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IDENTIFICATION OF THE SUBSTRATE BINDING REGION OF VMAT-2 USING IODOAMINOFLISOPOLOL AS A NOVEL PHOTOPROBE

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Running Title:

Fluorenone derivative as a useful VMAT2 photoprobe

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List of Abbreviations

IAmF: Iodoaminoflisopolol; AZIK: 7-Iodo-8-azido ketanserin; TBZ: Tetrabenazine

CCCP: carbonylcyanide-m-chlorophenylhydrazone

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ABSTRACT

Monoamines, such as serotonin, dopamine and norepinephrine, are sequestered into synaptic vesicles by specific transporters (VMAT2), utilizing energy from an electrochemical proton gradient across the vesicle membranes. Based on our previous studies using photoaffinity labeling techniques in characterizing the VMAT2 specific ligands ketanserin and tetrabenazine, this study describes the synthesis and characterization of a fluorenone based compound, iodoaminoflisopolol (IAmF), as a photoprobe to identify the substrate binding site(s) of VMAT2. Using vesicles prepared from rat VMAT2 containing recombinant baculovirus infected Sf9 cells, we show inhibition of [^{3}H] 5HT uptake and [^{3}H] TBZOH binding by aminoflisopolol and iodoaminoflisopolol. The interaction of [^{125}I] IAmF with VMAT2 is highly dependent on the presence of ATP and an intact proton gradient. We report a simple and novel method to distinguish between a ligand and substrate using classical compounds such as [^{3}H]5HT and [^{3}H]TBZOH by incubating the compound with the vesicles, followed by washes with isotonic and hypotonic solutions. Using this method, we confirm the characterization of IAmF as a novel VMAT2 substrate. Sf9 vesicles expressing VMAT2 show reserpine and tetrabenazine protectable photolabeling by [^{125}I]IAmF. [^{125}I] IAmF photolabeling of recombinant VMAT2, expressed in SH-SY5Y cells with an engineered thrombin site between TMs 6 and 7, followed by thrombin digestion retained photolabel in a 22KDa fragment indicating that iodoaminoflisopolol binds to the C-terminal half of the VMAT2 molecule. Thus, IAmF possesses a unique combination of VMAT2 substrate properties and a photoprobe and is therefore, useful to identify the substrate binding site of the vesicular transporter.

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The vesicular monoamine transporter-2 (VMAT2) is the main transporter protein involved in sequestration of cytoplasmic neurotransmitters such as dopamine, serotonin and norepinephrine into vesicles for storage and subsequent release (Erickson et al., 1996; Erickson and Varoqui, 2000; Henry et al., 1994; Peter et al., 1995) and is inhibited by reserpine, tetrabenazine (Scherman et al., 1983) and ketanserin (Darchen et al., 1988) . The energy for amine transport is derived from a proton gradient generated by ATP hydrolysis. The proton gradient is coupled, by an unknown mechanism, to the transport of biogenic amines into the synaptic vesicle against a steep concentration gradient (Rudnick, 1998; Schuldiner, 1994; Schuldiner et al., 1998). Two protons are released from the storage vesicle in exchange for one substrate molecule transported to the inside of the vesicle (Kanner and Schuldiner, 1987).

The rat synaptic vesicular monoamine transporter (rVMAT2) contains 515 amino acids and a hydrophobic analysis of rat VMAT2 predicts 12 alpha-helical transmembrane segments, with a predicted large lumenal loop between TM1 and TM2 (Erickson et al., 1992).

Photoaffinity labeling is an extremely useful technique, which enables the direct probing of a target protein through a covalent bond between a ligand and its binding protein. Using photoprobes, previous studies from our laboratory identified the ketanserin and tetrabenazine (TBZ) binding regions of VMAT2 (Sievert and Ruoho, 1997). Analyses of the binding site peptides showed that while the ketanserin photoprobe [^{125}I]-7-iodo-8 azido ketanserin [(^{125}I] AZIK] derivatized mainly the N-terminal region, the TBZ photoprobe [^{125}I]2-N-[(3'-iodo-4'-azidophenyl)propionyl]tetrabenazine [^{125}I]TBZ-

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AIPP, labeled both the N- and C-terminal portions of the VMAT2 molecule. The binding of both ketanserin and TBZ are not dependent on the proton gradient, indicating that they bind to similar conformational states of the VMAT2 molecule (Darchen et al., 1988). Another important inhibitor of VMAT2, reserpine, is believed to have a low affinity binding site (which does not require the proton gradient) and a high affinity site (which requires the presence of a proton gradient) (Weaver and Deupree, 1982). It is hypothesized that reserpine begins to be transported in the same way as substrates such as serotonin or dopamine, however, due to the bulkiness of the compound, reserpine becomes trapped in the protein such that it can neither be transported nor readily released. This leads to the formation of a 'dead-end" complex, which may explain the "irreversible" nature of this inhibitor (Rudnick et al., 1990).

Our previous studies have successfully used photoprobes to identify specific binding regions of interacting proteins and drug interaction with receptors (Guo et al., 2005; Guo et al., 2006; Sievert et al., 2002; Sievert and Ruoho, 1997; Wu et al., 2001). In a previous study, in an attempt to develop compounds capable of directly probing the catechol binding region of the β_2-adrenergic receptor (β_2AR), our laboratory synthesized novel benzophenone- and fluorenone-based β_2AR antagonists as photoaffinity probes (Wu and Ruoho, 2000; Wu et al., 2001). While the benzophenone-containing ligands bound with relatively modest affinity, one of the fluorenone-based compounds, 4-(2-hydroxy-3-isopropylaminopropoxy)-7-amino-6-iodofluorenone (iodoaminoflisopolol, IAmF), showed very high affinity for the β_2AR, inhibiting [^{125}I]ICYP binding with an apparent K_i of approximately 1×10^{-9} M (Wu and Ruoho, 2000). In comparison to the benzophenone ligands, the fluorenone ligands have one additional carbon-carbon bond that creates a

planar unsaturated ring system that leads to a large increase in receptor binding affinity. Fig1A and 1B shows the chemical structures of serotonin, ketanserin, reserpine, tetrabenazine and iodoaminoflisopolol (with the pharmacophore region in bold). Unlike previous photoaffinity ligands, an attractive and unique feature of the fluorenone derivative IAmF is that the large planar unsaturated ring, which is similar to the indole ring of serotonin and reserpine, serves as both the binding pharmacophore and the photoreaction center for this molecule (Fig 1E). Therefore, based on the characteristics of this compound, we tested and found aminoflisopolol inhibits [^{3}H]5HT uptake in chromaffin granules with a low micromolar *K*i. In this study, we present data demonstrating that iodoaminoflisopolol shows VMAT2 specific substrate like properties and is a photoprobe and therefore, is ideally suited to probe the substrate-binding site on the VMAT2 molecule.

MATERIALS AND METHODS

Expression of rVMAT2 in Sf9 cells

The full-length cDNA for the rat synaptic vesicle monoamine transporter (VMAT2) containing a C-terminal polyhistidine epitope was engineered into baculovirus DNA for expression in *Spodoptera frugiperda* (Sf9) insect cells (Sievert et al., 1998). Sf9 cells were grown in suspension cultures in Ex-cell 420 media (SAFC Biosciences, Lenaxa KS USA) at 25°C in flasks maintained at 110 rpm. Plaque-purified viral lysate was used for infection of log-phase Sf9 cells (density 1X10^6 cells/ml) and cells were harvested 48-60 hours later. Cells typically looked large and infected with no increase in cell number and showed less than 5% lysis.

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[^{3}H]5HT uptake

Sf9 cells infected with rVMAT2 containing baculovirus were harvested and suspended in sucrose-HEPES buffer (SH buffer)- (0.3M –10mM, pH 7.6), passed through a cell cracker 20 times and centrifuged at a low speed (3000rpm/5min) to obtain a crude vesicle preparation, which was used to assess [^{3}H] serotonin uptake. Uptake assays were performed by incubating the vesicle preparation in SH buffer at 32°C for 10min in the presence of ATP-Mg (10mM), thus allowing the formation of a proton gradient followed by the addition of [^{3}H] 5HT (20nM) (30Ci/ mmol) (PerkinElmer Life and Analytical Sciences, Inc. Wellesley, MA). The reaction was terminated after 7min followed by a rapid vacuum filtration through GF-B filters (Brandel, Gaithersburg, MD) using a cell harvester. The filters were washed three times with SH buffer and the radioactivity retained on the filter disks was determined in a liquid scintillation counter. The nonspecific uptake of [^{3}H]5HT was subtracted from total uptake by including samples containing reserpine (10 μM) (Sigma-Aldrich, St. Louis MO).

[^{3}H] TBZOH binding

Specific [^{3}H] TBZOH binding was determined by incubating the vesicular preparations with [^{3}H] TBZOH (20nM) (20Ci/ mmol) ((Sievert et al., 1998) in the absence or presence of TBZ (10 μM) (Sigma-Aldrich, St. Louis MO) at 32°C for 60min. Following the incubation, the samples were rapidly filtered through GF-B filters using a cell harvester. The filters were washed three times with SH buffer and the radioactivity retained on the filter disks was determined in a liquid scintillation counter.

In order to assess the nature of the interaction of [^{125}I] IAmF with VMAT2, the compound was incubated with vesicle preparation obtained from infected Sf9 cells in the

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presence or absence of ATP. Also, the effects of the V-type ATPase inhibitor, bafilomycin A1 (10μM) (Sigma-Aldrich, St. Louis MO) or a proton gradient releaser carbonylcyanide-m-chlorophenylhydrazone CCCP (1μM) (Sigma-Aldrich, St. Louis MO) on the interaction between [^{125}I]IAmF and the VMAT2 protein were studied. Specificity was determined by tetrabenazine inhibition. Similar experiments were performed with [^{3}H]5HT and [^{3}H]TBZOH to obtain typical results for the known substrate (serotonin) and known inhibitor (TBZ).

Binding and uptake experiments were performed in triplicates and statistical analysis of data was performed using Prism 4 software (GraphPad Software Inc, San Deigo, CA).

Iodination of Aminoflisopolol

The fluorenone compound, aminoflisopolol, originally synthesized and identified as a β-2 adrenergic antagonist (Wu and Ruoho, 2000), was radioiodinated using carrier free [^{125}I] NaI (PerkinElmer Life and Analytical Sciences, Inc. Wellesley, MA) in the presence of Chloramine T (Sigma Aldrich, St. Louis MO). The compound was extracted using ethyl acetate and streaked on a TLC plate. Upon chromatography in solvent system containing methanol: ethyl acetate: triethyl amine (10:10:1), the plate was exposed to an X-ray film and the radioactive band corresponding area was scraped off the plate. The product was extracted in methanol and estimated for radioactivity. Carrier-free [^{125}I] AZIK was synthesized following a protocol described earlier ((Sievert et al., 1998). Briefly, 7-Aminoketanserin was iodinated with [^{125}I] NaI in the presence of chloramine T and purified 7-amino-8-[^{125}I]iodoketanserin was converted to 7-azido-8-[^{125}I]iodoketanserin by reaction with ice-cold $NaNO_2$ and 1 M NaN_3 in the dark and

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extracted with ethyl acetate.

Photolabeling VMAT2 using [^{125} I] (IAmF) and [^{125}I] AZIK

Sf9 cells infected with recombinant baculovirus were used to prepare vesicles as described above. The tubes containing vesicle preparations were transferred to ice and [^{125}I] IAmF (1.5nM) or [^{125}I] AZIK (1nM) was added. Following incubation on ice for 5 min, the tubes were moved to a waterbath at 32°C for 2 min and were photolyzed for 5 s in ice water at a distance of 10 cm from a water-jacketed 1-kilowatt high-pressure mercury vapor lamp (AH-6 bulb, Advanced Radiation Corporation Santa Clara CA). Fig1E shows the proposed photoreaction and covalent modification mechanism of transporter derivatization by [^{125}I]IAmF during photolysis. Following photolabeling, vesicles were solubilized with equal volumes of 2% digitonin (Gallard Schlesinger, Carle Place NY) for 1 hour in cold room and the soluble fraction was collected by centrifugation at 14000Xg/ 30min at 4°C. Soluble fractions were electrophoresed on a 12% SDS-PAGE gel and exposed to a phosphorimager (Molecular Dynamics, GE Healthcare, WI). Since the rVMAT2 possessed a 6XHis-tag at its C-terminal, the transporter was partially purified using a Ni-NTA resin (Sigma-Aldrich, St. Louis, MO). In some experiments, VMAT2 specific antibody (or IgG as control) and Protein A beads were used sequentially to pull down VMAT2 from the digitonin solubilized fraction (Sigma-Aldrich, St. Louis, MO) following manufacturer's protocols. The beads were washed and bound proteins were eluted with 1X Laemmli buffer, electrophoresed on a 12% SDS-PAGE and exposed to a phosphorimager.

Photolabeling of recombinant VMAT2 in SH-SY5Y vesicles

SH-SY5Y cells (ATCC number CRL-2266) were grown in 10cm dishes in DMEM (Mediatech, Herndon VA) supplemented with 10% cosmic calf serum and penicillin-streptomycin to 50-70% confluence in a 37°C/ 5% CO_2 incubator. 25 μg of plasmid, encoding deglycosylated, HA-tagged hVMAT2 containing an engineered thrombin site between putative TM6 and TM7 (Thiriot et al., 2002) was transfected using the *Trans*IT-LT1 transfection reagent (Mirus Bio, Madison WI) as per manufacturer's recommendations and harvested 42 hours later. The cells were suspended in 1ml of sucrose-HEPES buffer (0.3M-10mM) (pH 7.6), passed through a 27-guage needle three times and centrifuged at 1200Xg for 5 min to obtain a crude vesicle preparation. This vesicle preparation was used for photolabeling using the procedure outlined above.

Thrombin digestion

Photolabeled vesicle preparations from SH-SY5Ycells, transfected with VMAT2 construct, containing thrombin site in between TM6 and TM7, were digested with 0.2 units of thrombin (Promega Corp. Madison WI) at 37°C for various times. Reactions were stopped with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and snap-frozen in dry ice. The samples were denatured with 50mM dithiothreitol in 1X Laemmli buffer and separated by electrophoresis on a 12% SDS-PAGE to observe thrombin digestion pattern. In another experiment, the electrophoresed samples were transferred to PVDF membrane and immunoblotted with C-terminal anti-His antibody (Invitrogen, Carlsbad, CA) using a Western blot protocol.

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RESULTS

Baculovirus infected Sf9 cells express functional VMAT2

Vesicles prepared from recombinant VMAT2 expressing baculovirus infected Sf9 cells show increased binding of [^{3}H] TBZOH, with increasing concentrations of the ligand. The non-specific binding (in the presence of 10μM TBZ) was typically less than 10% of the total ligand bound to the vesicles. *K*d and Bmax values were determined by nonlinear regression of the saturation curves as 28nM and 119 picomoles per mg protein respectively. Vesicle preparation from 2 or 3 day post-infected Sf9 cells expressing VMAT2 showed uptake of [^{3}H] serotonin, which was dependent upon the presence of ATP-Mg in the assay buffer (Fig 3A). This uptake was specifically inhibited by reserpine, thus confirming the presence of functional VMAT2 in the Sf9 cells. Non-infected Sf9 cells did not show any reserpine protectable substrate uptake or TBZ-protectable ligand binding (data not shown). This is the first report of an ATP-dependent, reserpine protectable VMAT2 mediated uptake in vesicles using the baculovirus expression system.

[^{125}I] IAmF shows VMAT2 substrate like properties

In a displacement study, increasing concentrations of aminoflisopolol or iodoaminoflisopolol was used to inhibit [^{3}H] TBZOH (20nM) binding or [^{3}H] 5HT uptake (30nM) in vesicle preparations obtained from 3-day post-infected Sf9 cells. Non-specific binding (in the presence of 10μM TBZ or 10μM reserpine) was subtracted from the respective values. Fig 2A and 2B show the inhibition of [^{3}H] TBZOH binding and [^{3}H] 5HT uptake by IAmF with K_i values of 8μM and 0.8μM respectively. On the other

hand, the highest concentration of aminoflisopolol (the non-iodinated precursor of IAmF) showed less than 50% inhibition of [^{3}H] TBZOH binding (Fig 2C), but showed a K_i value of 0.3μM when assayed for inhibition of [^{3}H] 5HT uptake (Fig 2D).

The low micromolar K_i of IAmF inhibition of [^{3}H] 5HT uptake is very similar to K_i values of other established VMAT2 substrates (Erickson et al., 1996) Also, the ratio of K_i binding/ K_i uptake is 10, supporting the substrate nature of IAmF. It has been observed in previous studies by Rothman's group that while substrates show an increased values of the ratio of K_i binding/ K_i uptake, inhibitors show values less than 3 (Rothman et al., 1999). Similar experiments in our lab established a value of 0.1 for a known inhibitor of VMAT2 ketanserin and 205 for a known substrate norepinephrine (data not shown). These observations led us to further address the possibility that IAmF is a substrate for VMAT2.

In order to assess the substrate characteristics of [^{125}I] IAmF, we tested the interaction of [^{125}I] IAmF with VMAT2 by incubating the compound with vesicles prepared from infected Sf9 cells in the absence or presence of ATP, bafilomycin A1 and CCCP. As seen in figure 3A, a typical substrate such as [^{3}H]5HT showed almost no specific uptake in the absence of ATP and in the absence of a proton gradient. [^{3}H]TBZOH binding, on the other hand, was not significantly affected under any condition. [^{125}I] IAmF interaction with VMAT2, like [^{3}H]5HT, was highly dependent on the presence of ATP and also on the proton gradient, as observed by loss of specific interaction in the absence of ATP or in the presence of ATP along with the V-type ATPase inhibitor bafilomycin (10μM) or CCCP, a proton gradient releaser (1μM).

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As further proof, we utilized a simple experiment to distinguish between a substrate (defined as a compound which is taken up into the lumen of the vesicle by an uptake mechanism) and an antagonist (i.e. a compound that only binds to the VMAT2 molecule) using classical compounds such as [^{3}H]5HT and [^{3}H]TBZOH. These two scenarios could be distinguished by comparing the radiolabel retention following washes with isotonic (SH buffer: 0.32M sucrose, 10mM HEPES, pH 7.6) or hypotonic (Lysis buffer: 1mM HEPES, pH 7.6). In the case of a substrate, wash with hypotonic buffer would cause lysis of the vesicles, leading to loss of the radiolabeled compound sequestered during the assay. However, little or no loss of the photolabel is expected by washing with hypotonic buffer, if the compound in question is only bound to the VMAT2 in the membrane. As expected, treating the VMAT2 expressing Sf9 vesicles with hypotonic buffer following [^{3}H]5HT uptake led to more than 80% loss of radiolabeled 5HT, while less than 15% loss of radiolabel was observed, when similar vesicles were treated with hypotonic buffer following [^{3}H]TBZOH binding (Fig 3B). In order to determine if [^{125}I] IAmF is a substrate or an antagonist, vesicles expressing VMAT2 were incubated with [^{125}I] IAmF in the presence of ATP-Mg and in the presence or absence of reserpine. One set of vesicles was washed with SH buffer during filtration, while another set was washed with lysis buffer. Reserpine protectable uptake was measured and compared from filters washed with SH buffer and from lysis buffer in 3 separate experiments (Fig 3B). Significant loss of radiolabel (77%) following washing of vesicles with lysis buffer indicated that [^{125}I] IAmF interacted with VMAT2 as a substrate.

Since our studies showed that [^{125}I]IAmF behaved as a substrate, i.e. is actively transported into the lumen of the vesicle, instead of binding to VMAT2 as an antagonist,

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we determined the K_m of VMAT2 for [^{125}I]IAmF in a transport assay. In this case, the carrier free hot compound was supplemented with cold IAmF to obtain concentrations from 250nM to 200μM and the corrected specific activity values were used to calculate uptake in Sf9 vesicles expressing VMAT2 as picomoles /mg protein /min. The data obtained was fitted to a non-linear curve fit using GraphPad Prism software to obtain a K_m and V*max* of 122 μM and 292 picomoles/mg protein/min respectively (Fig 4). In case of serotonin, saturation curves showed that VMAT2 in these vesicle preparations transport [^{3}H] 5HT with a K_m of 359nM and V*max* of 101 nanomoles / mg protein / min. Similar studies performed in the presence of 10μM Aminoflisopolol showed a significant decrease in K_m (583nM), with a small decrease in V*max* (81 nanomoles/ mg protein/min).

[^{125}I]IAmF is a VMAT2 photoprobe

Vesicles obtained from baculovirus infected Sf9 cells were photolabeled with carrier-free [^{125}I]IAmF (1.5nM) in the presence or absence of reserpine and TBZ (10μM). Fig 5A(i) shows a phosphorimager scan of reserpine and TBZ protectable [^{125}I] IAmF photolabeled VMAT2 as seen on a 12% gel. Fig 5A(ii) shows a scan of a parallel experiment using [^{125}I] AZIK as a photoprobe. [^{125}I]AZIK had been shown previously to photolabel VMAT2 specifically (Sievert et al., 1998) and had been determined to bind at the TBZ binding site (Darchen et al., 1988).

As represented in Fig 5(iii), we used vesicles prepared from SH-SY5Y cells, previously transfected with hVMAT2, which contain a thrombin cleavage site between putative TM6 and TM7. This construct had been shown to express a functional protein capable of

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substrate uptake and ligand binding (Thiriot et al., 2002). In Fig 5(iii), we also show that [^{125}I] IAmF is capable of photolabeling this protein in a reserpine and TBZ protectable manner.

Since the rVMAT2 expressed in Sf9 cells has a 6XHis tag, the photolabeled soluble fraction was passed through a Ni resin and rVMAT2 was partially purified by eluting with an imidazole (500mM) containing buffer. Fig 5B(i) shows a phosphorimager scan of photolabeled VMAT2 protein in a Ni column eluate. The presence of photolabeling in the Ni-imidazole elution further showed that [^{125}I] IAmF is able to specifically photolabel VMAT2. The panel on the right (in Fig 5B) shows lack of photolabeling in a vesicle preparation from uninfected Sf9 cells.

Photolabeled vesicle preparations, which were solubilized with equal volumes of 2% digitonin and then incubated sequentially with VMAT2 antibodies (or IgG) and Protein A bound sepharose, immunoprecipitated VMAT2 from the solubilized fraction. Bound proteins were eluted with SDS sample buffer and separated on a 12% gel. A phosphorimager scan (Fig 5C) shows that VMAT2 antibodies (and not IgG antibodies), are able to immunoprecipitate photolabeled VMAT2, thus further confirming that [^{125}I]IAmF specifically photolabeled VMAT2.

Thrombin digestion of photolabeled VMAT2

An [^{125}I] IAmF photolabeling experiment was performed in vesicles prepared from SH-SY5Y cells transfected with recombinant VMAT2 containing an engineered thrombin site in the loop between TMs 6 and 7 (Thiriot et al., 2002). Thrombin digestion of the photolabeled vesicle preparation retained the photolabel in a 22KDa fragment indicating that [^{125}I] IAmF cross-linked within the C-terminal half of the VMAT2 molecule (Fig

6(i)). The figure in panel (ii) shows a phosphorimager scan of reserpine protection of 22kDa fragment obtained after thrombin cleavage confirming that this fragment was obtained from the VMAT2 protein. Panel (iii) shows a C-terminal anti-His Western blot of an SDS-PAGE gel, showing the presence of 6X-His epitope in the full-length and 22KDa fragment of photolabeled VMAT2, following thrombin digestion.

DISCUSSION

Vesicular monoamine transporters are proteins that transport biogenic amines into storage vesicles so that neurotransmitters are available for exocytosis upon stimulation. This study describes the characterization of [^{125}I] IAmF as a tool to identify substrate binding sites on VMAT2 expressed in Sf9 cells. Specific [^{3}H]TBZOH binding and [^{3}H]5HT uptake is entirely due to the infected baculovirus expressed VMAT2, since uninfected Sf9 cells show negligible specific binding or uptake. Having established an efficient expression system in Sf9 cells, we characterized iodoaminoflisopolol as a possible ligand/ substrate for VMAT2. This fluorenone based compound has been successfully used as a photoprobe in our laboratory ((Wu and Ruoho, 2000) and a similar compound was used to covalently modify the N-terminus of parathyroid hormone receptor stably expressed in HEK-293 cells (Han et al., 2000). Initial studies in our lab showed that AmF could inhibit [^{3}H]5HT uptake in chromaffin granules with a K_i of 0.4μM.

In the current study, we used increasing concentrations of IAmF and/ or AmF to inhibit [^{3}H]5HT uptake and [^{3}H] TBZOH binding in VMAT2 containing Sf9 vesicles. IAmF and AmF showed K_i values for inhibition of substrate uptake in the same range as

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the K_m for serotonin. However, while the highest concentration of AmF did not inhibit more than 50% of [^{3}H]TBZOH binding, an inhibition curve of [^{3}H]TBZOH binding with the iodinated form IAmF yielded a K_i of 8μM. Previous studies have shown that known VMAT2 inhibitors such as tetrabenazine, ketanserine and reserpine are potent inhibitors of both [^{3}H]TBZOH binding and [^{3}H]dopamine uptake, whereas known VMAT2 substrates (dopamine, norepinephrine and serotonin) inhibit [^{3}H]TBZOH binding very poorly, but show inhibition of [^{3}H]dopamine uptake in the high nanomolar-low micromolar range (Partilla et al., 2006; Rothman et al., 1999). A comparison of K_i values of AmF and IAmF in inhibition studies, indicated that addition of an iodine group adds bulk and renders the compound a poorer substrate compared to the non-iodinated precursor compound, AmF.

The substrate characteristic of [^{125}I]IAmF is evident in its specific interaction with the VMAT2 protein (as evidenced by TBZ protectable interaction) only in the presence of ATP and an intact proton gradient. Additionally, following isotonic or hypotonic washes, [^{125}I] AmF behaved similar to [^{3}H]5HT as 77% of reserpine protectable uptake was hypotonic lysis sensitive, while a known antagonist/ligand ([^{3}H]TBZOH) maintained about 80% of bound radioactivity following washes in hypotonic buffer. These results agree with the hypothesis that [^{125}I]IAmF behaves as a substrate for VMAT2.

An earlier report showed stereoselective uptake of another β–2 receptor antagonist, atenolol, into storage granules isolated from PC12 cells and chromaffin granules. Interestingly, atenolol was also shown to be a substrate for VMAT2 (Bagwell et al., 1989) since the uptake was ATP-dependent, reserpine protectable and nigericin sensitive. In previous studies, propranolol ((Bright et al., 1985; Street et al., 1984) and

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atenolol ((Bright et al., 1985) had been shown to be accumulated in rat cortical synaptosomes and released upon stimulation by elevated K^+, Rb^+ or Cs^+ ions. These observations, together with the results of this study, indicate that low levels of β-receptor antagonists may, as a rule, be accumulated in synaptic vesicles and subsequently released into the synaptic cleft, to further inhibit β-adrenergic receptors.

Reserpine protectable uptake was studied in Sf9 vesicles using increasing concentrations of [^{125}I]IAmF. This nonlinear saturation curve yielded a K_m of 122μM, which further indicates that this compound has a relatively lower affinity for the transporter compared to the classical substrates such as serotonin. The apparent discrepancy between K_m for [^{125}I] IAmF uptake and K_i for [^{3}H]5HT inhibition, indicates that while mere binding of the [^{125}I] IAmF to the transporter is sufficient to inhibit [^{3}H] 5HT uptake, the larger K_m value indicates a slower transport across the vesicular membrane, thereby suggesting that the binding of the compound to the transporter is not the rate-limiting step of the transport process. The V*max* of [^{3}H]5HT uptake (nanomolar values) is much higher than the Vmax of [^{125}I] IAmF (picomolar values) indicating that the latter is transported more slowly across the vesicular membrane. Saturation experiments performed in the presence of 10μM aminoflisopolol showed a small change in V*max*, but an increase in K_m of [^{3}H]5HT uptake, indicating that aminoflisopolol inhibits [^{3}H]5HT in a competitive manner and therefore, is likely to bind to the same/ similar site(s) on the VMAT2 protein as does the substrate, serotonin.

Photolabeling experiments demonstrated that [^{125}I]IAmF bound specifically to the transporter, since only by close proximity of the photolabel and transporter can there be a covalent modification. The specificity of the photoprobe was confirmed by the absence of

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photolabeling in the presence of reserpine (10μM) and TBZ (10μM). Also, photolabeled VMAT2 in vesicle preparations from infected Sf9 cells (but not uninfected cells) could be partially purified through a Ni resin or immunoprecipitated by VMAT2 specific antibodies, which further confirmed the specific interaction of the photolabel with VMAT2.

The native VMAT2 molecule is not cleaved by thrombin since it does not possess a thrombin cleavage site (Thiriot et al., 2002). The construct used in these experiments has a HA epitope in the large loop between TM1 and TM2 and 6X-His epitope at the C-terminal, apart from a thrombin site in between putative TM6 and TM7. Therefore a thrombin site, which was engineered midway into the VMAT2 sequence between putative TM6 and TM7, upon thrombin digestion yields 2 unequal sized fragments of 35KDa and 22KDa. [^{125}I] IAmF photolabeling of VMAT2 expressed in SH-SY5Ycells, followed by thrombin digestion retained radioactivity in the 22KDa fragment, thus indicating that the C-terminal half of the molecule contained [^{125}I] IAmF interaction site, which is different from the region modified by the antagonist photoprobe, [^{125}I]AZIK. Previously, Thiriot et al (2002), showed that HA epitope was detectable in the 35KDa fragment following Western immnoblotting with anti-HA antibody. Therefore, the presence of HA epitope in the 35KDa fragment and presence of photoprobe and 6XHis epitope in the 22KDa fragment supports the conclusion that the photoprobe [^{125}I] IAmF interacts with the C-terminal region of the transporter. A small amount of radioactivity was detected at the 22kDa region in the 0 time point, which may be due to an immediate partial cleavage of VMAT2 by thrombin.

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Previous studies have used site-specific mutagenesis to determine the critical residues important for substrate and transport activity. These residues were chosen based on their importance in other related transporters such as the tetracycline transporter Tn10 (Merickel et al., 1995), since vesicular transporters are evolutionarily related to multidrug transporters of the Major Facilitator Superfamily (Saier and Paulsen, 2001); (Schuldiner et al., 1995). Random mutations in BMR, a member of large family of H^+/ substrate antiporters gave rise to four independent mutants exhibiting altered spectra of cross-resistance to various drugs. All these mutations were clustered in the region of TMs 9-11 (Klyachko et al., 1997) (the homologous proposed TMs are in the 22kDa region of VMAT2). Similarly, residues important for drug specificity and recognition lie in TMs 10-11 (p-glycoprotein) (Hafkemeyer et al., 1998), TMs 8-9 and TMs 1-3 (TetA) (Yamaguchi et al., 1993), TMs 9 and 10 (lactose permease) (Kaback, 1992). Merickel et al (1997) mutated several charged residues that were predicted to reside in transmembrane domains of VMAT2 and showed that Asp-33 was essential for substrate recognition. Also, Lys-139 and Asp-427 in TM2 and TM11 respectively form an ion-pair, which promotes high affinity interaction with the substrate. Schuldiner et al (1995) speculated that the region of TMs 9-11 is involved in substrate binding more than any other region, but not exclusively. In VMAT2, most of the mutations affecting apparent substrate binding were found to lie in the region of TMs 1-3, 9 and 12 (Parsons, 2000). Based on these studies, in future, we will test the hypothesis that [^{125}I]IAmF, as a substrate photoprobe, would derivatize one or more residues within the region of TMs 9 and 12.

Lactose permease, the most well studied member of the major facilitator superfamily, has 12 TMs, most of which pack circumferentially around TM 7 to form a central pore region. Based on lactose permease model, Parsons (2000) proposed similar packing of 12TMs of VMAT2 around TM7 and proposed a rotating domain model, in which a proton and neurotransmitter bind to opposite sites of the rotating domain formed by TM7 and TM10 after entering separate dead-end chambers. This model would favor our proposed substrate binding region (TM7-12), based on the results of this study.

Our previous studies show that human VMAT2 Cys 126 in loop 1/2 and Cys 333 in loop 7/8 form a disulfide bond which contributes to efficient monoamine transport (Thiriot et al., 2002). Therefore, based on mutagenesis and photolabeling studies, TMs I, 2, 7, 8, 10 and 11 are speculated to be in close proximity. Therefore, multiple TMs are likely to contribute to the formation of the substrate binding site.

Since IAmF is a substrate for VMAT2 (as opposed to TBZ and AZIK, which are inhibitors), this specific unique photoprobe therefore, provides an excellent tool to study and identify the substrate binding site on the VMAT2 protein in order to understand the mechanism of reuptake and sequestration of monoamines in storage vesicles.

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LEGENDS FOR FIGURES

Fig 1: The chemical structures of reserpine, tetrabenazine, 7-iodo 8-azido ketanserin, serotonin and IAmF with the pharmacophore (in bold). Also shown is the photochemical reaction of IAmF with VMAT2 and the proposed mechanism of covalent bond formation during the photolysis reaction (E).

Fig 2A: Binding of [^{3}H] TBZOH (20nM) was assayed in triplicates in 2 day post-infected Sf9 cell vesicle preparation in the presence of increasing concentrations of IAmF (A) and AmF (C). Binding in the presence of TBZ (10μM) was subtracted from each of the values. Uptake of [^{3}H] 5HT (30nM) was assayed in triplicates in a 2 day post-infected Sf9 cell vesicle preparation in the presence of increasing concentrations of IAmF (B) and AmF (D) The assay buffer contained 0.3M sucrose and 10mM HEPES. pH 7.6

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and 10mM ATP-Mg. Uptake in the presence of reserpine (10μM)was subtracted from each of the values.

Fig 3: (A) Radioactive 5HT, TBZOH and IAmF were allowed to interact with vesicles from infected Sf9 cells in the absence or presence of ATP (10mM) or in the presence of bafilomycin A1 (10μM) or CCCP (1μM). VMAT2 specific radioactivity retained on the filter is shown normalized to values obtained in the presence of 10mM ATP-Mg. (B) Demonstration of a method to distinguish between a substrate and ligand by allowing the compounds to interact with the Sf9 vesicles expressing rVMAT2, followed by isotonic or hypotonic buffer washes. Radioactivity retained on the filters is shown as normalized DPM values to the values obtained upon isotonic washes.

Fig 4: Saturation uptake of [^{125}I] IAmF into Sf9 vesicles expressing VMAT2. The specific activity of carrier-free [^{125}I]IAmF was reduced by adding appropriate amounts of cold IAmF to obtain high concentrations of the substrate. Vesicles were incubated with increasing concentrations of [^{125}I]IAmF in the presence of ATP-Mg for 15min at 32°C and filtered using vacuum filtration on to GF-B disks. Radioactivity retained on the filter was counted in a gamma counter. Uptake was calculated as picomoles per mg protein per min after correction of specific activity.

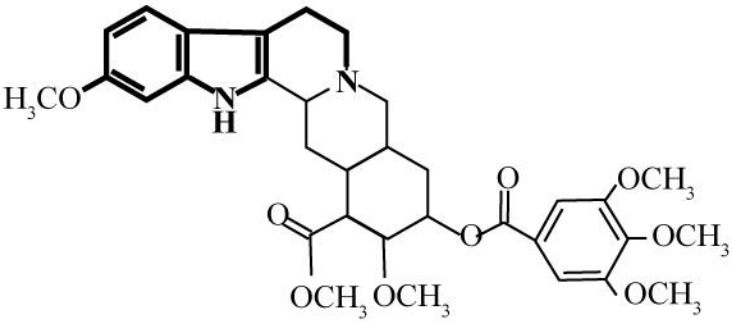
Fig 5 (A) Protectable photolabeling of VMAT2 by (i) [^{125}I] IAmF and (ii) [^{125}I] AZIK in Sf9 vesicles. Panel (iii) shows protectable photolabeling of VMAT2 by [^{125}I] IAmF in vesicles from transfected SH-SY5Y cells. (B) Vesicles from infected and uninfected Sf9 cells were photolabeled with [^{125}I] IAmF, digitonin solubilized and purified through a Ni-

NTA resin. Retention of [^{125}I] IAmF photolabeling of VMAT2 is shown by pre-incubation with TBZ or reserpine (10μM). Panel C shows immunoprecipitation of photolabeled VMAT2 from digitonin solubilized Sf9 vesicles using rVMAT2 specific antibodies (and not IgG) and Protein A beads.

Fig 6: (A) Time course of thrombin digestion of a [^{125}I] IAmF photolabeled vesicle preparation from SH-SY5Y cells transfected with hVMAT2 with an engineered thrombin site between TM6 and TM7 (Thiriot et al., 2002). The middle panel shows reserpine protection of the 22kDa fragment, generated from thrombin digestion of photolabeled transporter. (iii) Western immunoblotting of undigested and thrombin digested photolabeled transporter with C-terminal anti-His antibody. (B) shows the proposed sites of interaction of [^{125}I] IAmF with the VMAT2 molecule- also shown is the site of interaction of the inhibitor photolabel [^{125}I] AZIK which derivatized Lys 20 of VMAT2 (Sievert and Ruoho, 1997).

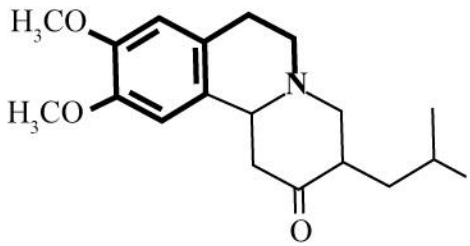
Fig 1

A



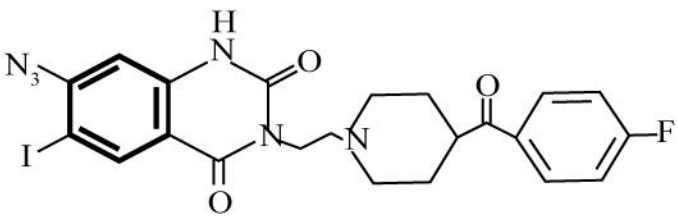
RESERPINE

B



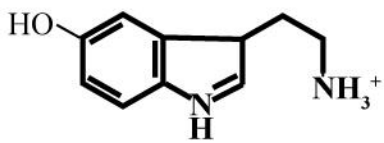
TETRABENAZINE

C



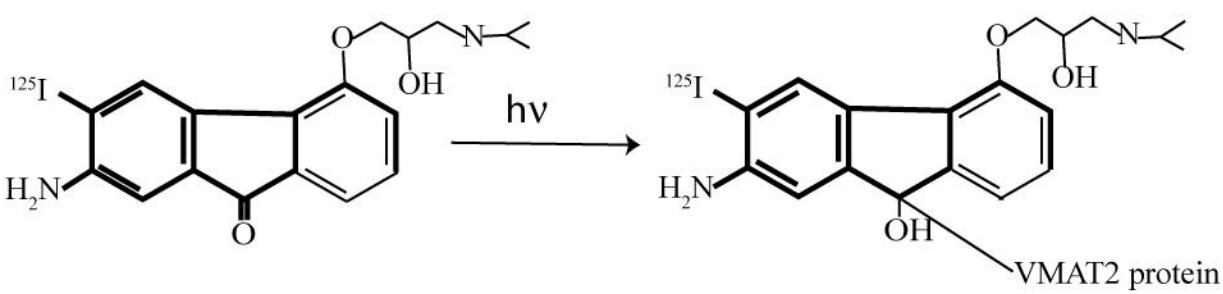
7-IODO 8-AZIDOKETANSERIN
(AZIK)

D



SEROTONIN

E

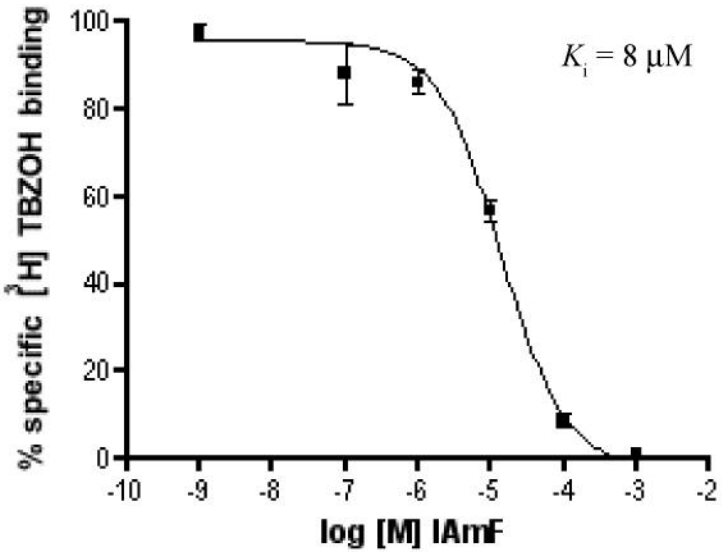


[^{125} I] IODOAMINO FLISOPOLOL
([^{125}I] IAmF)

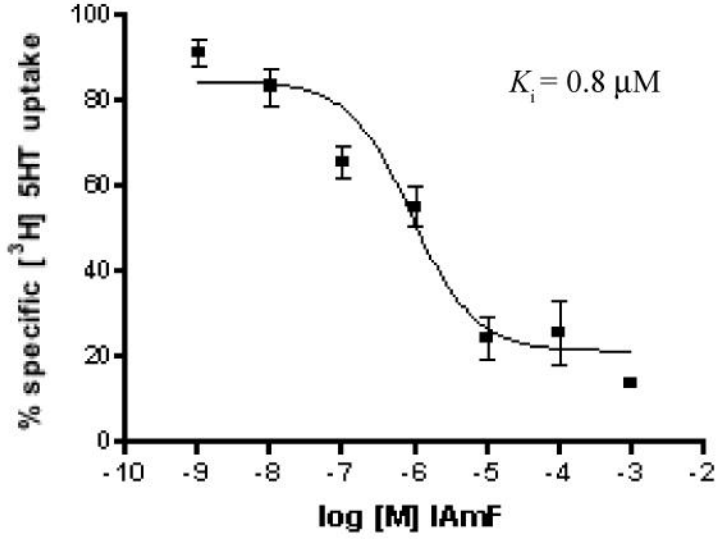
PHOTOREACTIVE PRODUCT

Fig 2

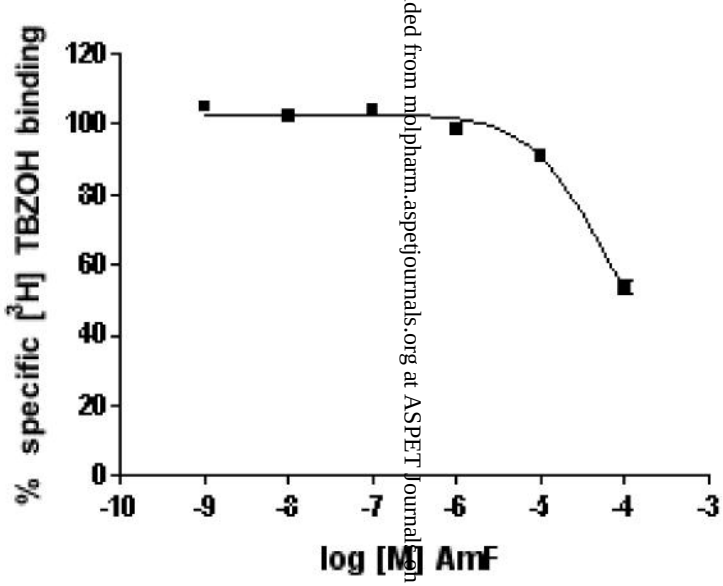
A



B



C



D

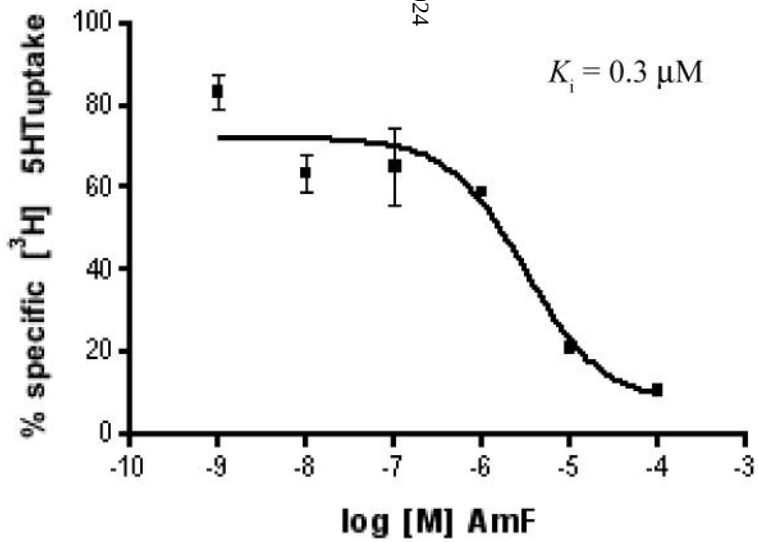
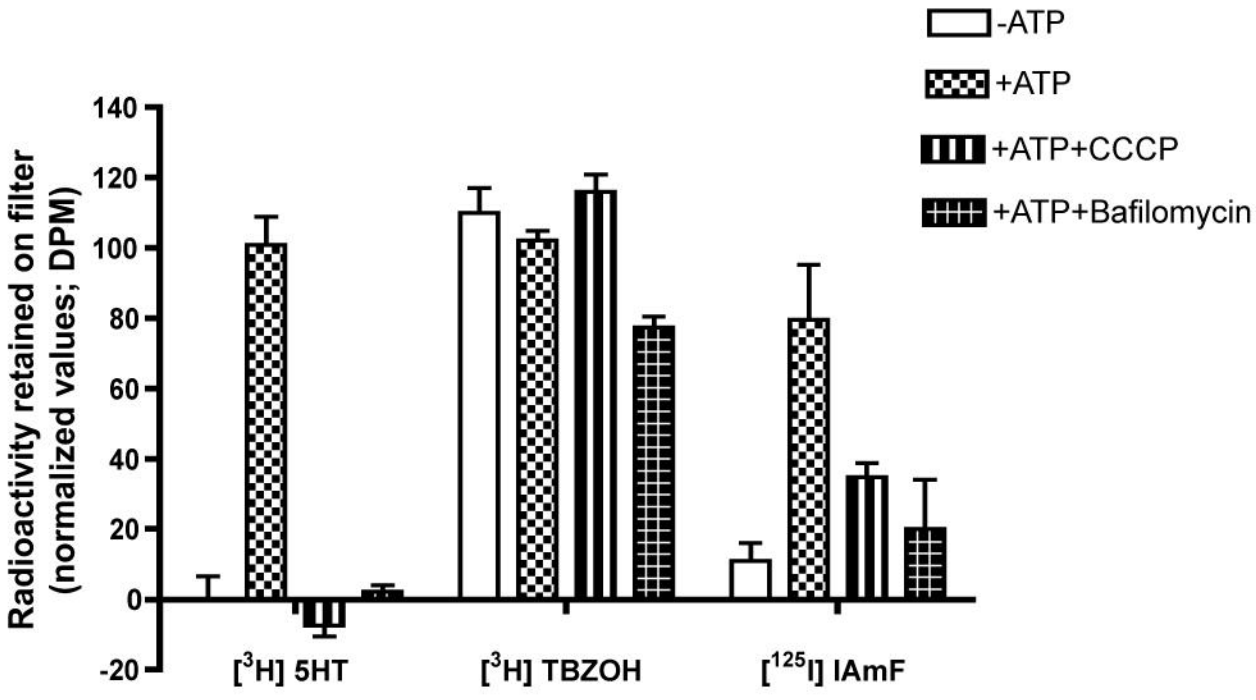


Fig 3

A


-ATP
+ATP
+ATP+CCCP
+ATP+Bafilomycin
Radioactivity retained on filter (normalized values; DPM)
140
120
100
80
60
40
20
0
-20
[3H] 5HT
[3H] TBZOH
[125I] IAmF

B

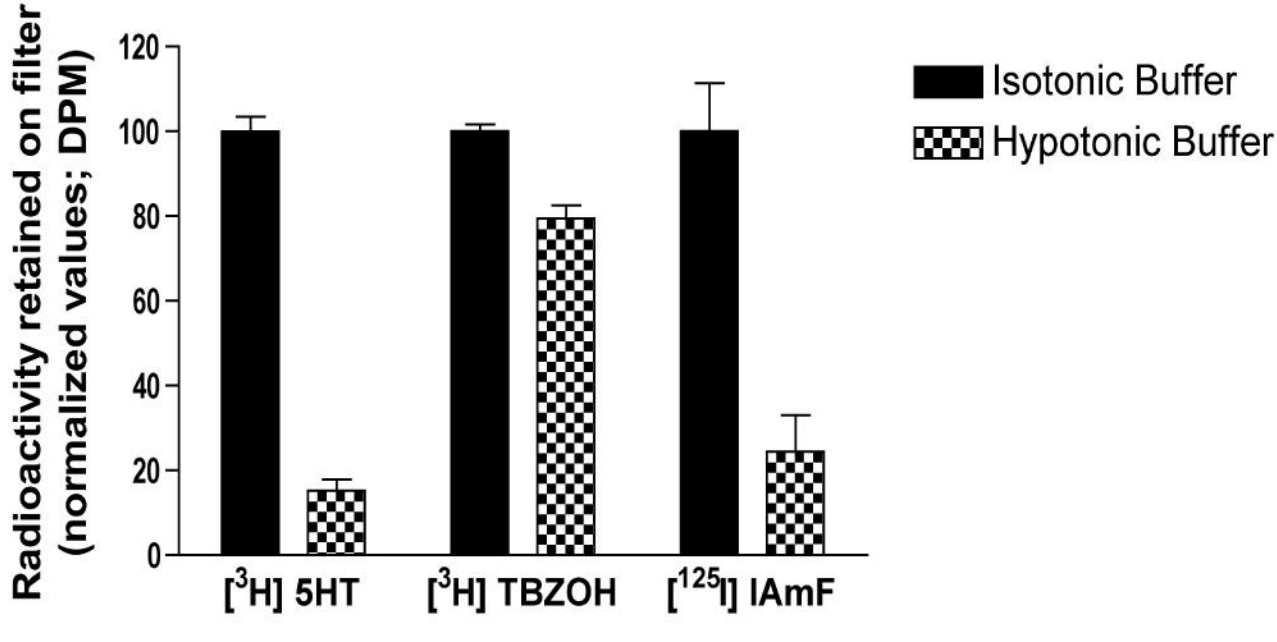

Isotonic Buffer
Hypotonic Buffer
Radioactivity retained on filter (normalized values; DPM)
120
100
80
60
40
20
0
[3H] 5HT
[3H] TBZOH
[125I] IAmF

Fig 4

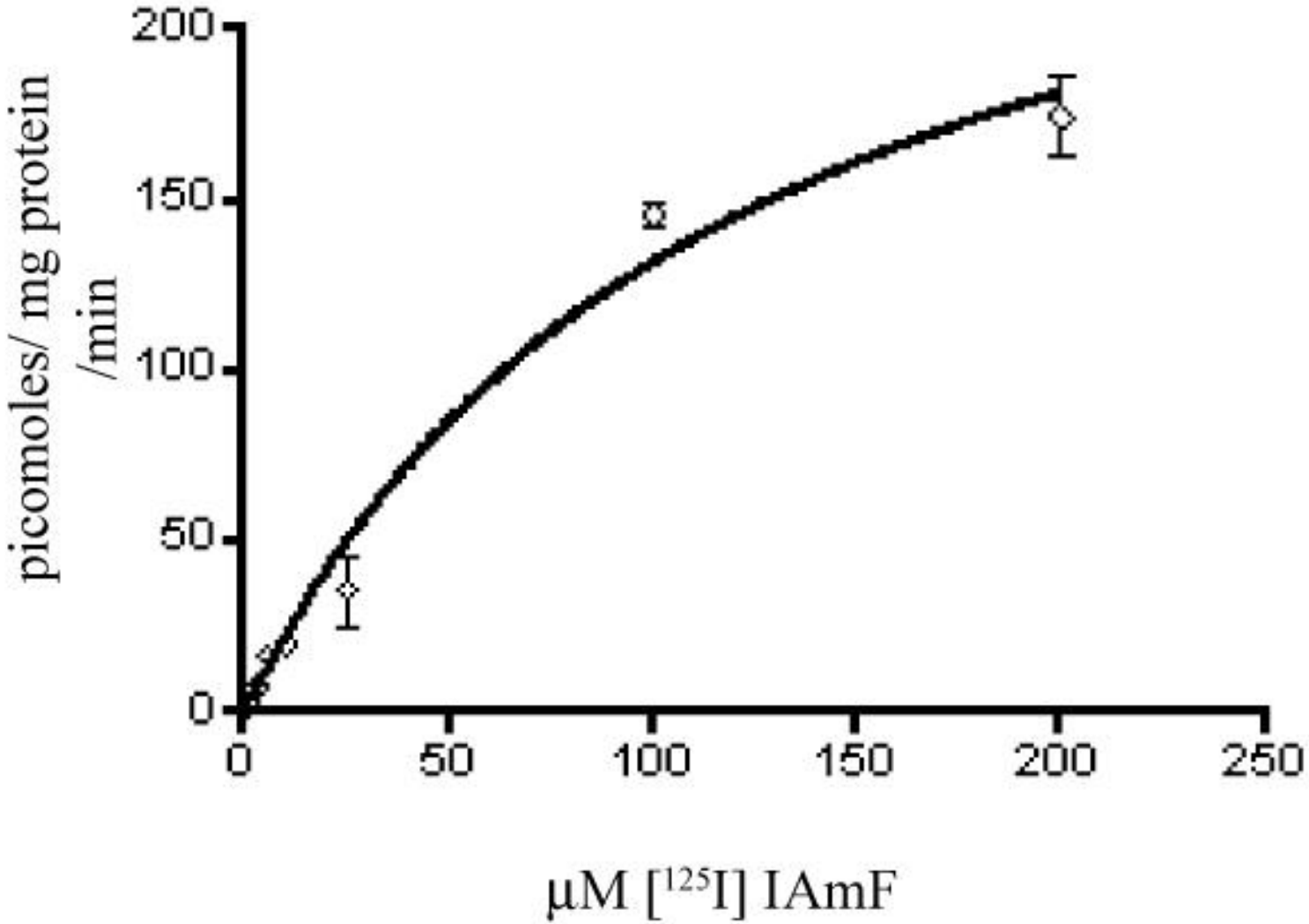

200
150
100
50
0
picomoles/ mg protein
/min
0
50
100
150
200
250
µM [125I] IAmF

Fig 5

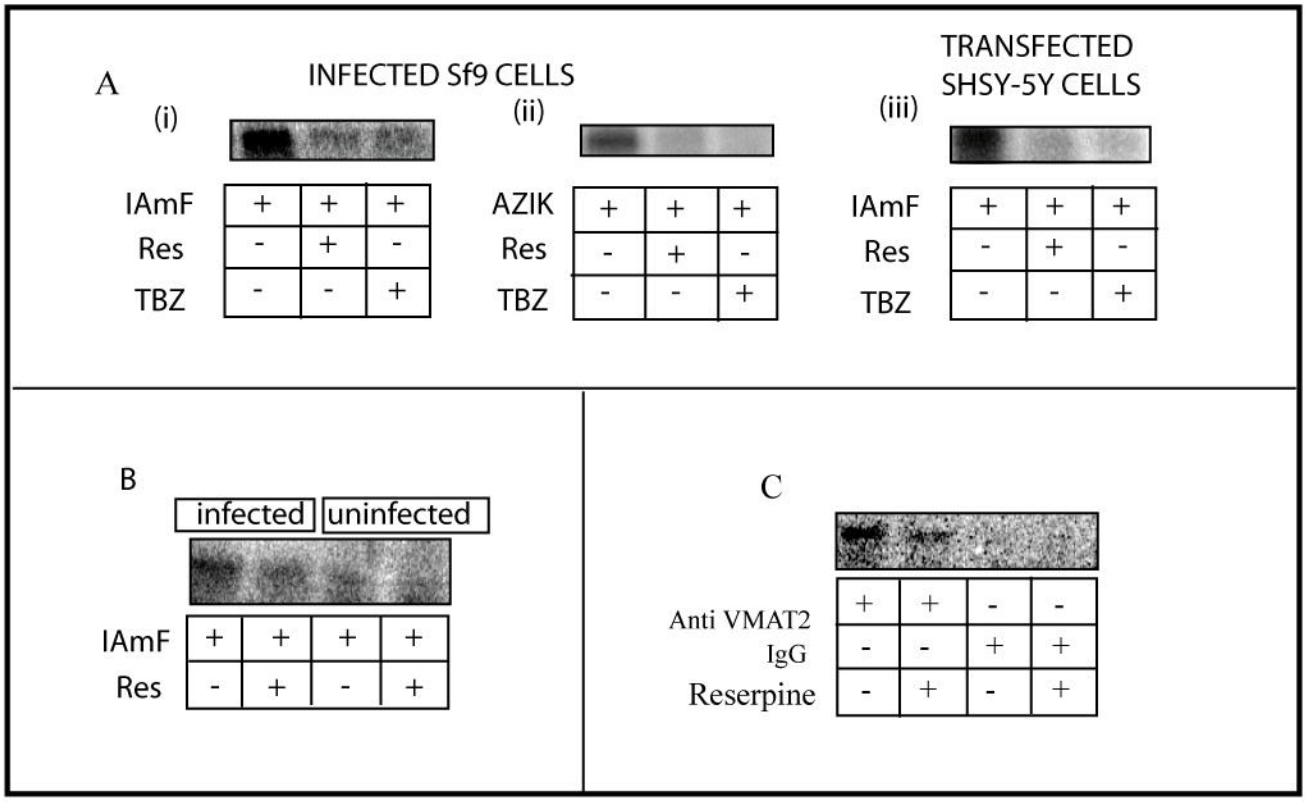

A
INFECTED Sf9 CELLS
(i)
IAmF + + +
Res - + -
TBZ - - +
(ii)
AZIK + + +
Res - + -
TBZ - - +
TRANSFECTED
SHSY-5Y CELLS
(iii)
IAmF + + +
Res - + -
TBZ - - +
B
infected
uninfected
IAmF + + + +
Res - + - +
C
Anti VMAT2 IgG + + - -
- - + +
Reserpine - + - +

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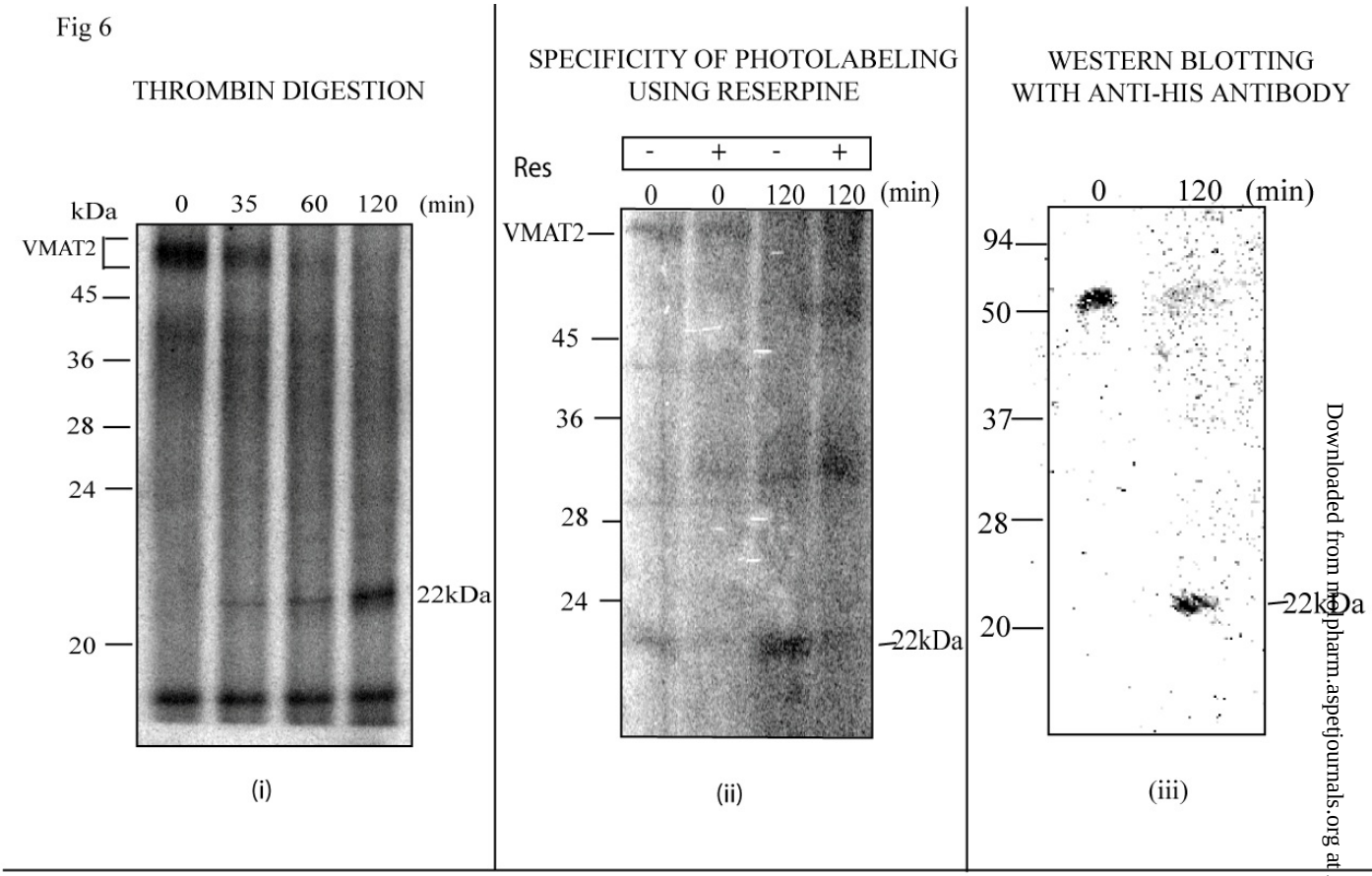
Fig 6



C

PROPOSED REGION OF INTERACTION OF [^{125}I] IAmF WITH THE VMAT2 TRANSPORTER