

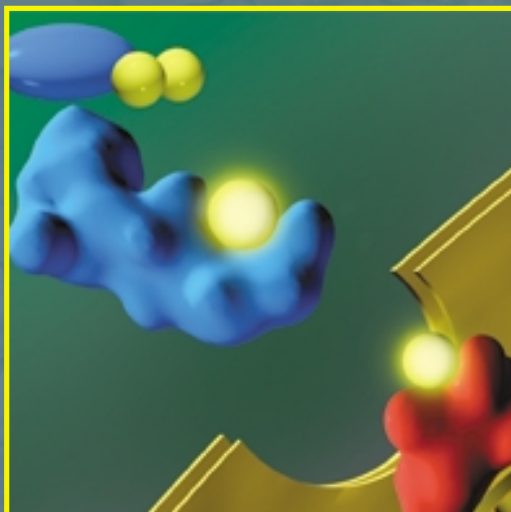
Celltransmissions

Newsletter for Cell Signaling and Neuroscience Research

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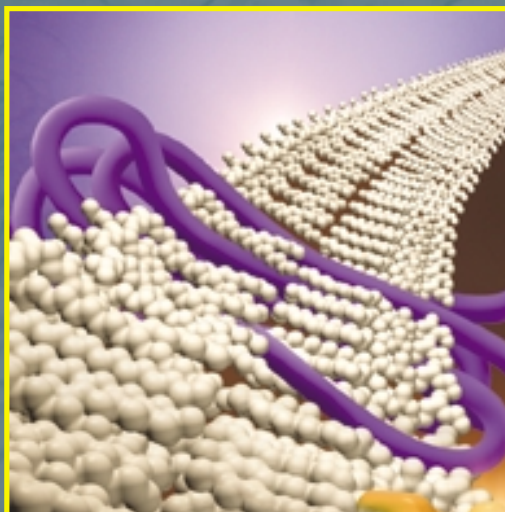
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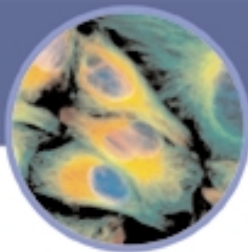
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Neurotransmitter Transporters: Two Busy Families

John Dunlop

Inactivation of the amino acid and biogenic amine neurotransmitters is achieved by their active removal from synapses by plasma membrane neurotransmitter transporters. Two distinct transporter gene families have been identified; the sodium-dependent glutamate transporter family and the sodium/chloride-dependent family encompassing the transporters for GABA (Prod. No. [A 2129](#)), glycine (Prod. No. [G 7403](#)), serotonin (Prod. No. [H 9523](#)), norepinephrine (Prod. No. [A 9512](#)) and dopamine (Prod. No. [H 8502](#)). Common to both families, substrate transport is driven largely by the energy of the sodium-electrochemical gradient and there is co-transport of sodium with substrate translocation. An important mechanistic difference between the two families is the counter-transport of potassium,

with respect to the functional properties of neurotransmitter transporters which go beyond the primary function of substrate binding and translocation. These include the dynamic regulation of the transporters by substrates (and other transporter ligands), phosphorylation and accessory proteins. This review will focus on the basic pharmacology, acute regulation, and identified protein:protein interactions of the neurotransmitter transporter family members. Finally, it should be noted that a number of transporters exhibit channel-like activity, distinct and uncoupled from the ionic requirements for substrate translocation, and potentially exist in monomeric and oligomeric forms. The functional significance of these two properties is not yet well understood.

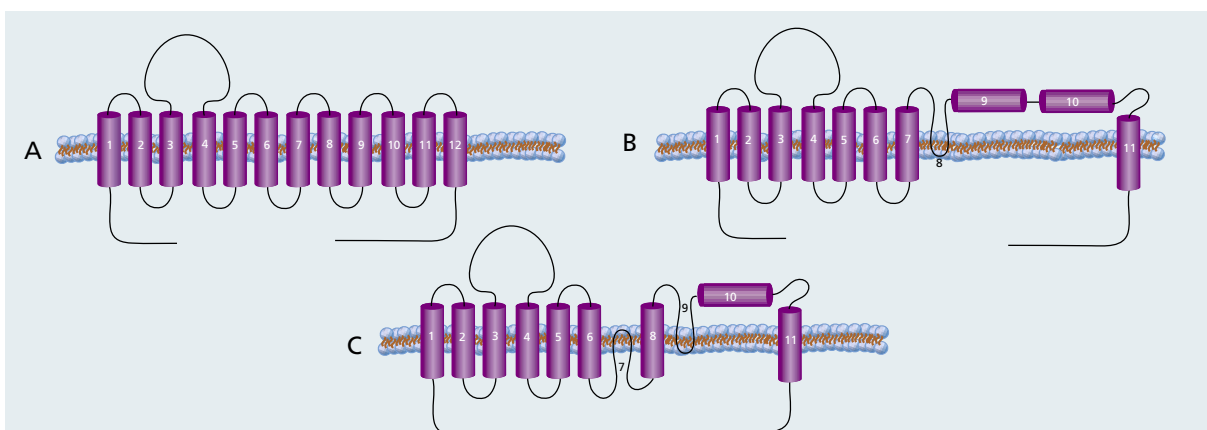


Figure 1. Hydropathy plots for the sodium/chloride-dependent family of transporters predicts 12 transmembrane (TM) spanning domains (A). Sites of potential N-linked glycosylation are localized to the second extracellular loop between TMs 3 and 4. Two distinct topology models for the glutamate transporters EAAT1 (B) and EAAT2 (C) have been proposed based on cysteine mutagenesis combined with accessibility determination using sulfhydryl modifying reagents. The model for EAAT1 (B) proposes 7 TM domains in the N-terminal half of the protein followed by a single re-entrant loop facing the extracellular face, and two linker domains placed on the extracellular face of the membrane by virtue of their high accessibility to membrane impermeant modifying reagents. The model for EAAT2 (C) proposes 6 TM domains in the N-terminal half of the protein followed by two re-entrant loops separated by a seventh TM domain with the first loop placed facing the cytoplasm and the second facing the extracellular face. This model also places a linker domain with high accessibility to membrane impermeant modifying reagents at the C-terminal end of the protein. Lastly, both models predict a final TM domain in the very distal C-terminus of the transporters. Models from references [1-3].

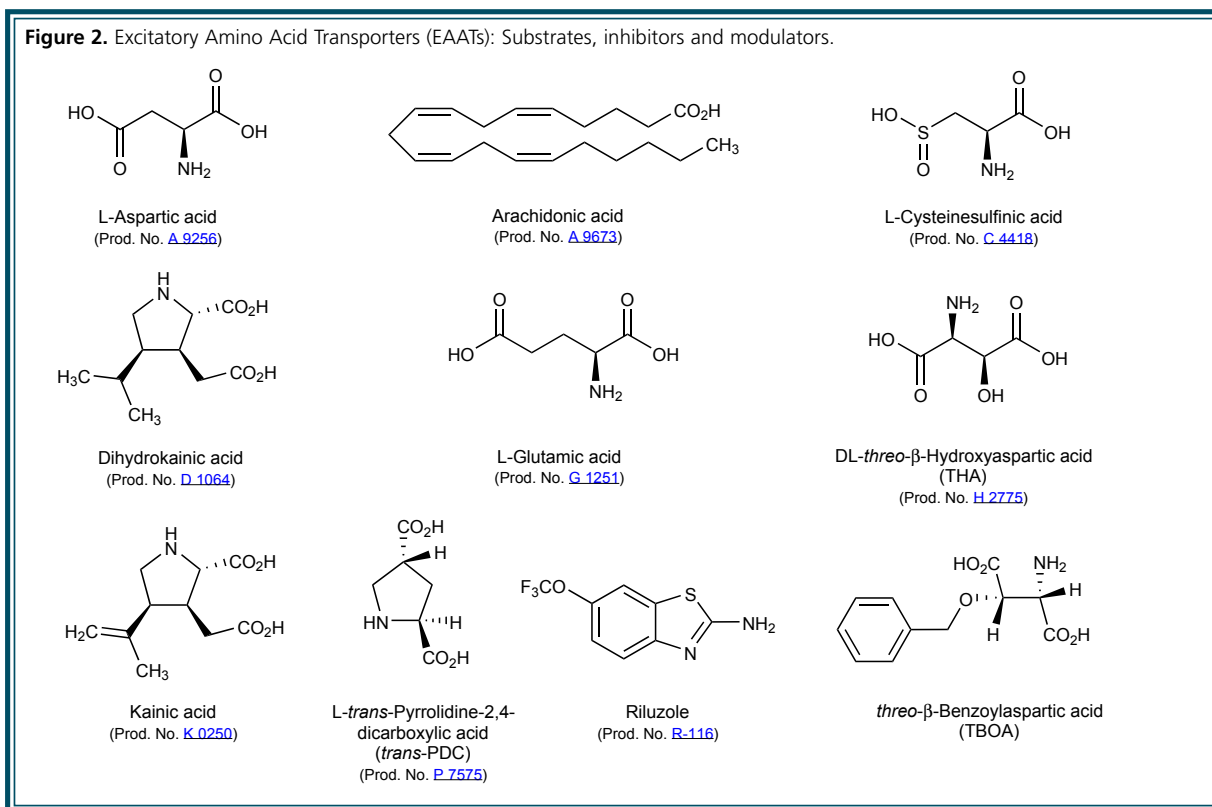
restricted to the glutamate transporters, and the co-transport of chloride, restricted to the sodium/chloride-dependent family. In addition, sequence homologies are high within each family, but are divergent between families. Moreover, the predicted membrane topology of the sodium/chloride-dependent family of transporters is consistent with 12 transmembrane-spanning domains (Figure 1A) [1]. However, there is controversy concerning the topology of the glutamate transporters; in particular, the C-terminal half of the protein (Figures 1B, 1C) [2,3]. Despite the diversity of substrates and the structural and mechanistic distinctions between families, several common family traits have emerged

About the Author

John Dunlop received his Ph.D. from the University of St. Andrews where he studied in the laboratory of Dr. Roger Griffiths and characterized the neurochemical properties of a number of sulfur-containing glutamic acid analogs. In 1992, he joined the Biochemical Pharmacology group at Wyeth Research in Taplow, UK where he established a number of *in vitro* neurotransmitter release and G protein-coupled receptor (GPCR) signaling assays. In 1995, he relocated to Wyeth-Ayerst (presently Wyeth Research) in Princeton, USA where he is currently a Principal Scientist in the Neuroscience division. He is currently responsible for a research group that focuses on drug discovery targets including GPCRs and glutamate transporters.

Neurotransmitter Transporters...(continued)

Figure 2. Excitatory Amino Acid Transporters (EAATs): Substrates, inhibitors and modulators.



Sodium-Dependent Glutamate Transporter Family: Excitatory Amino Acid Transporters (EAATs)

L-Glutamic acid (Prod. No. [G 1251](#)) is the predominant excitatory neurotransmitter in the mammalian central nervous system (CNS). Reuptake of glutamic acid is important not only for the termination of glutamatergic neurotransmission, but also for the maintenance of low extracellular glutamic acid levels below a threshold required to elicit excitotoxicity. The existence of multiple glutamate transporter subtypes was originally inferred by the heterogeneity of radio-labeled glutamate transport in synaptosomal preparations derived from various brain regions [4,5]. Almost simultaneously, three distinct glutamate transporters, referred to as GLAST (GLutamate/ASpartate Transporter), GLT-1 (GLutamate Transporter) and EAAC1 (Excitatory Amino Acid Carrier), were cloned [6-8], confirming the presence of multiple subtypes. Subsequently, human homologs for each of these subtypes were isolated and the designation excitatory amino acid transporter (EAAT) was adopted for the human clones EAATs 1-3, respectively [9]. Identification of two additional subtypes, EAATs 4 and 5 [10,11], completes the currently known siblings. The EAAT designation recognizes the multiple excitatory amino acid substrates including L-glutamic acid, L-aspartic acid (Prod. No. [A 9256](#)) and L-cysteine sulfinate (Prod. No. [C 4418](#)).

Pharmacological tools to discriminate between the EAATs are somewhat limited with only kainic acid (Prod. No. [K 0250](#)) and dihydrokainic acid (Prod. No. [D 1064](#)) available as selective inhibitors of the EAAT2 subtype [9]. Unfortunately, the use of the former compound as a selective EAAT2 blocker is limited by its ionotropic glutamate receptor activity. Of the five glutamate transporter subtypes, EAAT2, predominantly localized to astroglial cells, is the subtype commonly considered to represent the family quantitatively, both in terms of expression level and contribution to total uptake capacity. Generally speaking, the most frequently used uptake inhibitors, including L-trans-pyrrolidine-2,4-dicarboxylic acid (Prod. No. [P 7575](#)) [12], threo-hydroxyaspartic acid (Prod. No. [H 2775](#)) [13] and threo-β-benzoylaspartic acid (TBOA) [14], are essentially non-selective across the subtypes and therefore provide tools for the inhibition of total net uptake rather than the discrimination of individual transporter subtypes. One hallmark of EAAT2 is the enhancement of transport activity observed in the presence of arachidonic acid (Prod. No. [A 9673](#)) in both *Xenopus* oocytes and recombinant expression systems [15,16], although paradoxically, arachidonic acid decreases the transport activity of the purified transporter reconstituted in liposomes [17]. Recent data suggest that the glutamate transporter may be a target for the neuroprotective and anti-convulsant compound riluzole

Neurotransmitter Transporters...(continued)

(Prod. No. [R-116](#)). This agent, which possesses many potential modes of action including modulation of glutamate receptors and ion channel activity, has recently been shown to stimulate glutamate uptake in synaptosomes isolated from spinal cord [18].

Beyond the primary function of binding and translocation of substrate, glutamate transporters and their cousins in the sodium/chloride-dependent family are regulated at multiple levels, including by the substrate itself (and other transporter ligands), by phosphorylation via protein kinases and by their interaction with accessory proteins. These multiple levels of regulation may control the availability of functional transporters at the cell surface by both acutely affecting the transport activity and controlling their trafficking to and from the plasma membrane. The participation of the substrate L-glutamic acid in the regulation of glutamate transporters was first demonstrated for the GLAST (EAAT1) subtype where incubation of glial cultures in the presence of glutamic acid led to an increase in its cell surface expression and corresponding transport activity [19]. Similar regulation of EAATs 1-4 by glutamic acid has been observed, although increases in transporter activity following glutamic acid treatment are not always associated with increased cell surface expression of transporter protein [20].

Many studies have evaluated the effect of phosphorylation on the activity and cell surface expression of glutamate transporters, although conflicting results have arisen from many different laboratories. Generally, phorbol ester activation of protein kinase C (PKC) results in either an increase or decrease in transport activity which is sometimes associated with up-regulation or internalization of the transporter(s). GLAST is reported to be down-regulated following direct phosphorylation of the transporter protein after PKC activation with phorbol ester [21]. In the case of EAAT2, the data is most conflicting with reports of both PKC-mediated up- and down-regulation or even a lack of effect on both activity and cell surface expression [22-24]. Similarly, EAAC1 is rapidly up-regulated in C6 glioma cells following activation of PKC [25,26], yet it is down-regulated in *Xenopus* oocytes [27]. Up-regulation of EAAC1 in C6 glioma cells has also been reported in response to treatment with platelet-derived growth factor (PDGF, Prod. No. [P 8147](#)), suggesting that activation of tyrosine kinase and phosphatidylinositol 3-kinase (PI3K, Prod. No. [P 8615](#)) signaling cascades contribute to its regulation [28]. It seems clear that phosphorylation does play an important role in the regulation of glutamate transporters, although the use of different model systems has produced conflicting results, indicating that cellular context

has an impact on the outcome of such studies. With this in mind, it will be important to assess these aspects of regulation with transporters that have been expressed in a more native environment.

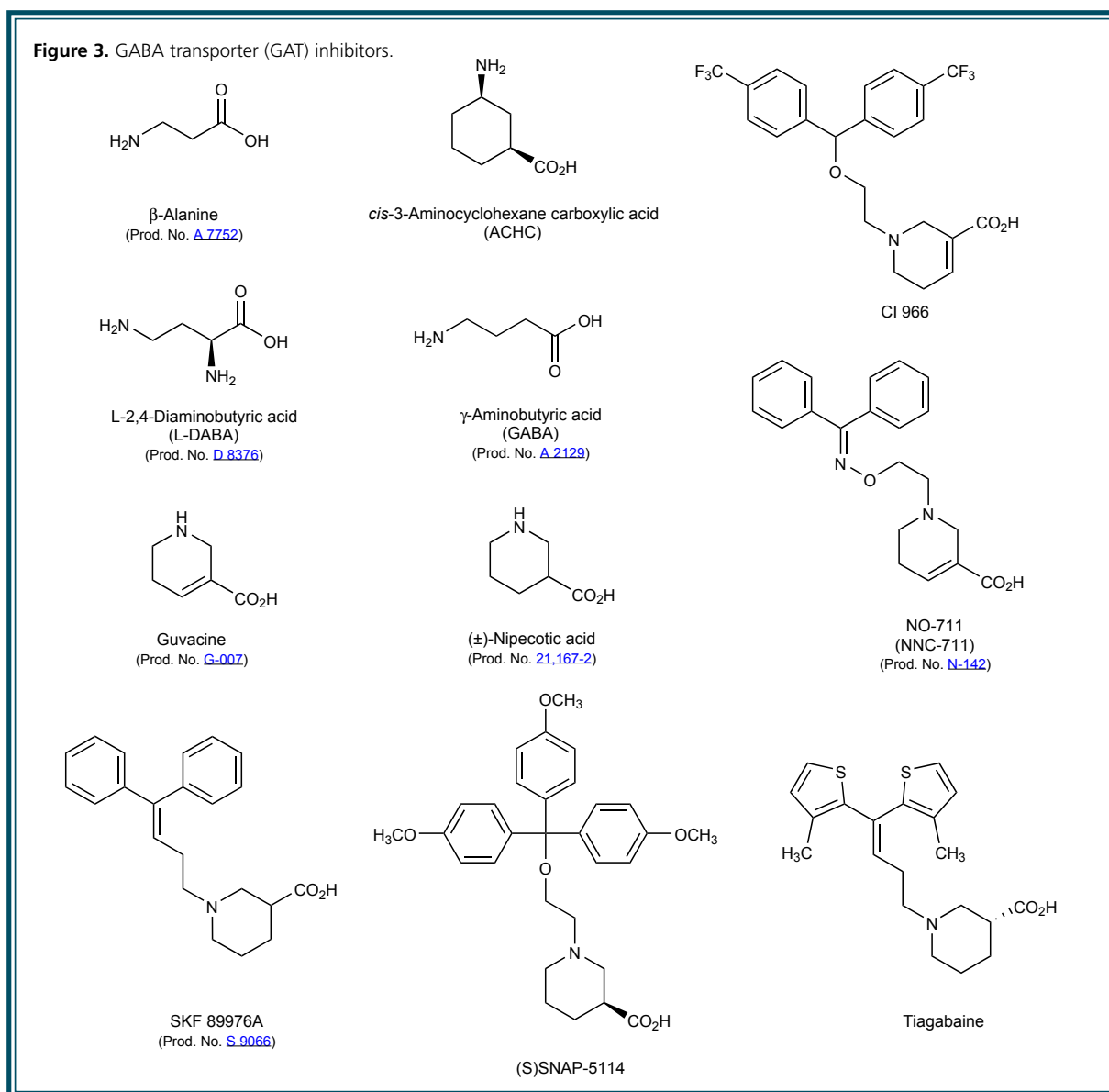
Recently, a number of glutamate transporter accessory proteins have been identified, adding further to the complexity of transporter regulation. Glutamate transport associated protein 3-18 (GTRAP 3-18) negatively regulates the functional activity of EAAT3 [29], while GTRAP41 and GTRAP48 are positive regulators of EAAT4, producing an increase in total EAAT4 transport capacity when co-expressed by a mechanism attributed to increased cell surface expression of the transporter protein [30]. The LIM protein Ajuba interacts with EAAT2 without influencing the functional transport activity and is proposed to act as a scaffolding or trafficking partner [31].

Sodium/Chloride-Dependent Family: GABA (γ -Aminobutyric Acid) Transporters (GATs)

GABA is the predominant inhibitory neurotransmitter in the mammalian CNS. Similar to the glutamate transporter family history, multiple GABA transporters were originally proposed based on the pharmacology and kinetics of GABA transport in a number of CNS derived preparations, particularly the differences between neuronal and astrocytic uptake [32]. Molecular cloning techniques have subsequently confirmed the existence of multiple GABA transporter subtypes. The GABA transporter, GAT-1, was the first neurotransmitter transporter to be cloned. The cDNA for GAT-1 was identified after purification of GABA transporter protein, partial amino acid sequencing and design of oligonucleotide probes to screen a rat brain cDNA library [33]. Subsequent, sequence homology screening has produced additional GABA transporter subtypes (in addition to those for glycine and biogenic amines as discussed below) including GAT-2 and GAT-3 [34], BGT-1, a betaine/GABA transporter [35] and TAUT, a taurine/GABA transporter [36]. The betaine and taurine transporters display high- and low-affinity for GABA, respectively.

GABA uptake into primary cultures of neurons and astrocytes has been shown to exhibit different pharmacological profiles based on the inhibitory potencies measured for a number of uptake blockers including nipecotic acid (Prod. No. [21.167-2](#)), guvacine (Prod. No. [G-007](#)), β -alanine (Prod. No. [A 7752](#)), 2,4-diaminobutyric acid (Prod. No. [D 8376](#)) and *cis*-3-aminocyclohexane carboxylic acid [32].

Neurotransmitter Transporters...(continued)



These agents and other more recently developed lipophilic derivatives with anti-convulsant properties, including tiagabine (NO-328), NNC-711 (NO-711, Prod. No. [N-142](#)), SKF-89976A (Prod. No. [S 9066](#)) and CI-966, have later been shown to exhibit selectivity for the GAT-1 subtype when studied in heterologous expression systems [37]. Agents selective for the other subtypes are limited, with the two nipecotic acid derivatives, NNC 05-2045 and NNC 05-2090, described as non-GAT-1 preferring based on synaptosomal uptake studies [38], and (S)-SNAP-5114 reported to display 4-fold selectivity for GAT-3 over GAT-2 [39].

Similar to EAAT2 in the glutamate transporter family, GAT-1 is the subtype commonly considered to represent the family quantitatively. Its regulation has been

studied in some detail revealing an important interaction between the neurotransmitter release and reuptake machinery. Phorbol ester treatment of *Xenopus* oocytes expressing GAT-1 induces an increase in the V_{max} for GABA transport, an effect attributed to the trafficking of intracellular transporters to the cell surface [40]. Syntaxin 1A, a protein present in the neuronal plasma membrane and involved in the docking and fusion of synaptic vesicles during the release process, interacts with GAT-1 as a positive regulator of its surface expression [41]. Paradoxically, syntaxin 1A negatively regulates GAT-1 functional transport activity, indicating that GAT-1 regulation by interaction with syntaxin 1A is complex. GABA appears to be involved in regulating this complex process since GABA transporter substrates up-regulate transporter

Neurotransmitter Transporters...(continued)

activity by a mechanism postulated to involve relieving the negative influence of syntaxin 1A on functional activity [42-45]. It is clearly a very exciting development to establish a partnership between a protein known to regulate release of the neurotransmitter and one responsible for its retrieval. GAT-2 and GAT-3 regulation has been documented in the context of targeted sorting in MDCK cells, with localization to the basolateral and apical surfaces, respectively [46]. Importantly, a C-terminal motif has been identified as a sorting determinant for GAT-2 and GAT-3, and in the case of GAT-3, a PDZ-like interaction domain has been noted, suggesting that protein:protein interactions might be important in the regulated expression of this subtype.

Glycine Transporters (GLYTs)

Glycine is a major inhibitory neurotransmitter in the spinal cord and brain stem, and a co-agonist of the NMDA subtype of excitatory glutamate receptor that is widely distributed throughout the CNS. Two glycine transporter subtypes have been cloned and designated GLYT1 and GLYT2 [47-50]. Tissue distribution studies support a correlation between GLYT1 expression with NMDA glutamate receptors and GLYT2 with the strychnine-sensitive inhibitory glycine receptor, suggesting the two distinct subtypes control the availability of glycine at its two principle sites of action. Pharmacological discrimination of GLYT1 and GLYT2 is achieved using sarcosine (N-methylglycine; Prod. No. [S 7672](#)) and the derivative ALX 5407 ((R)-(N-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine), both of which are selective for the GLYT1 subtype [51]. In addition, high concentrations of the tricyclic antidepressant amoxapine (Prod. No. [A-129](#)) inhibit GLYT2 to a greater extent than GLYT1, while doxepin (Prod. No. [D 4526](#)), amitriptyline (Prod. No. [A 8404](#)) and nortriptyline (Prod. No. [N 7261](#)) are equipotent at both subtypes [52].

Regulation of GLYTs has been less extensively characterized than for the EAATs or the GATs. The cloned GLYT1 transporter expressed in HEK cells is down-regulated following treatment with phorbol esters, indicating modulation by PKC [53]. In C6 glioma cells, the endogenous glycine uptake system is inhibited by treatment with arachidonic acid, although no similar reports have appeared with the cloned subtypes [54]. In common with the GAT-1 subtype, syntaxin 1A is an important binding partner for the glycine transporters [55]. Transient co-expression of either transporter with syntaxin 1A in COS cells resulted in a decreased capacity for glycine uptake. In synaptosomal preparations, depolarizing

conditions initiated cell surface trafficking followed by retrieval of GLYT2. Furthermore, the membrane insertion step was prevented when syntaxin 1A was inactivated by treatment with botulinum neurotoxin C (Prod. No. [B 1036](#)) [56]. These results suggest an important role once again for syntaxin 1A in the regulation of the neurotransmitter transporter life cycle.

Norepinephrine, Dopamine and Serotonin Transporters (NETs, DATs and SERTs)

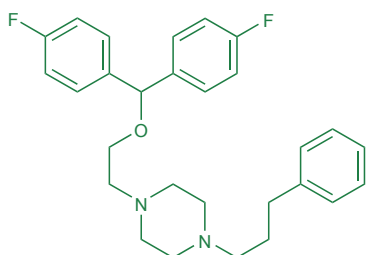
Unlike their amino acid relatives, a single transporter protein accounts for the reuptake of each of the biogenic amines norepinephrine, dopamine and serotonin. First to be isolated by expression cloning was the norepinephrine transporter (NET) [1], followed by the dopamine (DAT; Prod. No. [D-209](#)) [57,58] and the serotonin (SERT) [59] transporters using approaches combining both expression and homology cloning. Some degree of substrate promiscuity exists between these transporters, e.g., dopamine is also a substrate for NET while serotonin is also a substrate for DAT. A rich pharmacology exists for the biogenic amine transporters by virtue of their interaction with many drugs that are used in the treatment of psychiatric diseases and drugs of abuse such as cocaine (Prod. No. [C 5776](#)) and amphetamines (Prod. Nos. [A 5880](#), [A 1263](#)).

Pharmacologically, NET is characterized by its sensitivity to the tricyclic antidepressants desipramine (Prod. No. [D 3900](#)), nortriptyline (Prod. No. [N 7261](#)) and imipramine (Prod. No. [I 7379](#)), and its insensitivity to the serotonin reuptake inhibitor citalopram (Prod. No. [C 7861](#)) [1]. In comparison, potent inhibition by GBR 12909 (Prod. No. [D-052](#)) and mazindol (Prod. No. [M 2017](#); also a potent blocker of NET) are distinguishing pharmacological characteristics of DAT [57], while the selective serotonin reuptake inhibitors (SSRIs) fluoxetine (Prod. No. [F-132](#)), paroxetine, fluvoxamine (Prod. No. [F 2802](#)) and citalopram are potent blockers of SERT [59] and exert much weaker (or no) inhibitory activity on either NET or DAT. Compounds with mixed pharmacologies also exist, for example, venlafaxine, which blocks both NET and SERT and is used clinically to treat depression and generalized anxiety disorder. Cocaine and amphetamine interact with all three transporters with varying potencies exhibiting a preference, based on IC₅₀ values for inhibition of substrate transport, for NET followed by DAT and then SERT.

Neurotransmitter Transporters...(continued)

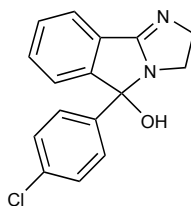
Figure 4. Biogenic amine transporter inhibitors.

DAT Preferring



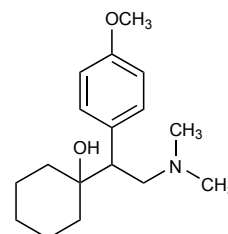
GBR 12909
(Prod. No. [D-052](#))

DAT/NET Preferring



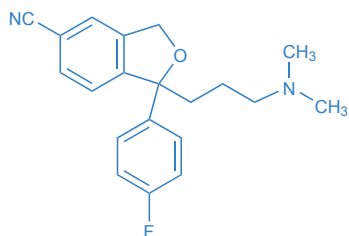
Mazindol
(Prod. No. [M-2017](#))

NET/SERT Preferring



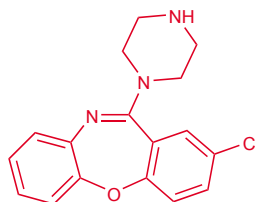
Venlafaxine

SERT Preferring

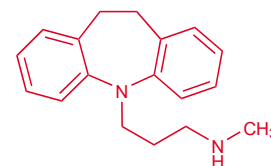


Citalopram
(Prod. No. [C-7861](#))

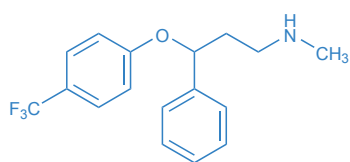
NET Preferring



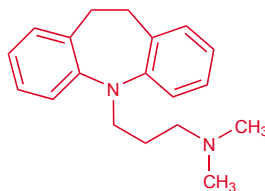
Amoxapine
(Prod. No. [A-129](#))



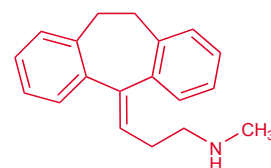
Desipramine
(Prod. No. [D-3900](#))



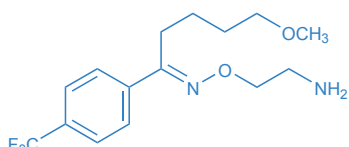
Fluoxetine
(Prod. No. [E-132](#))



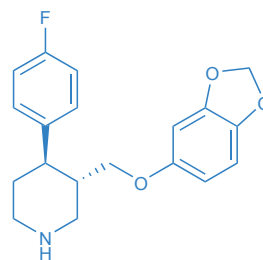
Imipramine
(Prod. No. [L-7379](#))



Nortriptyline
(Prod. No. [N-7261](#))



Fluvoxamine
(Prod. No. [F-2802](#))



Paroxetine

Neurotransmitter Transporters...(continued)

NET, DAT and SERT have similar regulatory mechanisms controlling both functional transport activity and cell surface expression to those described above for the amino acid neurotransmitter transporters. Using heterologous expression systems, it has been demonstrated that activation of PKC leads to a decrease in the transport activity of NET, DAT and SERT attributed to an internalization of the transporter protein(s) [60-62]. In the case of SERT, there is evidence for direct phosphorylation of the transporter protein [63]. In contrast, PKC-dependent internalization of DAT can occur in the absence of direct phosphorylation [64], implicating accessory phosphoproteins in this component of its regulation. Additional regulatory mechanisms for DAT have been revealed using inhibitors of tyrosine kinase [65] and PI3K [66], that both produce decreases in net transport activity associated with transporter removal from the cell surface. Conversely, activation of the PI3K signaling pathway following insulin treatment resulted in an increase in DAT activity and cell surface expression [66].

As described for the amino acid transporters, regulation of the biogenic amine transporters by their endogenous substrates is emerging as an important mechanism for controlling both their functional activity and cellular trafficking. Substrates for SERT, including serotonin, amphetamine and fenfluramine (Prod. Nos. [F 8507](#), [F-112](#), [F-113](#)), reduce the phosphorylation of transporter protein and internalization following activation of PKC by phorbol ester [67]. This provides for a use-dependent regulation of SERT expression (and function) by maintaining the transporter on the cell surface when extracellular clearance of available serotonin is required. Conversely, in the case of DAT, substrates such as dopamine and amphetamine reduce transporter activity by promoting internalization. In contrast, the inhibitor cocaine blocks this effect [68] and itself produces an increase in net uptake capacity by promoting cell surface expression [69]. Consistent with the negative regulation of DAT by substrates observed in the recombinant studies, both methamphetamine (Prod. No. [M 8750](#)) and methylenedioxymethamphetamine (MDMA, 'Ecstasy'; Prod. Nos. [M 6403](#), [M-139](#))

Neurotransmitter Transporter Research Tools Available from Sigma-RBI

Dopamine Transport (DAT) Inhibitors

| | |
|------------------------|---|
| D-044 | Amfolenic acid |
| B-138 | BTCP HCl |
| B-102 | Bupropion HCl |
| C-124 | β -CFT naphthalenesulfonate (WIN 35,428) |
| C-156 | D-CPT tartrate (WIN 35,065-2) |
| C-207 | 4'-Chloro-3 α -(diphenylmethoxy)tropane HCl |
| D-205 | 4',4'-Difluoro-3 α -(diphenylmethoxy)tropane HCl |
| D-209 | Dopamine transporter, human |
| D-052 | GBR 12909 diHCl |
| G 9659 | GBR 12935 diHCl |
| G-120 | GYKI 52895 |
| T 3146 | Trimipramine maleate |

Norepinephrine Transport (NET) Inhibitors

| | |
|------------------------|-------------------|
| A-129 | Amoxapine |
| D 3900 | Desipramine HCl |
| M 9651 | Maprotiline HCl |
| N-151 | Nisoxetine HCl |
| N 7261 | Nortriptyline HCl |
| P 8813 | Protriptyline HCl |
| T 7947 | Tomoxetine HCl |

Serotonin Transport (SERT) Inhibitors

| | |
|------------------------|---------------------|
| A-164 | Alaproclate HCl |
| C-155 | nor- β -CIT |
| C 7861 | Citalopram HBr |
| C 7291 | Clomipramine HCl |
| F-132 | Fluoxetine HCl |
| F 1553 | S(+)-Fluoxetine HCl |
| F 1678 | R(-)-Fluoxetine HCl |
| F 2802 | Fluvoxamine maleate |

| | |
|------------------------|--------------------------|
| F-133 | Norfluoxetine HCl |
| Q-109 | 6-Nitroquipazine maleate |
| T 6154 | Trazodone HCl |
| Z-101 | Zimelidine diHCl |

Mixed DAT/NET/SERT Inhibitors

| | |
|------------------------|-----------------------------|
| A 8404 | Amitriptyline HCl |
| D 4526 | Doxepin HCl |
| M 2017 | Mazindol |
| I-119 | Indatraline HCl (Lu 19-005) |
| I 7379 | Imipramine HCl |
| N 1530 | Nomifensine maleate |

GABA Transport Substrates

| | |
|------------------------|------------------|
| A 7752 | β -Alanine |
| B 3501 | Betaine HCl |

GABA Transport Inhibitors

| | |
|------------------------|----------------|
| D 8376 | L-DABA diHCl |
| G-007 | Guavacine HCl |
| N-142 | NO-711 HCl |
| 211672 | Nipecotic acid |
| V 8261 | Vigabatrin |

Excitatory Amino Acid Transport Inhibitors

| | |
|------------------------|---|
| D 1064 | Dihydrokainic acid |
| H 2775 | threo-Hydroxyaspartic acid |
| K 0250 | Kainic acid |
| P 7575 | trans-2,4-Pyrrolidine-2,4-dicarboxylic acid |

Glycine Transport Inhibitors

| | |
|------------------------|-----------|
| S 7672 | Sarcosine |
|------------------------|-----------|

Neurotransmitter Transporters...(continued)

decrease DAT function *in vivo* [70,71]. Taken together, these observations have important implications for the interpretation of drug action, since these agents can no longer simply be viewed as inhibitors of the transport process.

Dynamic regulation of the biogenic amine transporters likely involves many accessory proteins that regulate both their activity and their trafficking. A number of binding partners have been identified. Of particular significance with respect to an involvement in the targeting of transporter proteins is the demonstration that the PDZ domain containing protein PICK1 strongly interacts with both DAT and NET (weaker with SERT) in both recombinant expression systems and cultured neurons [72]. Whether PICK1 acts simply as a general targeting and anchoring partner, or also participates in the dynamic regulation of transporter function and expression, remains to be determined. Another identified partnership is between SERT and protein phosphatase 2A (Prod. No. [P 9989](#)). In this case, an interaction is believed to be involved in the regulation of transporter activity and surface expression which is influenced by the substrate serotonin [73]. Clearly, the identification of additional binding partners for SERT and other members of the neurotransmitter transporter families is an area of current focus and will undoubtedly lead to a greater understanding of the biology of these transporters.

Other Family Activities

Like most modern day families, both the sodium-dependent and sodium/chloride-dependent neurotransmitter families maintain hectic lifestyles. Add to this busy schedule the capacity for certain transporter subtypes to exhibit channel-like properties [74], distinct from their substrate binding and translocation function, and matters become even more complicated. Whether these various activities are achieved by each transporter protein working alone (monomeric), or in close collaboration with their counterparts (oligomeric forms), is an area likely to receive increased attention in the future. Recent observations that support the oligomeric assembly of SERT [75] and EAAT3 [76] offer the new challenge of ascribing the various functional activities of these transporters to these different structures. More family secrets undoubtedly await future discovery.

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New Product Highlights

R(-)-Fluoxetine and S(+)-Fluoxetine: Selective serotonin reuptake inhibitors

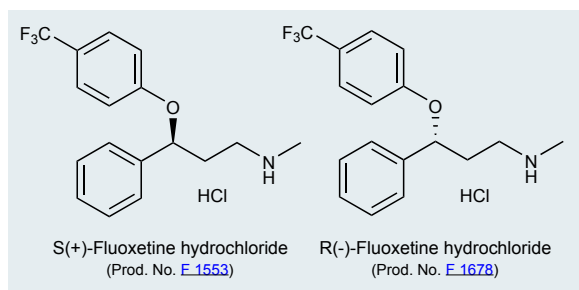
Fluoxetine (Prod. No. [F-132](#)), sertraline, paroxetine, fluvoxamine (Prod. No. [F 2802](#)) and citalopram (Prod. No. [C 7861](#)) are selective serotonin reuptake inhibitors (SSRIs) that have been used extensively in the treatment of anxiety and depression [1-3]. These drugs block the serotonin transporter (SERT), a high-affinity sodium/chloride-dependent neurotransmitter transporter, that terminates serotonergic neurotransmission by rapid reuptake of serotonin (5-hydroxytryptamine; 5-HT; Prod. No. [H 9523](#)) into serotonergic nerve terminals [4]. As a consequence, SSRIs increase the concentration of serotonin in the synapse and amplify signals sent by serotonergic neurons. The effects of fluoxetine (Prozac) as a SSRI were first reported in the scientific literature in 1974 [5]. Following its approval by the FDA in late 1987 for the treatment of depression, fluoxetine has become the world's most prescribed antidepressant drug [6]. Clinically, the drug is marketed as the racemate, i.e. equal amounts of the R(-) and S(+) enantiomers.

Both enantiomers inhibit serotonin reuptake with similar potencies and effectively produce functional responses associated with increased serotonergic neurotransmission [7-9]. For example, in synaptosomal preparations from rat cerebral cortex, concentrations required to induce 50% inhibition (IC_{50} value) of serotonin uptake were 34 nM (R,S), 40 nM (R) and 25 nM (S) [8]. Likewise, similar IC_{50} values of 4.6 nM (R,S), 3.9 nM (R) and 3.6 nM (S) were reported for the inhibition of serotonin uptake in human platelets [8].

However, some differences in the pharmacological profile of the enantiomers of fluoxetine have been observed. Thus, S(+)-fluoxetine is slightly more potent than R(-)-fluoxetine in blocking serotonin uptake *ex vivo* [7] and in preventing *p*-chloramphetamine-induced depletion of brain serotonin levels in mice [9,10]. In addition, while both enantiomers have been shown to exhibit relatively weak affinities for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT₃ serotonin receptor subtypes, R(-)-fluoxetine was shown to be 23-fold more potent at displacing [³H]-mesulergine binding to 5-HT_{1C} (now referred to as 5-HT_{2C}) receptors in

bovine choroid plexus [11]. In addition to its actions at serotonergic sites, fluoxetine has also been shown to interact with a range of ion channels, including HERG potassium channels [12], voltage-gated potassium channels [13] and volume regulated anion channels [14]. These actions may explain some of the side effects associated with the clinical use of fluoxetine.

To further explore the pharmacological effects of fluoxetine on a variety of serotonergic and non-serotonergic mechanisms, Sigma-RBI is pleased to introduce both S(+)-fluoxetine (Prod. No. [F 1553](#)) and R(-)-fluoxetine (Prod. No. [F 1678](#)). These novel research tools should prove useful in elucidating the mechanisms underlying both the therapeutic and the undesirable effects of fluoxetine.



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ALZHEIMER'S DISEASE AND NEURO-DEGENERATIVE DISEASE RESEARCH

[S 8190](#) **sAPP α (444-612)** (Secreted Amyloid Precursor Protein α 444-612), recombinant, expressed in *E. coli*.

[H 6161](#) **[Gly¹⁴]-Humanin**
Potent rescue factor which suppresses neuronal cell death.

APOPTOSIS

[A 5851](#) **Anti-Apoptosis Signal-Regulating Kinase 1 (ASK/MAPKKK5), C-Terminal** (rabbit)

[C 8487](#) **Anti-Caspase-3, Active** (rabbit)

[P 5367](#) **Anti-PAR-4 (Prostate Apoptosis Response 4)** (rabbit)

CELL STRESS

[M 8065](#) **O⁶-Methylguanine-DNA Methyl transferase (MGMT)**
DNA repair enzyme.

CELL CYCLE

[C 9987](#) **Anti-CDK3** (rabbit)

[C 0238](#) **Anti-CDK8** (rabbit)

[K 3888](#) **Kenpauillone**
Potent inhibitor of CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, CDK5/p25 and GSK-3 β .

CYTOSKELETON

[C 6987](#) **Anti-Cortactin (GK-18)** (rabbit)

[C 7112](#) **Anti-Cortactin (KE-20)** (rabbit)

[E 9653](#) **Monoclonal Anti-Endothelial Cells,**
Clone PIH2

[K 3513](#) **Anti-KIF3A** (rabbit)

[K 3638](#) **Anti-KIF17** (rabbit)

[M 2441](#) **Anti-MAGI-2 (Membrane-Associated Guanylate Kinase Inverted-2)** (rabbit)

[P 5117](#) **Monoclonal Anti-Peripherin,** Clone 8G2

[P 3118](#) **Monoclonal Anti-p115/TAP (Transcytosis Associated Protein),** Clone 5D6

[S 4191](#) **Monoclonal Anti-SLIPR/MAGI-3,**
Clone SLP-32

[T 1323](#) **Monoclonal Anti- ϵ -Tubulin,**
Clone TUB-11

G PROTEIN

[R 4777](#) **Monoclonal Anti-Ran,** Clone ARAN1

GENE REGULATION

[D 8690](#) **(R,R)-cis-Diethyl tetrahydro-2.8-chrysenediol**
Potent estrogen receptor β antagonist; potent partial estrogen receptor α agonist.

[M 6191](#) **GW9662**
Irreversible peroxisome proliferator-activated receptor γ (PPAR γ) antagonist. *Sold for research purposes only, pursuant to an agreement with GlaxoSmithKline.*

[R 3902](#) **Monoclonal Anti-hnRNP (Heterogenous Nuclear Ribonuclear Proteins) M1-4,**
Clone HL-374

[R 3777](#) **Monoclonal Anti-hnRNP (Heterogenous Nuclear Ribonuclear Proteins) M3-4,**
Clone HL-372

[R 2902](#) **Monoclonal Anti-Retinoid X Receptor γ (mRXR γ),** Clone 1373

[T 1698](#) **Tetradecylthioacetic acid (TTA)**
Peroxisome proliferator-activated receptor α (PPAR α) agonist.

ION CHANNELS

[D 9190](#) **DCEBIO**
Facilitates chloride secretion in epithelia by activating potassium and chloride currents.

[D 8190](#) **3',4'-Dichlorobenzamil hydrochloride** (L-594,881)
Inhibits sodium/calcium exchanger, sodium transport and sarcoplasmic reticulum calcium release channel.

[F 9677](#) **Felodipine** (Plendil)
L-type calcium channel blocker.

[F 3176](#) **FS-2, *Dendroaspis p. polylepsis***
L-type calcium channel blocker.

[M 5441](#) **Mibefradil dihydrochloride** (Ro 40-5967)
T-type calcium channel blocker; anti-hypertensive.

[P 4243](#) **S-Petasin**
L-type voltage-dependent calcium channel blocker.

LIPID SIGNALING

[L 9539](#) **L-655,240**
Thromboxane A₂ receptor antagonist.

[L 2790](#) **Anti-Lipid Phosphate Phosphatase 2 (LPP2)** (rabbit)

[L 2915](#) **Anti-Lipid Phosphate Phosphatase 3 (LPP3)** (rabbit)

[T 9567](#) **Thiolactomycin**
Antibiotic; bacterial myristate synthesis inhibitor.

[U 1508](#) **U-75302**
BLT₁ leukotriene receptor agonist.

MULTI-DRUG RESISTANCE

[M 6565](#) **Monoclonal Anti-MRP-1 (MDR related protein-1)**, Clone P2A8/6

NEUROPEPTIDES

[P 4118](#) [**CPP¹⁻⁷**, **NPY¹⁹⁻²³**, **Ala³¹**, **Aib³²**, **Gln³⁴**]-**Pancreatic polypeptide (human)**
Potent and selective Y₅ neuropeptide Y receptor agonist.

[U 1008](#) **Urocortin III (human)**, synthetic

[U 0883](#) **Urocortin III (mouse)**, synthetic

NEUROTRANSMISSION

[B 9308](#) **BP 897**
Partially selective D₃ dopamine receptor agonist.
Sold with the permission of INSERM and BIOPROJET.

[C 6862](#) **CB 34**
Selective, high affinity ligand at peripheral benzodiazepine receptors.

[C 1112](#) **CP-55,940**
Cannabinoid receptor agonist; equipotent at CB₁ and CB₂ cannabinoid receptors.

[D 9815](#) **Doxazosin mesylate**
α₁ Adrenergic receptor blocker; related to prazosin.

[F 1678](#) **R(-)-Fluoxetine hydrochloride**
Selective serotonin reuptake inhibitor.

[F 1553](#) **S(+)-Fluoxetine hydrochloride**
Selective serotonin reuptake inhibitor.

[I 9531](#) **Imiloxan hydrochloride**
Selective α_{2B}-adrenoceptor antagonist.

[M 6316](#) **MRS 1754**
Selective A_{2B} adenosine receptor antagonist.
Manufactured and sold under exclusive license from Adenosine Therapeutics, LLC.

[S 2941](#) **SKF 75670 hydrobromide**
Atypical D₁ dopamine receptor agonist.

[S 3191](#) **SKF 83565 hydrobromide**
Atypical D₁ dopamine receptor agonist.

[S 2816](#) **SKF 83959 hydrobromide**
Atypical D₁ dopamine receptor agonist.

[S 3316](#) **SKF 89145 hydrobromide**
D₁ dopamine receptor agonist.

[S 3066](#) **SKF 89626 hydrobromide**
D₁ dopamine receptor agonist.

[T 9573](#) **Anti-Phospho-Tyrosine Hydroxylase (TH) (pSer⁴⁰)**, Rat (Rabbit)

[V 0389](#) **Anti-VGLUT-1 (Vesicular Glutamate Transporter 1)** (rabbit)

NUCLEAR SIGNALING

[A 8975](#) **Anti-AFX (FOXO4)** (rabbit)

[C 5112](#) **Monoclonal Anti-CUG Binding Protein 1** (CUG-BP1), Clone HL-1190

[F 2303](#) **Anti-FKHR (FOXO1)** (rabbit)

[F 2178](#) **Anti-FKHRL1 (FOXO3a)** (rabbit)

[H 9286](#) **Anti-Acetyl Histone H3 [Ac-Lys⁹]** (rabbit)

[H 9161](#) **Anti-Acetyl & Phospho Histone H3 (Ac-Lys⁹, pSer¹⁰)** (rabbit)

[H 9536](#) **Anti-Histone Deacetylase 4 (HDAC4) (DM-15)** (rabbit)

[H 9411](#) **Anti-Histone Deacetylase 4 (HDAC4) (ML-19)** (rabbit)

[P 7493](#) **Anti-PCAF (p300/CREB Binding Protein Associated Factor)** (rabbit)

[S 8316](#) **Monoclonal Anti-SUV39H1 Histone Methyltransferase**, Clone 44.1

PHOSPHORYLATION

[E 6029](#) **Anti-Phospho-EGFR [pTyr⁸⁴⁵]**, Human, (rabbit)

[G 1918](#) **Gö 6983**
PKC α and PKC β inhibitor.

[P 6493](#) **Anti-Phospho-PKR [pThr⁴⁵¹]**, Human (rabbit)

[P 9868](#) **Anti-Protein Phosphatase 5 (PP5)** (rabbit)

KITS

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[NTS-II](#) **Nerve Terminal Staining Kit**
Used to detect and study synaptic vesicle recycling in neuronal synapses and neuromuscular junctions. NTS-II utilizes AM1-43 and the sulfonated β-cyclodextrin Advasep-7.

[NTS-V](#) **Nerve Terminal Staining Kit**
Used to detect and study synaptic vesicle recycling in neuronal synapses and neuromuscular junctions. NTS-V utilizes SynptoRed C2 and the sulfonated β-cyclodextrin Advasep-7.

New Product Highlights

Antibody to Acetylated Proteins: A measure of transcriptional regulation

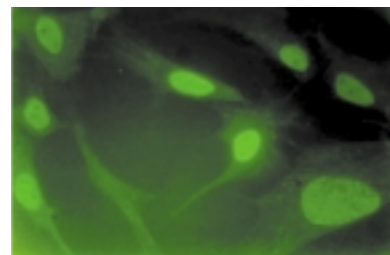
The organization of chromatin into higher order structures is required for chromosome function and epigenetic gene regulation [1]. Nucleosomal histones H2a, H2b, H3 and H4 form octameric core histone around which DNA winds. The conserved N-terminal tails of histones, particularly of H4 and H3, have been functionally characterized and are targets for acetylation, phosphorylation and methylation [1-5]. These modifications are thought to regulate chromatin structure and to modify the histone deposition that accompanies DNA replication, mitosis and DNA repair.

Acetylation of lysine is an important reversible modification that controls protein activity. Histone acetylation of N-ε-lysine residues destabilizes the nucleosome structure through charge neutralization and renders the DNA more accessible to transcription factors. The level of histone acetylation correlates directly with localized transcriptional activity of a particular domain. Histones are acetylated by histone acetyltransferases (HATs), including Gcn5p, PCAF, p300/CBP and TAF_{II}250 [6-7], and are deacetylated by histone deacetylases (HDACs) [8].

Protein acetylation regulates the activity of certain transcription factors including p53, GATA-1, E2F, TFIIIEβ, TFIIIF, and EKLF [9,10]. In response to UV or γ-irradiation, the tumor suppressor p53 becomes a target for acetylation by the transcription factors p300/CBP and PCAF, which also function as co-activators for p53-mediated transcriptional activation [9,11].

Sigma-RBI is pleased to offer an antibody to acetylated proteins (Prod. No. [A 5463](#)) which was generated to the amino-terminus of human histone H4, with N-acetylated-ε lysines at positions 5, 8, 12 and 16. Although this sequence is identical in many species of histone H4 and not found in other histones, the antibody recognizes several acetylated proteins, including acetylated histone H4 (12 kDa), H1 and H2b, but not acetylated histone H3. Applications include immunoblotting, immunocytochemistry, indirect immunofluorescence and dot blotting.

Nuclear staining of chicken fibroblast cells. Cells were methanol/acetone-fixed and stained with Anti-Acetylated Proteins (Prod. No. [A 5463](#)) at 1:1,000, followed by goat anti rabbit IgG (H+L), FITC conjugate (Prod. No. [F 9887](#)).



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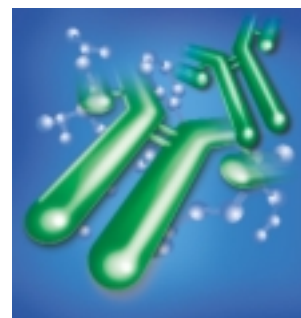
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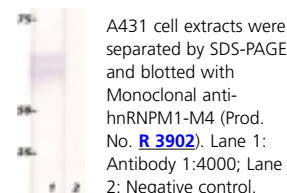
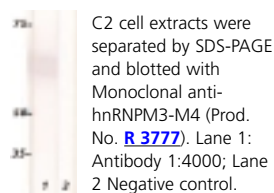
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Monoclonal Antibodies to hnRNP: RNA splicing tools

In the nucleus, RNA polymerase II transcripts are complexed with many different proteins. These proteins are called heterogeneous nuclear ribonucleoproteins (hnRNPs) and are involved in transcription, pre-mRNA processing, cytoplasmic mRNA translation and turnover [1]. hnRNPs can be isolated by immunoprecipitation or by sucrose gradient fractionation of cell extracts. These hnRNPs consist of protein groups A to U. Many of these protein groups consist of more than one isoform. Groups L and M are among the most abundant groups of proteins and also migrate closely on 2D gels. The hnRNP M1-M4 proteins are members of the RNP consensus sequence family of RNA-binding proteins that also includes the hnRNPs A1, A1/B1, C1/C2, I and L. The M proteins possess an unusual auxiliary domain, which consists of a highly repeated methionine/arginine rich (MR) hexapeptide motif. They are localized in the nucleus with a general nucleoplasmic staining pattern similar to that of other hnRNP complex proteins (A1, C1/C2, K/J and U proteins) [2]. Antibodies specific for hnRNP M proteins applied to HeLa nuclear extracts inhibited *in vitro* splicing, suggesting that these proteins are involved in RNA splicing [3].

Sigma-RBI now offers two antibodies to hnRNP M proteins: Monoclonal anti-hnRNP M1-M4, Clone HL374 (mouse IgG1 isotype) (Prod. No. [R 3902](#)) generated against hnRNP M1-4 (M19 Fusion Protein) [4] and Monoclonal Anti-hnRNP M3-M4, Clone HL372 (mouse IgG2b isotype) (Prod. No. [R 3777](#)) generated against hnRNP M3-M4 (M19 Fusion protein) [4]. Monoclonal Anti-hnRNP M1-M4 recognizes human, bovine, porcine, rabbit, mouse and rat hnRNP M1-M4 (approx. 65-70 kDa) [4]. Monoclonal Anti-hnRNP M3-M4 recognizes human, bovine, porcine, rabbit, mouse and rat hnRNP M3-4 (approx. 68 kDa). Applications include the detection of hnRNP M1-M4 and M3-M4 by immunoblotting and immunoprecipitation [4].



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New Product Highlights

Vanilloid receptor research tools from Sigma-RBI

Vanilloid receptors are predominantly expressed on C and A δ fibers projecting to the dorsal horn of the spinal cord, as well as in trigeminal ganglion neurons projecting to the spinal nucleus of the trigeminal tract [1]. Vanilloids, such as capsaicin (Prod. No. [M 2028](#)), elicit a biphasic action on sensory neurons characterized by an initial excitatory phase (pain and/or inflammation) followed by desensitization. Vanilloid receptor 1 (VR1) is essential for normal thermal nociception and for thermal hyperalgesia induced by inflammation [2]. Consequently, VR1 receptor antagonists may be useful in the study of inflammatory hyperalgesia and pain [3]. Sigma-RBI provides these important research tools for vanilloid receptor research.

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2. Caterina, M.J. et al., *Science*, **288**, 306-313 (2000).
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[A 2098](#) **Arvanil**

Activator of cannabinoid and vanilloid receptors; potent inhibitor of anandamide accumulation.

[I 1279](#) **Isovelleral**

VR1 Vanilloid receptor agonist.

[I 9281](#) **5'-Iodoresiniferatoxin**

VR1 Vanilloid receptor antagonist.

[O 0257](#) **Olvanil**

VR1 Vanilloid receptor agonist.

[P 9983](#) **Phorbol 12,13-didecanoate 20-homovanillate**

Resiniferatoxin-type vanilloid phorbol ester with capsaicin-like selectivity for VR1 vanilloid receptors.

[P 0234](#) **Phorbol 12,13-dinonanoate 20-homovanillate**

Resiniferatoxin-type vanilloid phorbol ester with capsaicin-like selectivity for VR1 vanilloid receptors.

[S 0441](#) **SB-366791**

VR1 Vanilloid receptor-1 antagonist.

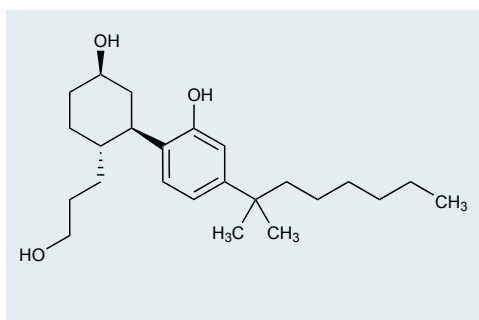
[S 8186](#) **Scutigeral**

Novel vanilloid receptor agonist with affinity for VR1 vanilloid and D₁ dopamine receptors.

[V 1510](#) **Anti-Vanilloid Receptor-Like Protein 1, Rabbit, IgG**

fraction of antiserum.

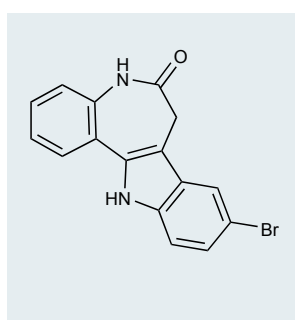
More New Products Available from Sigma-RBI



CP 55,940
Prod. No. [C 1112](#)

Non-selective CB₁/CB₂ cannabinoid receptor agonist; more potent than Δ^9 -THC.

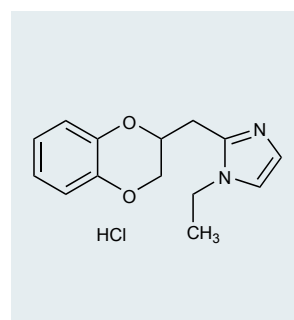
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Patel, S. and Hillard, C.J., *J. Pharmacol. Exp. Ther.*, **297**,
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Kenpauillone
Prod. No. [K 3888](#)

Potent inhibitor of cyclin-dependent kinases CDK1, CDK2 and CDK5.

Zaharevitz, D.W., et al., *Cancer Res.* **59**, 2566-2569 (1999); Schultz, C., et al., *J. Med. Chem.*, **42**, 2909-2919 (1999).



Imiloxan HCl
Prod. No. [I 9531](#)

α_{2B} -Adrenoceptor antagonist.

Michel, A.D., et al., *Br. J. Pharmacol.*, **99**, 560-564 (1990); Takano, Y., et al., *Eur. J. Pharmacol.*, **219**, 465-468 (1992).

New Product Highlights

[cPP¹⁻⁷,NPY¹⁹⁻²³,Ala³¹,Aib³²,Gln³⁴]-Pancreatic Polypeptide (human): An agonist for the Y₅ neuropeptide Y receptor

Neuropeptide Y (NPY; Prod. Nos. [N 5017](#), [N 3266](#)) is the most abundant neuropeptide in brain. It consists of 36 amino acid residues that share high sequence homology with pancreatic polypeptide (PP; Prod. Nos. [P 9903](#), [P 6410](#)) and peptide YY (PYY; Prod. No. [P 1306](#)). The various biological effects of NPY and its homologs are mediated by the activation of at least five types of G protein-coupled receptors, designated as Y₁ (Prod. No. [N-186](#)), Y₂, Y₄, Y₅ and y₆. There has been particular interest in the Y₁ and Y₅ receptors, since they have been associated with the stimulatory effects of NPY on food intake [1,2].

Sigma-RBI is pleased to introduce the first potent and selective agonist for the Y₅ neuropeptide Y receptor, [cPP¹⁻⁷,NPY¹⁹⁻²³,Ala³¹,Aib³²,Gln³⁴]-pancreatic polypeptide (human) (Prod. No. [P 4118](#)). This polypeptide exhibits high affinity for the human Y₅ neuropeptide Y receptor expressed in HEK-293 cells, possessing an

IC₅₀ value of 0.24 nM as compared with an IC₅₀ value of 0.6 nM obtained for NPY [3]. In contrast, the synthetic polypeptide exhibited IC₅₀ values of 530, >500 and 51 nM, respectively, versus human Y₁, Y₂, and Y₄ receptors expressed in BHK cells [3]. In *in vivo* experiments, [cPP¹⁻⁷,NPY¹⁹⁻²³,Ala³¹,Aib³²,Gln³⁴]-pancreatic polypeptide (human) induced long-term stimulation of food intake in rats when administered centrally, thereby supporting the hypothesis that the Y₅ receptor as an orexigenic receptor [3].

In summary, [cPP¹⁻⁷,NPY¹⁹⁻²³,Ala³¹,Aib³²,Gln³⁴]-Pancreatic Polypeptide (human) will serve as an important research tool for studying the Y₅ neuropeptide Y receptor and its role in regulating food intake.

References:

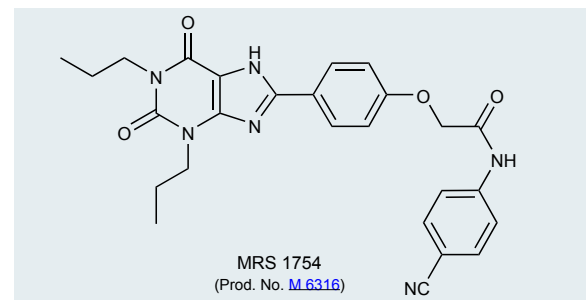
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2. Pralong, F.P., et al., *FASEB J.*, **16**, 712-714 (2002).
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MRS 1754: A selective A_{2B} adenosine receptor antagonist

Adenosine (Prod. No. [A 9251](#)) receptors have been implicated in the etiology of various cardiovascular, inflammatory and neurological diseases. Certain Alkylxanthine derivatives, such as caffeine (Prod. No. [C 8960](#)) and theophylline (Prod. No. [T 1633](#)), are antagonists at adenosine receptors with the latter compound having been used in the treatment of asthma. Four adenosine receptor subtypes have been identified and are referred to as A₁, A_{2A}, A_{2B} and A₃. A_{2B} adenosine receptors stimulate adenylyl cyclase, increase intracellular calcium release and are involved in the control of vascular tone, cell growth and gene expression, mast cell degranulation and intestinal water secretion [1]. While A_{2B} adenosine receptor antagonists such as ZM 241385 and I-ABOPX have been identified, these compounds are not selective and display significant interaction with other adenosine receptor subtypes.

Sigma-RBI is pleased to introduce MRS 1754 (Prod. No. [M 6316](#)), a selective, high-affinity A_{2B} adenosine receptor antagonist. [³H]-MRS 1754 bound to human A_{2B} and A_{2A} adenosine receptors expressed in HEK-293 cells with K_D values of 1.13 nM and >50 nM,

respectively [2]. In contrast, it did not bind to membranes expressing rat or human A₁ or A₃ receptors. MRS 1754 also displaced both [³H]-ZM 241385 and [¹²⁵I]-I-ABOPX from human A_{2B} adenosine receptors with K_i values of 1.97 nM [2], in addition to displacing [³H]-MRS 1754 from the same receptors with a K_i value of 1.45 nM [2]. MRS 1754, therefore, represents a highly selective tool with which to study the physiology of A_{2B} adenosine receptors.



Manufactured and sold under exclusive license from Adenosine Therapeutics, LLC.

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New Product Highlights

Monoclonal Antibody to Endothelial Cells (CD146): A tumor suppressor in breast carcinoma

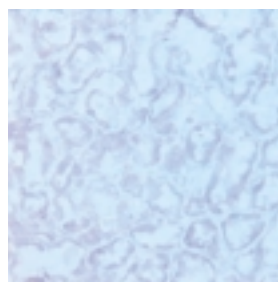
CD146 (also known as A32, MCAM, Mel-CAM, MUC18, and S-Endo-1) was originally defined as a marker of melanoma progression. It is a cell-cell adhesion receptor that mediates calcium-independent homotypic endothelial cell adhesion. CD146 is a component of the endothelial junction and is located outside the adherens junction, but is not restricted to the junction [1]. It is a cell-surface glycoprotein that belongs to the immunoglobulin super-gene family. It has a molecular size of 130 kDa in its reduced form (118 kDa unreduced), with N-linked glycosylation accounting for fifty percent of the apparent molecular weight. In some cells CD146 carries a sulfate-3-glucuronyl moiety.

CD146 is expressed in a wide range of normal tissues, including endothelium, smooth muscle, Schwann cells, ganglion cells, cerebellar cortex, basal cells of bronchial epithelium, parathyroid glands, skeletal muscle, lens epithelium, and glial cell fibers in the central nervous system in early embryos. In malignant tumors, CD146 is expressed in melanomas, angiosarcomas, Kaposi's sarcomas, leiomyosarcomas, mucoepidermoid carcinomas of salivary gland, placental-site trophoblastic tumors, and choriocarcinomas. However, other carcinomas, sarcomas, lymphomas, leukemias, and neuroendocrine tumors have been shown not to express CD146 [2].

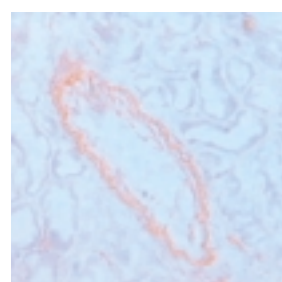
CD146 is significantly elevated in osteoarthritis, psoriatic arthritis and rheumatoid arthritis [3]. It may represent an activation marker of keratinocytes and may be involved in cutaneous inflammatory tissue reaction [4]. Its expression can promote tumor progression in human melanoma through enhanced interaction between melanoma cells and endothelial cells [5]. Such expression allows melanoma cells to separate from the epidermis and invade the basement membrane zone and dermis. Melanoma cells lacking CD146 are poorly invasive, possible due to their loss of gap junctional communication [6]. However, in breast carcinoma, CD146 may act as a tumor

suppressor. Overexpression of CD146 in breast carcinoma cells results in more cohesive cell growth and in formation of smaller tumors in nude mice [7].

Sigma-RBI is pleased to offer Monoclonal Anti-endothelial cells, clone P1H12 (Prod. No. [E 9653](#)) that has been produced using human umbilical cord cells (HUVEC) as immunogen. Monoclonal anti-endothelial cells recognizes cultured microvascular and large-vessel endothelial cells (MVED and HUVEC, respectively) and endothelial cells in tissues and in the circulation [8]. The antibody may be used for immunocytochemistry in live or fixed cells [8-10], immunohistochemistry [8,9], flow cytometry [10], immunoprecipitation and ELISA. It also may be used for enrichment of endothelial cells from circulation [8-10] and tissues [2].



Control



Monoclonal Anti-Endothelial Cells (CD146)

Staining of blood vessels in human kidney. Sections of frozen human kidney were stained with Monoclonal Anti-Endothelial Cells, Clone P1H12 (Prod. No. [E 9653](#)) 1:100, followed by Anti-Mouse Fab, Biotin conjugate (Prod. No. [B 7151](#)) and ExtrAvidin-HRP (Prod. No. [E 2886](#)) and AEC substrate (Prod. No. [A 6926](#)).

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New Product Highlights

GW9662: An irreversible PPAR γ antagonist

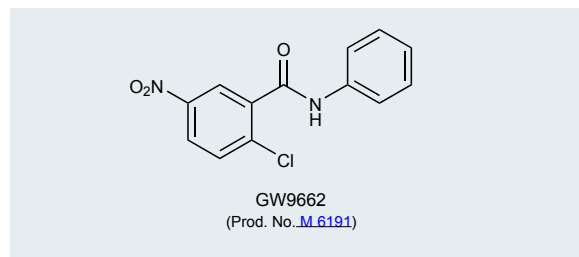
Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors that play an important role in many cellular functions, including lipid metabolism (storage and catabolism of dietary fats), cell proliferation, differentiation, adipogenesis and inflammatory signaling [1,2]. Currently, three PPAR subtypes have been identified and are referred to as PPAR α , PPAR β (also known as PPAR δ) and PPAR γ . PPAR γ is the most studied of the three subtypes, on account of its role in adipocyte differentiation as well as its involvement in glucose and lipid metabolism [2]. Thus, this receptor has become an exciting therapeutic drug target for various disease states, in particular diabetes, atherosclerosis and hypertension [2].

A range of synthetic and naturally occurring substances activate PPAR γ , including antidiabetic thiazolidinediones (troglitazone and ciglitazone; Prod No. [C 3974](#)), polyunsaturated fatty acids such as linoleic acid (Prod. No. [L 1268](#)), linolenic acid (Prod. No. [L 2376](#)) and arachidonic acid (Prod. No. [A 9673](#)) and 15-deoxy- Δ prostaglandin J₂. Other activators include components of oxidized low-density lipoprotein, such as 13-hydroxyoctadecadienoic acid (13-HODE; Prod. No. [H 9146](#)) and 15-hydroxyeicosatetraenoic acid (15-HETE; Prod. No. [H 1142](#)), as well as tetradecylthioacetic acid (TTA; Prod. No. [T 1698](#)). Unfortunately, with the exception of BADGE (Prod. No. [D 3415](#)), PPAR γ antagonists are not readily available to researchers.

Sigma-RBI is now pleased to offer GW9662 (Prod. No. [M 6191](#)), a potent, irreversible and functionally selective PPAR γ antagonist that is not structurally related to BADGE. Displaying nanomolar to micromolar affinity, GW9662 selectively antagonizes PPAR γ in multiple cell types including adipocytes,

macrophages and hepatic stellate cells [1-4]. In human aortic smooth muscle cells, GW9662 was utilized to implicate PPAR γ as a mediator of TGF- β (Prod. No. [T 3698](#))-induced connective tissue growth factor expression through direct interference of the Smad3 signaling pathway, a finding that may help to explain why PPAR γ is significantly activated after vascular injury [3]. GW9662 also prevented the IL-4 (Prod. No. [I 4629](#))-induced inhibition of osteoclast formation in the low micromolar range (1-2 μ M) [5]. This ability to attenuate osteoclast differentiation may explain why certain PPAR γ -targeting antidiabetic drugs exhibit antiresorptive effects on bone in diabetic patients.

These and other findings suggest that GW9662 will serve as an important research tool in the elucidation of the role of PPAR γ in various metabolic diseases.



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Application Note

Detecting Apoptosis In Primary Monolayer Cultures of Rat Hepatocytes Using Annexin-V FITC Apoptosis Kit

Mekala Gunaratnam and M. Helen Grant

Primary cultures of hepatocytes are widely used as *in vitro* model systems to study the metabolism and toxicity of novel xenobiotics, particularly in the pharmaceutical industry. As part of these investigations, it is important to be able to determine the mechanism of cell death induced by foreign chemicals and their reactive metabolites. Cell death occurs either by necrosis or apoptosis. The latter is associated with a characteristic cellular morphology involving condensation and fragmentation of the nucleus, cleavage of DNA, cell shrinkage and blebbing of the cell membrane [1]. In apoptosis, cells do not lyse, but fragment into membrane-enclosed particles, or apoptotic bodies, which are phagocytosed and cleared by macrophages *in vivo*. In contrast, during necrosis, cells swell, their membranes rupture and cell contents spill out into the adjacent environment causing inflammatory reactions due to the release of toxic reactive intracellular constituents. Unlike necrotic cell death, apoptosis does not initiate an inflammatory response and thus, tends not to lead to widespread tissue damage. Strict control of apoptosis is critical to the health of tissues, and its dysregulation has been implicated in the development of cancers and degenerative diseases. Moreover, the role of apoptosis in the toxicity of many foreign chemicals is becoming increasingly evident [2].

The Annexin-V FITC Apoptosis Kit (Prod. No. [APO-AF, A2214](#)) was designed to detect apoptotic cells by flow cytometry, and involves staining cells with pro-

pidium iodide (Prod. No. [P 4170](#)) and annexin-V labelled with fluorescein isothiocyanate (FITC) (Prod. No. [A 9210](#)). Annexins are a group of proteins that bind, by a calcium-dependent process, to phosphatidylserine (PS) present on the surface of cells undergoing apoptosis [3,4]. PS is normally sequestered on the inner leaflet (cytoplasmic side) of the plasma membrane. However, during apoptosis, membrane phospholipid asymmetry is lost and PS is exposed on the outer leaflet (extracellular side) where it can interact with Annexin V. Using this detection kit, the fluorescently-labelled annexin gives a bright green fluorescence around the periphery of apoptotic cells where binding has taken place. In cells where the membrane is damaged, propidium iodide penetrates the membrane and produces a red fluorescence in the nucleus. Thus, necrotic cells with damaged membranes stain with propidium iodide. In the later stages of apoptosis, cells also undergo damage to the cell membrane and would be stained with both annexin-V and propidium iodide. They appear with bright green staining around the cell periphery and red staining inside the cells, particularly in the nuclei. In contrast, healthy intact cells show only background fluorescence for both dyes.

We describe here an alternative use of the Sigma Annexin-V FITC Apoptosis Kit to detect apoptosis in primary cultures of hepatocytes, visualizing cell morphology with confocal laser-scanning microscopy (CLSM). This method requires a minor, but crucial modification to one of the reagents described in the Technical Bulletin MB-390 that accompanies this kit. We found that the propidium iodide solution (Prod. No. [P 4864](#)), provided at a concentration of 100

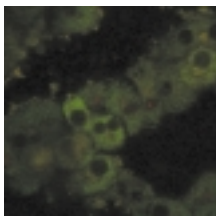


Figure 1. Unfixed hepatocytes exposed to 0.8 µg/ml propidium iodide for 1 min.

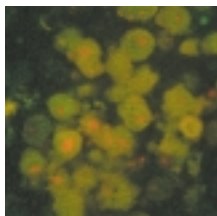


Figure 2. Unfixed hepatocytes exposed to 10 µg/ml propidium iodide for 1 min.

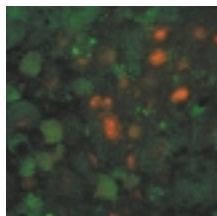


Figure 3. Unfixed hepatocytes exposed to 50 µg/ml propidium iodide for 1 min.

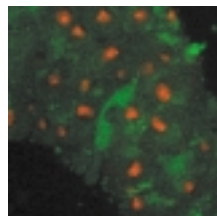


Figure 4. Unfixed hepatocytes exposed to 100 µg/ml propidium iodide for 1 min.

About the Authors

M. Helen Grant received her Ph.D. in Pharmacology in 1983 from the University of Aberdeen in the UK. She began her research in metabolism and toxicity in hepatocytes during her post-doctoral studies in the laboratory of Professor Sten Orrenius at the Karolinska Institute in Sweden. She joined the staff of the University of Strathclyde in Glasgow, UK in 1990, and is currently a Reader in Bioengineering. One of her major research interests is in the interaction of cells with biomedical materials and foreign chemicals, with particular interest in the role of glutathione in the metabolism and toxicity of xenobiotics. **Mekala Gunaratnam** is a final year Ph.D. student working under the supervision of Dr. M. Helen Grant within the department of Pharmacology at the University of Aberdeen. Her thesis has involved the study of hexavalent chromium toxicity and has been sponsored by the UK Medical Research Council.

Detecting Apoptosis in Primary Monolayer Cultures...(continued)

$\mu\text{g/ml}$, rapidly kills hepatocytes even at a reduced exposure time of 1 min. To resolve this issue, we titrated the concentration of propidium iodide to determine a concentration that was non-toxic. Figures 1-4 show the sensitivity of control unfixed 24 hr cultures of rat hepatocytes to propidium iodide at concentrations of 0.8, 10, 50 and 100 $\mu\text{g/ml}$. The exposure time to the propidium iodide was 1 min, and the rest of the protocol remained unchanged as described in the bulletin. Figure 1 shows the cells exposed to 0.8 $\mu\text{g/ml}$ propidium iodide and indicates that the dye did not penetrate the cell membrane from which it can be concluded that the cells remained viable. Figures 2-4 show that, at increasing concentrations, the propidium iodide penetrated the cell membrane, disrupted cell morphology and killed the cells. Primary hepatocytes are more sensitive to the toxicity of xenobiotics, including propidium iodide, than most cell lines adapted to *in vitro* culture for use in cell biology/immunology research. This is because liver cells contain xenobiotic metabolizing enzymes that can activate chemicals and produce reactive toxic forms. The use of 0.8 $\mu\text{g/ml}$ propidium iodide for an exposure time of 1 min does not kill hepatocytes, allowing discrimination between apoptosis and necrosis when used in combination with annexin V-FITC.

We have employed this modified method to investigate the mode of cell death induced by hexavalent chromium (Cr VI, Prod. No. [O 2673](#)) during its toxic interaction with hepatocytes [5]. Cr VI has been shown previously to cause apoptosis in fibroblasts and lung cells [6,7]. Isolated hepatocytes [8] were preincubated in Chee's medium with 5% fetal calf serum for 4 hr before media replacement and exposure to 0, 5 or 25 μM Cr VI for 24 hr. Cells were then stained with annexin V-FITC as described above and viewed using CLSM. In control cells, which were not exposed to Cr VI, some green fluorescence was detected. However, this green signal was very weak and due to auto-fluorescence of the hepatocytes (Figure 5). This image was recorded at almost twice the sensitivity (voltage setting 719) of that of figures 6 and 7 (voltage settings 442 and 585, respectively) so that the outline of the cells could be clearly seen. The cells were in colonies and displayed a characteristic flattened morphology with prominent nuclei. Cells treated with 5 μM Cr VI showed a clear bright green fluorescent ring around the periphery of the cells

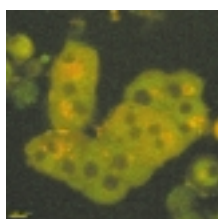


Figure 5.
Control hepatocytes in culture for 24 hr.

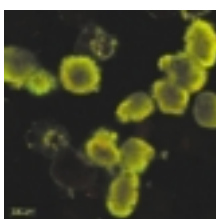


Figure 6.
Hepatocytes exposed to 5 $\mu\text{g/ml}$ Cr VI for 24 hr.

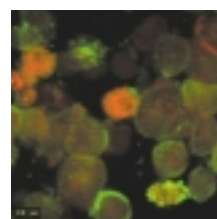


Figure 7.
Hepatocytes exposed to 25 $\mu\text{g/ml}$ Cr VI for 24 hr.

(Figure 6). This was seen in approximately 40% of the cell population. These cells had lost their cell to cell contact and had separated and rounded up. When the Cr VI concentration was increased to 25 μM , cells with red staining were visible (Figure 7). The small (approximately 1-2 μm diameter) green fluorescing buds on some of the cells represent the apoptotic bodies reflecting later stages of apoptosis. After treatment with Cr VI, the cells had shrunk in size. Control cells had areas between 850-880 μm^2 and Cr VI treated cells had areas between 160-240 μm^2 .

The peripheral staining pattern of Annexin-V FITC in Cr VI-treated hepatocytes demonstrates the exposure of PS on the outer membrane surface, indicating early stage apoptosis [3,4]. Further evidence of apoptotic cell death is the shrinkage of the cells, the appearance of apoptotic bodies [8] and the loss of cell to cell contacts [9]. These findings show that, at low concentrations (5 μM), Cr VI causes apoptosis, whereas, at higher concentrations (25 μM) it can also cause necrosis. Defining the mode of cell death caused by metal ions such as Cr VI is an important initial step in further elucidating the extent and nature of the *in vivo* toxicological response.

Thus, titration of the propidium iodide reagent, coupled with a shorter exposure time, permits the use of this method in the detection of Cr VI-induced apoptosis in hepatocytes. Our data indicate that the Sigma Annexin V-FITC Apoptosis Kit, applied with the modifications described, can be used in toxicological studies for the detection of apoptosis induced by a wide range of hepatotoxins. It can also be used in drug screens for the development of chemotherapeutic agents that induce apoptosis, as well as for identifying agents that can be co-administered to protect normal hepatocytes from apoptosis.

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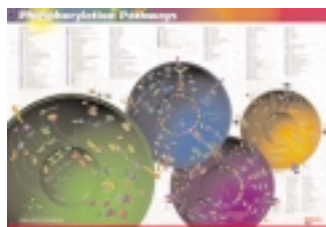
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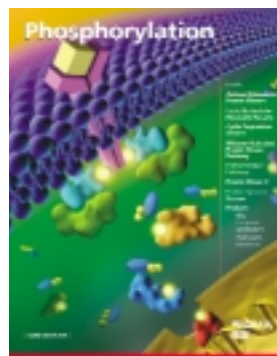
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