowded patent area. Recent progress in this area is highlighted by the thiadiazole (**14**), a selective inhibitor of MMP-3 [43], the pyrimidine-2,4,6-trione (**15**) [44] and the MMP-2 selective thiirane (**16**) [45] which was designed using a mechanism based approach (see Figure 2).

Miscellaneous Natural Products

Screening has led to the discovery of both synthetic and natural product MMP inhibitors. The latter include tetracyclines such as doxycycline (Prod. No. **D 9891**) and minocycline (Prod. No. **M 9511**), for which it has been found that chemical modification can separate MMP activity from antibiotic activity [46], i.e. CMT-1 (**17**). Actinonin (Prod. No. **A 6671**; **18**, see Figure 2) [47] has been identified as an MMP inhibitor and is a suc-cinyl hydroxamic acid that bears close structur-al similarity to compounds obtained by sub-strate based design. Although significant advances have been made in inhibitor design, it is still not clear how to design compounds that specifically inhibit individual MMPs in spite of the available structural data. This remains a major challenge for MMP inhibitor medicinal chemistry.

Preclinical and Clinical Evaluation of MMP Inhibitors

MMP inhibitors have been evaluated in a wide range of animal models of human disease, and a number of 'second generation' orally-bioavailable MMP inhibitors have been evaluated in the clinic, including BB-2516 (**4**), BAY 12-9566 (**11**), AG3340 (**9**), CGS-27023A (**8**), D2163 (**12**) and Ro 32-3555 (**7**). The principal indications for which MMP inhibitors have been evaluated in the clinic are arthritis and cancer. A key issue with several of these agents is the dose limiting side effect of musculoskeletal pain and inflammation (observed with both BB-2516 and AG3340). The mechanism underlying this effect is poorly understood, but it is reversible and can be managed in the clinic by "drug holidays" [48,49].

Degradation of the extracellular matrix is a characteristic of both rheumatoid arthritis and osteoarthritis [1]. Adjuvant arthritis is an arthritis-like syndrome that can be induced in rodents following injection with Freund's complete adjuvant and a number of MMP inhibitors exhibit efficacy in this model. BB-94 (**1**) given intraperitoneally from onset of symptoms significantly reduced paw edema, bone degradation and cartilage breakdown [50]. BB-1101 (**3**) given orally also reduced paw oedema and bone degradation [51]. The reduction in paw swelling was attributed in part to the anti-inflammatory effect of inhibiting TACE [51]. The relative contributions of MMP inhibition and TACE inhibition will remain unclear until compounds that are specific for TACE and MMPs are tested. A thiol MMP inhibitor that also inhibits TACE was orally active in the adjuvant arthritic rat model [42]. The sulfonamide hydroxamate CGS 27023A (**8**) inhibited cartilage proteoglycan loss in the rabbit following direct injection of stromelysin into the knee joint [39]. The collagenase selective compound Ro 32-3555 (**7**) inhibited the degradation of articular carti-lage in a rat monoarthritis model induced by an intra-articular injection of *Propioni-*

bacterium acnes [52]. Interestingly, Ro 32-3555 did not show an effect in an adjuvant arthritis model [52]. However, the compound inhibited both cartilage and bone changes in a mouse model of osteoarthritis [53]. Ro 32-3555 was in development as a potential treatment for rheumatoid and osteoarthritis. The compound was reported to be well tolerated in rheumatoid arthritis patients at all doses tested (25-150 mg) for over 28 days with no evidence of musculoskeletal-related events [54]. However, development has been subsequently discontinued due to an unfavorable risk-benefit profile. Phase I trials have been initiated with BB-2827, an MMP inhibitor targeted for the treatment of arthritis. This compound is free of tendonitic liability in animal studies and, in contrast to Ro 32-3555, exhibits efficacy in an adjuvant arthritis model. The thiol derivative D1927 is also being developed for arthritis by Celltech Chiroscience, and Roche Biosciences are developing a "deep pocket selective" MMP inhibitor, RS-130,830, for osteoarthritis [54]. For chronic disease-modifying therapy for treatment of arthritis, it is clear that an MMP inhibitor must be free of side effects. Thus, the results of the trials of these structurally diverse compounds will be of great interest.
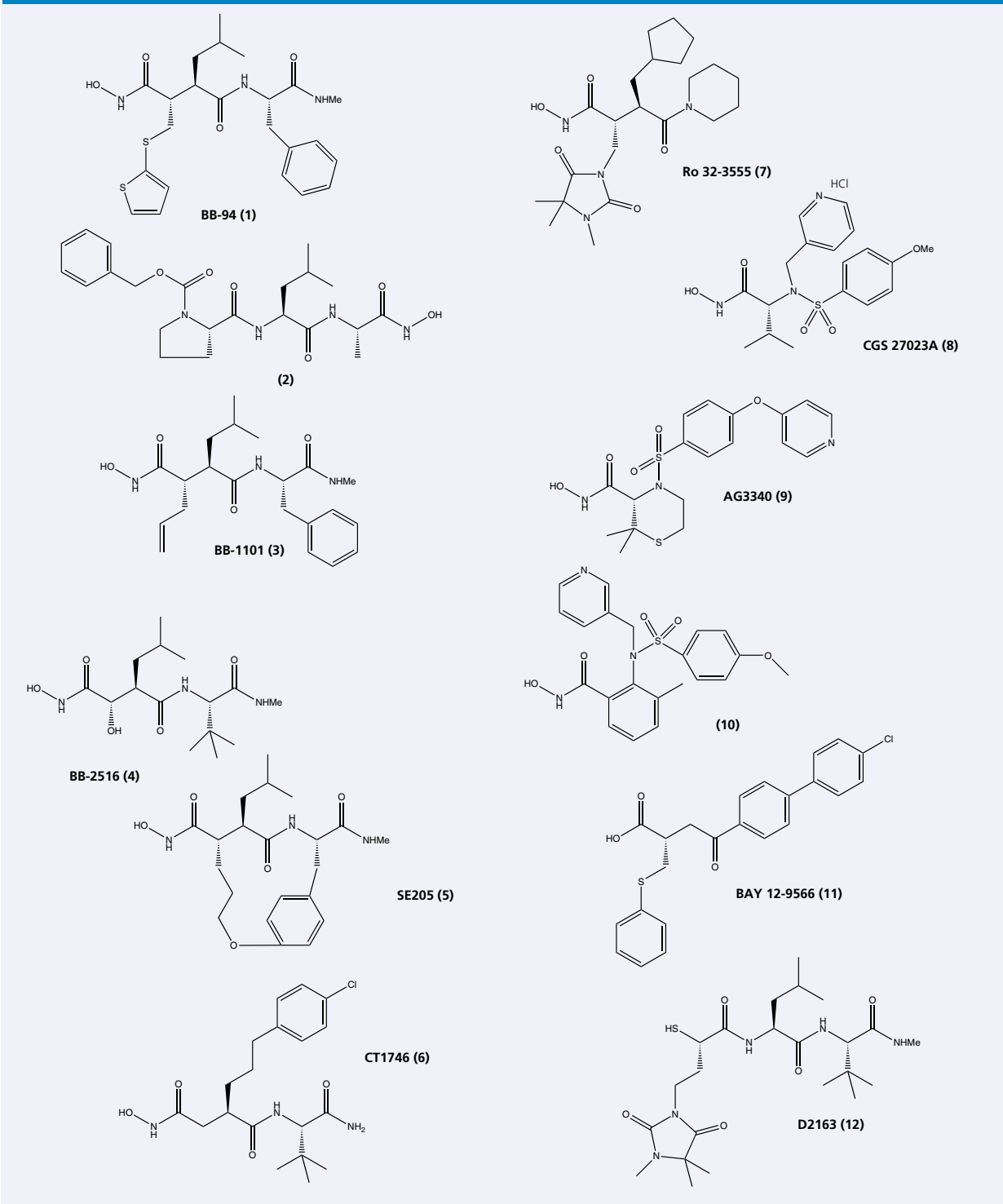
The ability of MMP inhibitors to restrict invasive tumor growth and metastasis has been demonstrated in a wide variety of animal cancer models. In a rat mammary carcinoma model, effective suppression of micro-metastatic disease has been demonstrated with the broad spectrum MMP inhibitor BB-94 (**1**) [55]. Other studies have shown inhibition of the growth of human carcinomas established as xenografts either subcutaneously or by orthotopic implantation in the tissue of the human primary site. Both BB-94 (**1**) [56] and the 'selective' inhibitor CT1746 (**6**) [57] have been shown to inhibit the local invasive growth and spread of orthotopically implanted human colorectal carcinoma. BB-94 was shown to inhibit the local regrowth of MBA-MD-435 human breast carcinoma following resection in nude mice [58], and to reduce
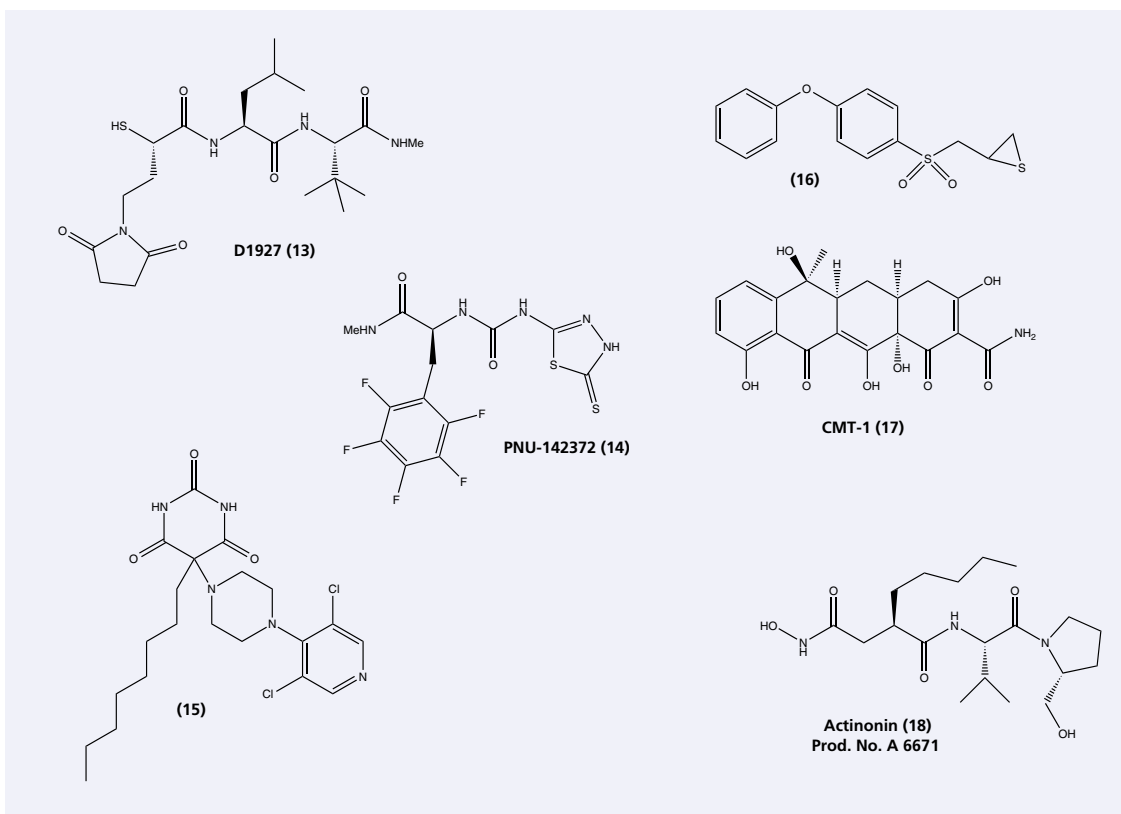
tumor growth and prolong survival in a xenograft model of human pancreatic cancer [59]. The "deep-pocket" MMP selective inhibitor AG3340 (**9**) has shown a range of effects in animals including inhibition of tumor growth in models of Lewis lung carcinoma and human non-small cell lung cancer [40]. The "deep-pocket" selective inhibitor BAY 12-9566 (**11**) has shown similar activity in models of Lewis lung carcinoma and B16 melanoma [41]. Both broad spectrum (BB-94) [60] and "selective" MMP inhibitors (BAY 12-9566) [41] have shown anti-angiogenic activity when tested in a Matrigel implant model.

A number of MMP inhibitors have been and are being evaluated in cancer clinical trials. Since this class of agent is non-cytotoxic, conventional oncology measures, such as cytoreductive tumor responses, could not be used to establish activity. In early trials with BB-2516, cancer antigens were used as surrogate markers of disease stabilization. Using this approach, a combined analysis of a series of six similarly designed phase II trials has revealed a dose-dependent reduction in the rate of rise of these serum markers [48,49]. In a separate study, patients with advanced gastric cancer were examined endoscopically before and after 4 weeks treatment with BB-2516 given at 25 mg once daily or 50 mg twice daily. Treatment at both doses was shown to be associated with changes in the macroscopic and histological appearance of the tumors consistent with an increase in the quantity of fibrotic stromal tissue. The changes were very similar to those seen in various cancer models and several of the patients appeared to benefit from these alterations in tumour/stroma ratio. In a Phase III trial of BB-2516 in patients with advanced gastric cancer there was a modest, but not significant, improvement in survival in patients treated with BB-2516 as compared to placebo [49].

In other trials reported to date for BB-2516, the clinical end-points have not been met and a dose-limiting "tendinitic" side effect has been observed. Trials with AG3340 have been halted in patients with advanced malignancy

Figure 2. Structures of Matrix Metalloproteinase Inhibitors

BB-94 (1)

(2)

BB-1101 (3)

BB-2516 (4)

SE205 (5)

CT1746 (6)

Ro 32-3555 (7)

CGS 27023A (8)

AG3340 (9)

(10)

BAY 12-9566 (11)

D2163 (12)

D1927 (13)

(16)

PNU-142372 (14)

CMT-1 (17)

(15)

Actinonin (18)
Prod. No. A 6671

due to lack of efficacy, but trials in earlier stage disease are being expanded. Trials with BAY 12-9566 have been stopped since the compound performed worse than placebo in small cell lung cancer. Interestingly, BAY 12-9566 did not cause a 'tendinitic' side effect as observed with both BB-2516 and AG3340.

MMP inhibitors have also been studied in combination with cytotoxic chemotherapies in animal cancer models. In these studies, the anti-tumor effects appear to be additive without additional marked toxicity. On the basis of these experimental results, and from theoretical considerations, it would seem that the use of MMP inhibitors in combination with chemotherapies in patients with minimal or microscopic disease may be of benefit.

Conclusion

The treatment of human diseases through the inhibition of MMPs has been an area of great excitement in the pharmaceutical industry over the past decade. Clinical trials reported to date have been a disappointment in terms of efficacy and, in some cases, dose limiting side effects. However, the potential for an MMP inhibitor with good pharmacokinetic profile and appropriate MMP selectivity is such that the area will remain of great interest to the pharmaceutical industry. The challenge is to learn more about the complex interaction and balance of MMPs, both in disease states and in maintaining homeostasis, and to generate more structural information for the design of selective inhibitors with improved pharmaco-kinetic profiles and reduced toxicity.

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New Products for Cell Signaling & Neuroscience

APOPTOSIS

P 4359 Pifithrin-α
Reversible inhibitor of p53-mediated apoptosis and p53-dependent gene transcription such as cyclin G, p21WAF1 and mdm2 expression.

P 4236 Cyclic Pifithrin-α
Stable analog of Pifithrin-α, with similar biological activities and lower cellular toxicity.

CELL CYCLE

M 8515 Monastrol
Potent, cell-permeant inhibitor of mitosis.

ION CHANNELS

D 0439 δ-Dendrotoxin
Blocks outward K^+ current; blocks voltage-gated K^+ channels.

MULTI-DRUG RESISTANCE PRODUCTS

C 7803 **Cytochrome P450 Isozyme 1A2**
C 7817 **Cytochrome P450 Isozyme 2C9**
C 9195 **Cytochrome P450 Isozyme 2D6 , His Tagged**
C 9573 **Cytochrome P450 Isozyme 2E1**
C 3831 **Cytochrome P450 Isozyme 3A4**
N 5516 **NADPH-P450 Reductase**
C 1427 **Cytochrome b5**
S 1677 **Sulfotransferases 1A1*2**
S 8928 **Sulfotransferases 1A2*1**
S 6054 **Sulfotransferases 1A3**
S 8309 **Sulfotransferases 1E**
S 8434 **Sulfotransferases 2A1**
G 5524 **Glutathione Transferase Human Recomb A1-1**
G 4151 **Glutathione Transferase Human Recomb M1-1**
G 1902 **Glutathione Transferase Human Recomb P1-1**
U 7254 **UDP-Glycosyltransferase 1A1**
U 7379 **UDP-Glycosyltransferase 1A6**
U 7504 **UDP-Glycosyltransferase 1A7**
U 7629 **UDP-Glycosyltransferase 1A10**
U 8379 **UDP-Glycosyltransferase 1A3**
U 8504 **UDP-Glycosyltransferase 2B7**

NEUROPEPTIDES AND HORMONES

A 6583 Ala-Pro-Arg-Thr-Pro-Gly-Gly-Arg-Arg (S5)
Substrate for meiosis-activated myelin basic protein kinase (p44mpk).

A 2793 Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu
Specific substrate for casein kinase II.

G 3902 Ghrelin, Human
Endogenous ligand for the orphan growth-hormone secretagogue receptor which stimulates the release of growth hormone from the pituitary.

G 8903 Ghrelin, Rat
Endogenous ligand for the orphan growth-hormone secretagogue receptor which stimulates the release of growth hormone from the pituitary.

N 8138 Neuromedin U-25
Porcine spinal cord peptide which exhibits potent hypertensive effect and uterine smooth muscle stimulation in rats.

N 8648 Neuropeptide Y Antagonist (1229U91)
High affinity and selective Y_1 Neuropeptide Y receptor antagonist.

P 2235 Protein Kinase C Fragment 19-31 amide
Inhibitor of protein kinase C.

NEUROTRANSMITTERS

A 3595 AC915 oxalate
Selective sigma-1 ligand; useful for masking sigma-1 sites in sigma-2 binding assays.

B 4555 Benazoline oxalate
I_1 Imidazoline binding site agonist.

D 7938 DBO 83 dihydrochloride
Novel nicotinic acetylcholine receptor agonist.

D 1064 Dihydrokainic acid
Non-transportable L-glutamate and L-aspartate uptake inhibitor.

F 1041 FAUC73
Potent, selective nonaromatic D_3 dopamine receptor agonist.

G 3416 Guanidinyl-Naltrindole ditrifluoroacetate (GNTI)
Selective kappa opioid receptor antagonist.

H 8034 HE-NECA
A_{2A} and A_3 adenosine receptor agonist.

I 0154 IEM-1460
Selective inhibitor of a subtype of AMPA receptors that are Ca^{2+} permeable and lacking $GluR_2$ subunit; tool for functionally discriminating between AMPA receptors of different subunit composition.

L 8401 LE 300
Potent, selective D_1 dopamine receptor antagonist.

M 3184 MG 624 iodide
Selective antagonist at neuronal nicotinic αBgtx receptors containing the α7 subunit.

M 1809 MRS 1523
Selective A_3 adenosine receptor antagonist selective for rat.

M 7684 MRS 2159 trisodium salt
$P2X_1$ purinoceptor antagonist.

P 2607 PRE-084 hydrochloride
Sigma-1 agonist.

R 8900 Ro 8-4304 hydrochloride
Non-competitive, voltage-independent NR2B glutamate receptor antagonist.

M 5560 (S)-MAP4 hydrochloride
mGluR4, mGluR6 and mGluR7 metabotropic glutamate receptor antagonist.

For more details of these products, including key references and structures, visit our website at www.sigma-aldrich.com/cellsignaling.

ANTIBODIES FOR APOPTOSIS

A 7549 **Anti-Apoptosis-Inducing Factor** (AIF) (rabbit)
B 5897 **Anti-Bak** (rabbit)
D 7810 **Anti-DAXX** (rabbit)

ANTIBODIES FOR CELL CYCLE

P 1484 **Anti-p21WAF1/Cip1**, clone CP74 (mouse)
P 2859 **Anti-p300/CBP**, clone NM11 (mouse)
T 1192 **Anti-Thymine Dimer**, clone H3 (mouse)

ANTIBODIES FOR CELL STRESS

A 7674 **Anti-AOP1** (Antioxidant-like protein 1), clone AOP-38 (mouse)
C 0979 **Anti-Catalase**, clone CAT-505 (mouse)

ANTIBODIES FOR GENE REGULATION

A 4095 **Anti-phospho-ATF2** (phosphothreonine 69,71), clone AFT-22P (mouse)
D 7438 **Anti-DP2**, clone BC2 (mouse)
E 8901 **Anti-E2F1**, clone KH20 (mouse)
E 9026 **Anti-E2F1** (rabbit)
E 8776 **Anti-E2F2**, clone CC11 (mouse)
E 8651 **Anti-E3F3**, clone PG30 (mouse)
E 8526 **Anti-E2F4**, clone WUF3 (mouse)

ANTIBODIES FOR G PROTEINS

F 6925 **Anti-Farnesyl** (rabbit)

ANTIBODIES FOR ION CHANNEL RESEARCH

A 5560 **Anti-Aquaporin 1** (rabbit)
A 7310 **Anti-Aquaporin 2** (rabbit)
A 0303 **Anti-Aquaporin 3** (rabbit)
A 8310 **Anti-ASIC2** (rabbit)
B 4180 **Anti-BAPTA** (rabbit)
C 5105 **Anti-Calcium Channel** ($\alpha2/\alpha1$ subunit) (rabbit)
C 4980 **Anti-Calcium Channel** (cardiac $\alpha1C$ subunit) (rabbit)
C 8206 **Anti-Calcium Channel** ($\gamma2$ subunit) (rabbit)
C 9347 **Anti-Chloride Channel CLC-1** (rabbit)
C 7597 **Anti-Chloride Channel CLC-K** (rabbit)
H 2769 **Anti-HCN2** (rabbit)
P 3709 **Anti-Potassium Channel IsK** (rabbit)
P 6102 **Anti-Potassium Channel Kir2.1** (rabbit)
P 9978 **Anti-Potassium Channel Kir2.3** (rabbit)
P 0856 **Anti-Potassium Channel ROMK1** (rabbit)
P 0981 **Anti-Potassium Channel TASK-1** (rabbit)
P 1106 **Anti-Potassium Channel TASK-2** (rabbit)
S 0645 **Anti-Sodium Channel PN1** (Scn9a) (rabbit)
S 0438 **Anti-Sodium Channel Scn8a** (PN4) (rabbit)
T 8276 **Anti-TRPC1** (rabbit)
T 5067 **Anti-TRPC3** (rabbit)

ANTIBODIES FOR NEUROSCIENCE

G 1166 **Anti-Glutamic Acid Decarboxylase 65** (GAD65), clone GAD-6 (mouse)
C 4598 **Anti-Chapsyn-110** (PSD93) (rabbit)
P 5110 **Anti-Presenilin 1 [303-316]** (rabbit)
P 4985 **Anti-Presenilin 1 [31-46]** (rabbit)
S 1172 **Anti-Syntaxin 1** (rabbit)
S 5547 **Anti-Syntaxin 3** (rabbit)
S 9924 **Anti-Syntaxin 4** (rabbit)

ANTIBODIES FOR PHOSPHORYLATION

C 9603 **Anti-c-Cbl** (rabbit)
G 2791 **Anti-GRB2**, clone GRB-232 (mouse)
P 8859 **Anti-Protein Phosphatase 2 X/C Interior** (rabbit)
P 8984 **Anti-PTP** μ, clone SBK10 (mouse)
P 9109 **Anti-PTP-PEST**, clone AG25 (mouse)
P 7484 **Anti-Serine/Threonine Protein Phosphatase 1β** (rabbit)
P 7609 **Anti-Serine/Threonine Protein Phosphatase 1γ1** (rabbit)
P 8109 **Anti-Serine/Threonine Protein Phosphatase 2 A/A** (rabbit)
P 8234 **Anti-Serine/Threonine Protein Phosphatase 2 A/B α** (rabbit)
P 5359 **Anti-Serine/Threonine Protein Phosphatase 2 A/B γ** (rabbit)
P 8359 **Anti-Serine/Threonine Protein Phosphatase 2 A/B' pan 2** (rabbit)
P 8609 **Anti-Serine/Threonine Protein Phosphatase 2C** (rabbit)
P 8734 **Anti-Serine/Threonine Protein Phosphatase V/C** (rabbit)
S 1687 **Anti-SLP-76**, clone AS55 (mouse)
S 1562 **Anti-SSeCKS** (sheep)
V 7879 **Anti-VAMP** (sheep)

ANTIBODIES FOR RECEPTOR RESEARCH

E 2764 **Anti-Endothelin Receptor B** (rabbit)
G 4416 **Anti-GABA$_A$ Receptor** ($\alpha1$ subunit) (rabbit)
G 4291 **Anti-GABA$_A$ Receptor** ($\alpha3$ subunit) (rabbit)
P 3610 **Anti-P2X$_3$ Purinergic Receptor** (rabbit)
P 1231 **Anti-P2Y$_4$ Purinergic Receptor** (rabbit)
E 5017 **Anti-EDG-2, C-terminal** (rabbit)
E 1891 **Anti-EDG-3, N-terminal**, clone AS60 (mouse)
E 4141 **Anti-EDG-3, C-terminal**, clone AS61 (mouse)
E 3141 **Anti-EDG-4, N-terminal**, clone AS62 (mouse)
E 1767 **Anti-EDG-4, C-terminal**, clone AS63 (mouse)
E 4892 **Anti-EDG-5, N-terminal**, clone AS64 (mouse)
E 4767 **Anti-EDG-5, C-terminal**, clone AS65 (mouse)
V 1631 **Anti VIPR1**, clone AS58 (mouse)
V 6257 **Anti VIPR2**, clone AS69 (mouse)

continued from page 11

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- Chloride Channels
- Potassium Channels
- Sodium Channels

Intracellular Signaling Enzymes/Receptors
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New Product Highlights

Metrazoline and Benazoline: Potent and Selective Ligands at Imidazoline Binding sites

Although the molecular structure of imidazoline binding sites remains to be elucidated, there is much indirect evidence to suggest the existence of two subpopulations of these sites referred to as Imidazoline Type 1 (I_1) and Imidazoline Type 2 (I_2). I_1 binding sites bind both Clonidine (Prod. No. **C 7897**) and Idazoxan (Prod. No. **I 6138**), whereas I_2 binding sites are sensitive only to idazoxan [1]. I_1 binding sites are thought to couple through a G protein to the enzyme phospholipase C. This coupling generates diacylglycerol, phosphocholine, arachidonic acid and eicosanoids as second messengers [1]. I_2 binding sites are not coupled to G proteins and may modulate cellular activity by affecting both potassium channel function and monoamine oxidase activity [1].

Sigma-RBI is pleased to introduce Metrazoline (Prod. No. **M 5685**) and Benazoline (Prod. No. **B 4555**), two potent and selective imidazoline binding site ligands with affinities in the nanomolar range. In binding assays performed in rat liver using [^{3}H] idazoxan, benazoline and metrazoline displayed pK_i values of 8.82 and 9.55, respectively [2]. Moreover, both ligands show remarkable selectivity for imidazoline sites as compared with their activity at α_2-adrenoceptors [2]. *In vitro* data indicate that benazoline can modulate forskolin-stimulated cAMP accumulation in cell lines expressing I_1 binding sites [3].

Imidazoline binding sites may play a role in the regulation of blood pressure [1,4]. Recent evidence suggests that those imidazoline ligands with lower α_2-adrenoceptor activity serve as effective antihypertensive agents, while also eliciting fewer adverse side-effects [4]. Also, alterations in imidazoline binding sites have been implicated in a variety of CNS disorders, including depression, opioid addiction, glial tumors and neurodegenerative diseases, such as Huntington's disease and Parkinson's disease [5].

The improved selectivity of metrazoline and benazoline suggests that these compounds are unique and exciting new research tools with which to further investigate the pharmacology and physiological role(s) of these enigmatic binding sites.

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Ephrins: Regulators of Axonal Growth and Migration

Sigma-RBI has introduced several new Ephrin and Ephrin receptor antibodies to our cell signaling and neuroscience product line enabling researchers to better study axonal growth and pathfinding.

Ephrin receptor tyrosine kinases and their ligands, the ephrins, are important mediators of vascular and neural morphogenesis. As developing axons navigate through the nervous system, their growth and guidance is aided by a variety of environmental signals leading to changes in cellular behavior. Ephrins and Ephrin receptors are key environmental cues that guide a migrating neuron, acting as cell contact-mediated repellents that influence axon pathfinding.

The following antibodies to Ephrins and Ephrin receptors are now available from Sigma-RBI:

Antibodies to Ephrins:
E 7150 **Anti-Ephrin-A1, Mouse**
E 7275 **Anti-Ephrin-A4, Human**
E 7400 **Anti-Ephrin-A5, Human**
E 7525 **Anti-Ephrin-B2, Mouse**
E 7650 **Anti-Ephrin-B3, Human**

Antibodies to Ephrin Receptors:
E 6650 **Anti-Eph-A1, Human**
E 1026 **Anti-Eph-A2, Mouse**
E 6775 **Anti-Eph-A3, Mouse**
E 6900 **Anti-Eph-A4, Mouse**
E 7025 **Anti-Eph-B1, Rat**
E 5892 **Anti-Eph-B1 (EXT), Human**
E 6392 **Anti-Eph-B1 (SAM), Human**
E 7517 **Anti-Eph-B1 (CY), Human**

Structures of Metrazoline and Benazoline

Metrazoline oxalate
Prod. No. M 5685

Benazoline oxalate
Prod. No. B 4555
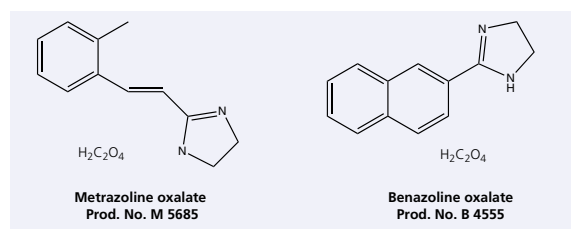
New Product Highlights

Pifithrin-α: A Reversible Inhibitor of p53-Dependent Transcription and Apoptosis

Mutations resulting in the inactivation or deletion of the p53 tumor suppressor gene are associated with a variety of tumors [1,2]. Genomic instability, rapid tumor progression and often refractivity to various cancer therapy modalities characterize this functional loss of p53. In animal models of p53 deficient mice, the frequency of spontaneous cancer is significantly increased. In this context, p53 is generally considered as an essentially beneficial check on cell proliferation and the loss of p53 function is viewed as an adverse event.

The role of p53 in normal tissues during radiation or chemotherapy may not be benign [3]. One of the causes of severe side effects in cancer therapy is p53-mediated apoptosis in normal tissues. This is consistent with the high level of expression of p53 in tissues that are most susceptible to the side effects of these cancer therapies. Some examples are hemopoietic organs, the immune system and gastro-intestinal epithelia. Thus, the temporary inhibition of p53 function in normal tissues is a potential therapeutic adjunct for reducing the toxicity of anticancer treatment for tumors that lack p53 [3,4]. Other potential clinical uses for p53 inhibitors include the control of ischemia-induced pathology in the heart and brain.

Pifithrin-α (PFT-α; Prod. No. **P 4359**) was described by Komarov, et al. [4] as a reversible blocker of p53-dependent apoptosis and transcription. PFT-α was shown to enhance survival in cells expressing wild type p53 after treatment with apoptosis-inducing agents such as doxorubicin (Prod. No. **D 1515**), etoposide (Prod. No. **E 1383**), cytocine arabinoside (Prod. Nos. **C 1768**, **C 6645**) and UV irradiation. This protection is lost in cells expressing the dominant negative p53 mutant gene or in p53-null mice. Conversely, protection is conferred by the transient expression of p53 in p53 deficient cell lines. PFT-α also inhibited the transactivation of p53-responsive genes after induction with doxorubicin and UV treatment. These included the expression of a LacZ-reporter gene under the control of a p53–responsive promoter and Cyclin G, p21/waf1 and mdm2, all known to be p53 responsive. Most importantly, the protective effect of PFT-α is transient and occurs in animal models without increasing.the incidence of cancer.

Pifithrin-α bromide
Prod. No. P 4359

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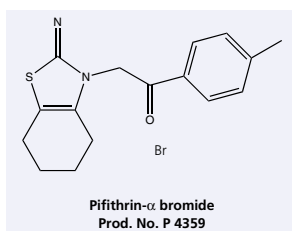
Monastrol: A Potent Inhibitor of Mitosis

Most small molecules that target the mitotic machinery interact with tubulin. Monastrol (Prod No. **M 8515**) differs in having no effect on microtubules. Monastrol was discovered by Mayer et al. [1] using a whole-cell immunoblot technique to detect phosphonucleolin as a marker for mitotic arrest. The mitotic phenotypes produced include chromosome misalignment, loss of spindle pole organization and alterations in spindle shape. In immunofluorescence staining, the normal bipolar spindle was replaced by a mono-astral microtubule array surrounded by a ring of chromosomes the observation for which the molecule was named. Monastrol is cell permeable producing reversible inhibition of mitosis with an IC_{50} of 14 mM in mammalian cells. *In vitro* studies indicate that monastrol inhibits Eg5-kinesin-driven microtubule motility. This inhibition is selective and does not affect non-kinesin-driven motility even though there is 55% homology between motor domains of Eg5 and conventional kinesins. This observation is correlated with the lack of effect on organelle localization in interphase cells.

Monastrol represents a new class of tools for the study of mitosis and may serve as a drug lead in anti-cancer therapy. It is also the only kinesin inhibitor that is cell permeable.

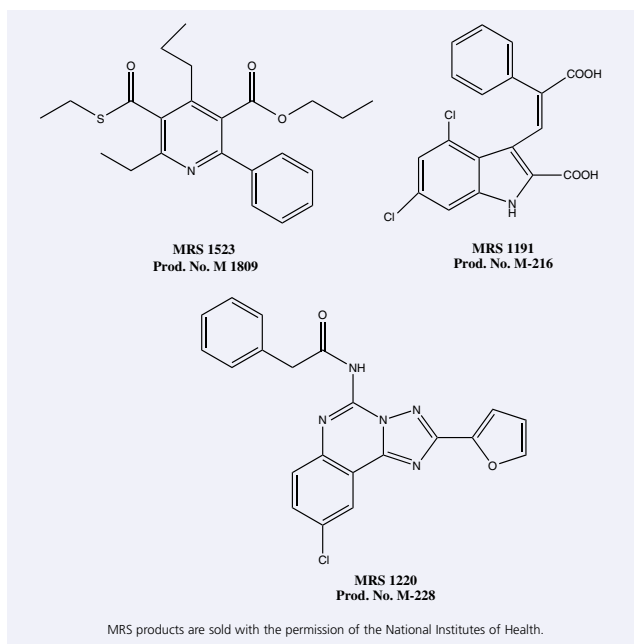1. Mayer, T.U. et al. *Science* **286**, 971-974 (1999).

Monastrol
**Prod. No. M 8515**
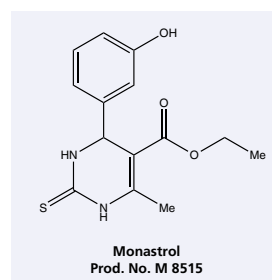
New Product Highlights

MRS 1523 and HE-NECA: New Ligands for Adenosine Receptor Research

Adenosine is known to mediate a wide variety of physiological effects through its interaction with four receptor subtypes referred to as A_1, A_{2A}, A_{2B} and A_3. All four subtypes are members of the G protein superfamily characterized by the presence of seven transmembrane spanning domains. Modulation of adenosine receptors by selective agonists and antagonists has potential for the treatment of various cardiovascular, inflammatory and neurological diseases. Accordingly, new selective adenosine receptor ligands are eagerly awaited.

A_3 adenosine receptor antagonists may be of use in treating inflammatory diseases. However, a persistent problem in developing A_3 antagonists has been the variation in affinity encountered between different species with the affinity at rat receptors generally being orders of magnitude lower than human receptors. MRS 1523 (Prod. No. **M 1809**) is the first relatively potent and selective A_3 adenosine receptor antagonist that is relatively selective for non-primate species, notably rat [1]. In radioligand binding studies, MRS 1523 displayed K_i values of 18.9 nM and 113 nM vs. human and rat A_3 receptors, respectively [2]. As such, MRS 1523 possesses advantages over MRS 1191 (Prod. No. **M-216**), which is selective for both human and rat A_3 receptors and MRS 1220 (Prod. No. **M-228**) which is A_3 selective only in humans [3].

MRS 1523
Prod. No. M 1809

MRS 1191
Prod. No. M-216

MRS 1220
Prod. No. M-228

MRS products are sold with the permission of the National Institutes of Health.

When designing agonists, substitution of the 2 position of NECA (Prod. No. **A-014**) with alkynyl chains results in a decrease in A_1 potency and a concomittant increase in potency at A_{2A} receptors [4]. Of a series of 2-substituted derivatives tested at rat receptors, HE-NECA (2-hexynyl-NECA; 2-hexyn-1-yl-adenosine-5'-ethyluronamide; Prod. No. **H 8034**) displayed 36-fold selectivity at A_{2A} vs. A_1 receptor, albeit that this selectivity was less pronounced at the human receptor subtypes. Although HE-NECA also possesses significant affinity at rat A_3 receptors [4], it should prove to be a useful tool for characterizing the effects mediated by A_{2A} adenosine receptors.

NECA
Prod. No. A-014

HE-NECA
Prod. No. H 8304
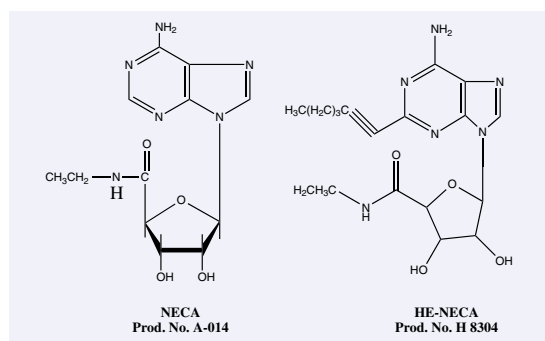
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Protein Kinase C Isoforms

In humans, at least 11 different PKC polypeptides have been identified. These isoforms include: α, βI, βII, γ, δ, ε, ζ, η, o, μ, and ι, which can be grouped into three subfamilies. The isoforms differ in their primary structure, tissue distribution, subcellular localization, mode of action *in vitro*, response to extracellular signals and substrate specificity. PKC α, βI, βII, and γ form the first family and their activities are Ca^{2+}- and phospholipid-dependent, while PKC δ, ε, η, and o comprise the second family and are Ca^{2+}-independent, but phospholipid-dependent. PKC ζ, μ, and ι form the third family and are not activated by either phorbol esters or diacylglycerol.

P 1782	**PKC** α	P 8538	**PKC** δ
P 1787	**PKC** β **I**	P 1164	**PKC** ε
P 3287	**PKC** β **II**	P 0194	**PKC** ζ
P 9542	**PKC** γ	P 0540	**PKC** η

Key
α (alpha), βI (beta I), βII (beta II), γ (gamma), δ (delta), ε (epsilon), ζ (zeta), η (eta), o (theta), μ (mu), ι (iota)

Nitroindolinyl-Caged L-Glutamate – A New Tool for Investigating Glutamate-Gated Ion Channels

John Corrie, David Ogden, Marco Canepari and George Papageorgiou

Reagents that release biologically-active compounds when subjected to a brief pulse of near-UV light (typically ≥ 300 nm) are colloquially termed caged compounds and have been available in increasing variety since the description of caged ATP in 1978 [1]. The range of bioeffectors that can be photo-released includes Ca^{2+}, nucleotides, amino acids, glucose, inositol trisphosphate and many others [2-5]. Important parameters of caged compounds are the rate and the efficiency of product release following the light pulse. It is unsatisfactory if the biological process being investigated is limited by the rate of liberation of the effector species from its caged precursor. Ideally, caged precursors need to also be resistant to hydrolysis so that release of the bioeffector occurs only as a result of the light flash.

Despite much effort from a number of research groups, reagents capable of releasing L-glutamate rapidly (i.e. on a sub-millisecond time scale) and efficiently from hydrolysis-resistant precursors have been elusive. Recently, we have described a new caged L-glutamate that is stable in aqueous solution ($t_{1/2}$ is 6 h for hydrolysis at pH 12, 30°C) and undergoes rapid photorelease ($t_{1/2} \leq 0.26$ ms) upon irradiation with a pulse of near-UV light [6]. This compound is a 7-nitroindolinyl amide derivative of the γ-carboxylate of L-glutamate and is referred to as NI-caged L-glutamate (Prod. No. **G 3291**; see Figure 1, Compound **1**).

With all caged compounds, it is necessary to assess whether the by-products of photolysis have any physiological effects. This can be best achieved using a related compound that has the same photochemistry, but does not release the relevant bioeffector. In the present study, a suitable control compound is the phosphate (Figure 1, Compound **2**), that releases inert 5-phosphonoxypentanoate upon photolysis, together with the same 7-nitroso-indole by-product. In any study with caged compounds, it is also important that the caged precursor is inactive in the physiological systems at the concentrations at which it is to be used. In the present case, the most likely side-effect would be that the NI-caged L-glutamate might bind to glutamate recep-tors and antagonize the actions of the L-glutamate released by photolysis. Other undesirable effects are also possible, for example, activity on presynaptic mechanisms.

The biological effects of L-glutamate released from NI-caged L-glutamate were tested on rat cerebellar granule and hippocampal neurons in primary culture and Purkinje neurons in brain slices. L-Glutamate, photoreleased onto ionotropic AMPA glutamate receptors present on cerebellar granule cells, produced currents that rose within the flash duration ($t_{1/2}$ 0.7 ms). Similarly, using appropriate antagonists, it was determined that ionotropic NMDA and metabotropic mGluR glutamate receptors were also selectively activated by L-glutamate released from NI-caged L-glutamate. The

Figure 1. Structures of NI-caged L-glutamate (1) and NI-caged 5-phosphonoxypentanoate (2). Irradiation of 1 with a pulse of near-UV light results in the rapid release of L-glutamate. Irradiation of 2 releases inert 5-phosphonoxy-pentanoate. The box indicates the photocleavable protecting group.
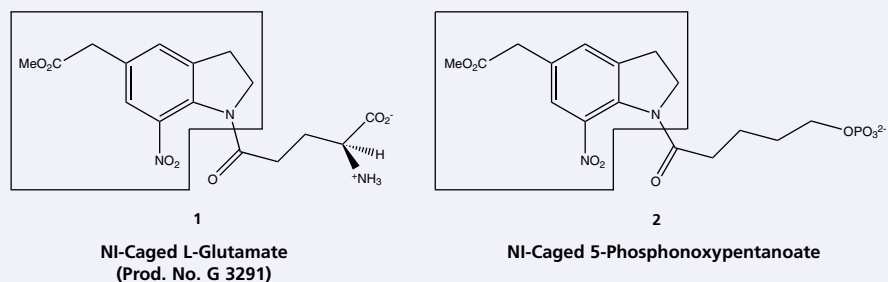
1
NI-Caged L-Glutamate
(Prod. No. G 3291)

2
NI-Caged 5-Phosphonoxypentanoate

Figure 2. Effects of NI-caged L-glutamate on a rat hippocampal pyramidal neuron in primary culture, voltage clamped at –80 mV. Two superimposed traces from the same neuron are shown following the release of 30 µM L-glutamate from both low (0.2 mM) or high (0.6 mM) concentrations of NI-caged L-glutamate. Photolysis by 1 ms near-UV pulse occurred at the time indicated by the upward artifact on the trace. The release of L-glutamate was limited to 30 µM by adjusting the flash intensity with a neutral-density filter. NI-caged L-glutamate was stored as a frozen 60 mM aqueous stock and diluted in Ringer as required. Precautions against UV exposure of NI-caged L-glutamate during microscopy were necessary, although these were easily achieved by using illumination wavelengths >450 nm.
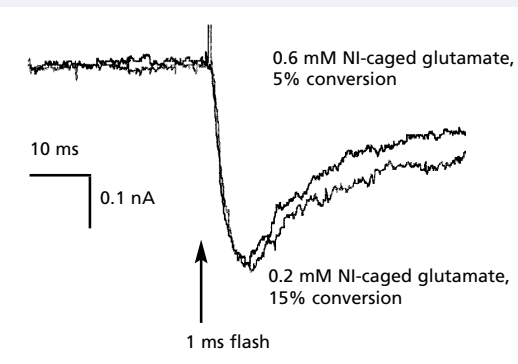
efficiency of photorelease was estimated as 15% when produced by a commercial xenon flash lamp coupled optimally into the microscope path. The light flash parameters (280-360 nm, 1 ms flash) were selected because they produce 40% photolysis of caged ATP under similar conditions [4].

Figure 2 shows the AMPA receptor-mediated effects of releasing 30 µM glutamate from either 0.6 or 0.2 mM NI-caged L-glutamate onto the same hippocampal neuron, voltage clamped at –80 mV. The flash intensity was adjusted to release the same concentration of L-glutamate. The similar amplitudes and time-courses of the superimposed responses show that the presence of the large excess of NI-caged L-glutamate had no effect on the receptors. In hippocampal pyramidal neurons, the rise-time of the current was slowed due to the activation of dendritically located receptors. To further assess possible adverse factors, NI-caged L-glutamate was tested rigorously for actions at glutamatergic synapses. No blockade of iontophoretic glutamate pulses was seen at AMPA or NMDA glutamate receptors in the presence of 1 mM NI-caged L-glutamate when tested on cultured granule cells or hippocampal pyramidal cells.

Similarly, there was no block of the metabotropic mGluR1 glutamate receptor tested on cerebellar Purkinje neurons in slices, and no effect on the response to climbing fiber stimulation or on the paired pulse depression at that synapse. Thus, NI-caged L-glutamate does not behave as an antagonist at these receptors and does not alter presynaptic release parameters. Further experiments indicated that photorelease of the inert control phosphate **2** had no effect on the responses to ionophoretically-applied glutamate at each receptor type, whether applied before, during or after the glutamate pulse. These data indicate a lack of effect of photolysis intermediates or products on glutamate receptors.

In summary, NI-caged L-glutamate is a stable and pharmacologically inert source of rapidly photoreleasable glutamate at the concentrations tested in this study (≤ 1 mM). It should prove to be a useful tool for both kinetic investigations of glutamate receptor activation and studies where spatial control of glutamate release is important.

NI-caged L-glutamate is manufactured and sold under license from the Medical Research Council, London, UK.

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About the Authors

John Corrie received his Ph.D. in organic chemistry in 1971 from the Australian National University. Since 1988, he has been at the National Institute for Medical Research (NIMR) in London in the Division of Physical Biochemistry. His research has centered on the development of new caged compounds and studies of their photolysis mechanisms, and on the development and application of reactive fluorophores for protein labeling.

David Ogden has worked in the field of ion channel physiology since 1971, firstly at University College, London then at Kings College, London. Since 1989, he has been at NIMR where his work with photolysis has been part of a collaborative program to develop photolytic techniques for application to intracellular ion channels and investigation of synaptic processes in the CNS.

George Papageorgiou and Marco Canepari are postdoctoral researchers at NIMR in organic synthesis and synaptic physiology, respectively.

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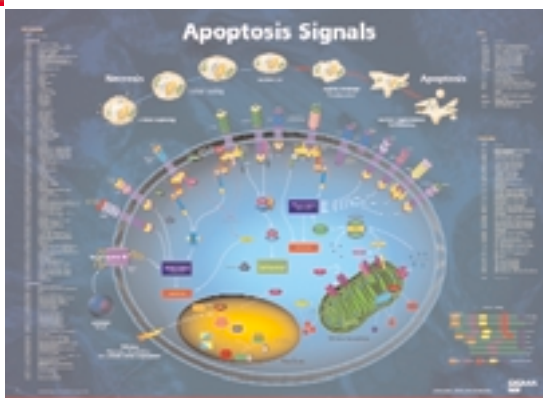
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