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**Interaction of Prolyl Oligopeptidase with α-Synuclein *in vivo* and *in vitro***

Anne-Marie Lambeir

Laboratory of Medical Biochemistry, University of Antwerp - Campus Drie Eiken, Universiteitsplein 1 building S, B-2610 Wilrijk, Belgium

Phone +32-3-2652549, Fax +32-3-2652685, email anne-marie.lambeir@ua.ac.be

Running title: Prolyl Oligopeptidase and α-Synuclein

**Abstract**

Prolyl oligopeptidase (PO) interacts with α-synuclein *in vitro*. It is a weak interaction that induces a nucleation prone conformation of α-synuclein. PO accelerates aggregation and fibril formation of α-synuclein in a process that can be reversed by specific inhibitors and is also influenced by an impairing mutation in the PO active site. There is evidence that PO and α-synuclein also interact inside the cell, especially in conditions where the expression of α-synuclein is high. Specific PO inhibitors reduce the number of cells with α-synuclein inclusions in a cellular model of Parkinson's disease. If these interactions also exist in the human brain, PO may be a target for treatment of Parkinson's disease and other synucleinopathies. Whether PO also contributes to the normal physiological functions of α-synuclein remains an open question, but there are some intriguing parallels between the proposed functions of both proteins that deserve further investigation.

**Keywords**

Amyloid, peptidase, neurodegeneration, neurotransmission, Parkinson's disease, prolyl oligopeptidase, synuclein.

## 1. Introduction

α-synuclein is a fairly abundant brain protein. Since its discovery in 1997 as a major factor in the pathology of Parkinson's disease (PD), research has expanded immensely (reviewed, amongst others, by Surguchov [1], Uversky [2] and Bisaglia [3]). There is good evidence that α-synuclein aggregation contributes to the pathology of PD. Toxic α-synuclein aggregates are involved in the loss of dopaminergic neurons in the substantia nigra, leading to the typical motor and behavioral symptoms of PD. α-synuclein is a major component of Lewy bodies and Lewy neurites [4], not only found in the brains of PD patients but also in other neurodegenerative diseases collectively known as "synucleinopathies" -Alzheimers, multiple system atrophy, amyotropic lateral sclerosis, neurodegeneration with brain iron accumulation, Down syndrome, Gaucher disease [1]. In human, there are three synucleins, called α, β, and γ. Only α-synuclein forms aggregates. Besides a role in pathology, α-synuclein has been attributed a wide array of other functions in the healthy brain. Most of these relate to trafficking and release of neurotransmitter vesicles in the synapses. α-synuclein knock-out mice are viable and grow up without any disease phenotype. However, upon further investigation, they appear to have defects in dopamine related activities [5]. α-synuclein is a relatively new protein in evolution, with no prokaryotic precursor. It is believed to have arisen when more and more complex synaptic transmission and plasticity processes developed in eukaryotic organisms [6].

Prolyl oligopeptidase (PO), on the other hand, is a very "old" enzyme that occurs in most organisms of the tree of life [7]. It is strongly conserved among species. PO was discovered in human placenta as an oxytocin degrading enzyme, long before the discovery of α-synuclein [8]. PO is present in most cells and tissues, but recent studies have focused on the brain [9-12]. The reason for this is that PO inhibitors were shown to have a positive effect on learning and memory in scopolamine induced amnesia models in rats and mice. Several reviews discuss the effect of PO inhibitors on experimental brain injury and neurodegeneration [13-15]. Generally, the mode of action of the inhibitors was attributed to a prolongation of the active half-life of neuropeptides, some of which are involved in learning, memory and cognition. A few inhibitors were tested in humans, in phase I and II clinical trials [16, 17], but , perhaps due to lack of a clear indication, these studies were abandoned. PO research received a new impetus with the discovery that PO knock-out counteracts the effect of lithium on signaling pathways in the model organism *dictyostelium discoidum* [18]. Subsequently, the groups of A. Mudge and A. Harwood joined forces and showed that PO inhibitors also reverse the effect of lithium and mood stabilizing drugs on growth cone collapse in developing sensory neurons in cell culture [19]. This work moved the actions of PO from the extracellular space (where neuropeptides are released) to the cytosol where, amongst others, known targets of lithium (glycogen synthase kinase-3 and inositol monophosphatase) and growth cone associated proteins (e.g. growth associated protein-43, GAP-43) are located. PO was back as a drug target and a new burst in inhibitor development followed [20-27]. Extrapolating mostly from the *dictyostelium* work, the prevailing hypothesis for a decade was that PO peptidase activity was somehow involved in the regulation of $IP_3$ signaling, but a specific peptide substrate was never identified [28, 29]. More recently, however, there is new evidence that PO has other actions in the brain, that do not require a catalytically competent active site, but instead are based on protein-protein interactions [30]. It is at this stage that PO and α-synuclein research converge.

The remaining of this review covers recent findings that support the hypothesis that in certain circumstances the old housekeeping peptidase PO, due to its protein binding properties, interferes with the intricate actions of the synucleins that regulate neurotransmission.

## 2. Proline specific cleavage of α-synuclein fragments

Neuropeptides typically are released in the synaptic cleft from neurotransmitter vesicles, but, unlike neurotransmitters, there is no re-uptake. The prevailing idea is that they are rapidly inactivated by extracellular proteases. Intrigued by the apparent discrepancy between the cytosolic localization of PO and the sequestration of its putative substrates in secretory vesicles, Brandt and collaborators looked for other types of peptides that may exist in the cytoplasm [31]. Using MS/MS peptide sequencing techniques, they identified C-terminal fragments of α-synuclein and β-synuclein, among several other peptides derived from intracellular proteins. The peptides found to be PO substrates in this work, varied between 7 and 20 amino acids, consistent with previous observations that PO does not cleave peptides longer than 30 amino acids. The place of the α-synuclein derived peptide (that was cleaved by PO) in the full length sequence is shown in Figure 1.

There is good evidence that proteolysis plays a role in the formation of toxic α-synuclein aggregates and fibrils. The non-Aβ component (NAC) fragment (α-synuclein$^{61-95}$) was originally identified in plaques of Alzheimer patients [32]. Several investigators [33-35, and references therein] reported C-terminally truncated fragments, as well as NAC, with a higher propensity to form fibrils *in vitro* and *in vivo*. Moreover, C-terminally truncated forms of α-synuclein form complexes that serve as nuclei for fibril formation by full length α-synuclein. Whereas increased aggregation is associated with truncated N-terminal peptides and the NAC domain, C-terminal fragments were reported to regulate proteolysis of full length α-synuclein [35].The most likely origin of both N-terminal and C-terminal fragments is from turnover of α-synuclein by the proteasome [36]. When the proteasome is overloaded or inhibited, accumulation of toxic α-synuclein aggregates occurs. Apart from the proteasome, m-calpain and several matrix metalloproteinases (MMP) have been implicated in the formation of truncated forms (Figure 1). An interesting feature of the α-synuclein sequence is the C-terminus (aa 101 -140) which is rich in negatively charged amino acids, small side chains (Ser and Thr) and contains 5 prolines. Proteins with such a “PEST” sequence are assumed to be rapidly turned over in the cell and PO has often been pinpointed in this context [37]. However, the half-life of α-synuclein is not particularly different from that of other cytosolic proteins [34].

Since proteolysis by the 20S proteasome and MMP-1 releases relatively short, proline rich peptides from the C-terminal part, PO might play a role in the final stages of α-synuclein turnover in the brain.

## 3. PO accelerates α-synuclein aggregation *in vitro*

As expected, PO does not cleave full length α-synuclein *in vitro*. Instead, prolonged incubation of purified recombinant α-synuclein with high concentrations of PO accelerates the aggregation [38]. The rate enhancement depends upon the PO concentration. Active site inhibitors, which bind to the catalytic serine side chain, reverse the acceleration. The acceleration does not occur with a

catalytically impaired PO mutant; but the aggregation curves are different from the control experiment, indicative of an altered interaction between PO and α-synuclein.

Purified α-synuclein spontaneously aggregates and forms beta-fibrils at 37°C when stirred or shaken for several days. The time course of the aggregation can be followed by turbidity measurements and fibril formation by the appearance of thioflavin T fluorescence [39, 40]. An example of the effect of PO on α-synuclein aggregation is shown in Figure 2. The aggregation process of α-synuclein is characterized by a nucleation phase, represented by a lag phase in the turbidity versus time plot. The nucleation phase is followed by the propagation phase, causing the sigmoidal burst in turbidity and approach to equilibrium at longer incubation times. During the propagation phase large aggregates are formed and transformed into beta-fibrils. Analysis of aggregation (such as in Figure 2) and thioflavin fluorescence curves leads to the conclusion that PO mainly increases the nucleation rate (a shorter lag time) without a significant effect on the propagation rate (little change in the slope of the central part of the curve). These results indicate that PO interacts with the monomeric forms of α-synuclein. Based upon the concentrations of synuclein required to see the effect, it is a low affinity interaction with a $K_d$ in the micromolar range [41].

It is not uncommon for intrinsically unstructured proteins, like α-synuclein, to have transient, low affinity interactions with a fairly large number of binding partners [42]. The long list included in the review of 2008 [1] already needs expanding by a number of new α-synuclein binding proteins, for example the prolyl isomerases called FK506 binding proteins [43] and components of the endoplasmatic reticulum–associated degradation system (ERAD) [44]. It is noteworthy that PO also has a growing list of protein binding partners in the cell, including tubulin [45] and two intrinsically unstructured proteins, the growth associated protein GAP-43 and α-synuclein [30]. In the latter case it appears that PO stabilizes a conformation of α-synuclein that promotes the formation of nucleating centers for aggregation. The aggregates formed in presence of PO are indistinguishable from the typical thioflavin-staining beta-fibril formed by α-synuclein alone (Figure 2) [41]. A question now presents itself: is such a transient, nucleation promoting interaction also relevant *in vivo*?

**4. PO inhibitors affect α-synuclein aggregation in living cells**

α-synuclein is an abundant protein, it amounts to 1% of the soluble cytosolic protein fraction [1, 46 and references therein]. It is well accepted that toxic α-synuclein aggregates only form when the α-synuclein concentration is exceptionally high, for instance in familial forms of PD where gene duplication or triplication has occurred. Mitochondrial oxidative stress, ER-protein aggregation stress and proteolytic stress (when the proteasome is blocked) also induce elevated levels of α-synuclein [1, 2, 44]. It therefore appears quite plausible that the α-synuclein concentration rises above the critical concentration for aggregation in certain stress conditions. PO is considered as quite an abundant peptidase in neurons, at least in comparison with other proline specific peptidases [13-15, 31]. PO activity can be measured in cell homogenates using fluorogenic or chromogenic substrates, such as *Z*-gly-pro-*p*-nitroanilide. In lysates of SH-SY5Y neuroblastoma cells the specific activity of PO is around 10 mU/mg (unpublished results). Using the specific activity of recombinant porcine PO (12 U/mg) and a molecular weight of 80 kDa, this amounts to approximately 0.1% of the soluble protein in cell homogenates, with an estimated concentration of 5-50 nM in the cytoplasm. Thus there is a 50-fold molar excess of α-synuclein over PO in normal conditions. Keeping in mind that α-synuclein is

increased under various stress conditions, this brings the concentration of PO and α-synuclein in the range used for the *in vitro* studies, where a maximal increase in aggregation rate is observed with 20 nM PO [38].

Recently researcher from the university of Leuven described a neuronal cell culture model for synucleinopathy [43]. These recombinant SH-SY5Y cells overexpress α-synuclein. In normal cell culture conditions only about 20% of the cells have intracellular α-synuclein aggregates, visualized by thioflavin S staining. Oxidative stress increases the percentage of α-synuclein aggregate containing cells and induces apoptosis [43]. PO inhibitors, such as *Z*-pro-prolinal or UAMC-00021 [38], counteract these effects[1] [41]. An example of this is shown in Figure 3. In α-synuclein overexpressing neuroblastoma cells, as previously observed in wild type SH-SY5Y cells and other neuronal cell types, PO can be observed in particles located in the perikaryon. PO levels and subcellular distribution is not significantly affected by oxidative stress, but the perinuclear distribution is lost after nocodazole treatment, which disrupts the microtubules [45]. A similar phenomenon has been described for α-synuclein aggregates, which appear in aggresome-like particles, transported to the perinuclear space via dynein-mediated retrograde transport along the microtubules [47]. In aggregate containing α-synuclein overexpressing SH-SY5Y cells, PO immunoreactivity sometimes colocalizes with α-synuclein (not shown). During the PSP-meeting in Halle (Germany, May 2010), T. Myöhänen reported similar results with neuroblastoma cells expressing the human pathological A30P α-synuclein mutant[2]. Colocalization of PO and α-synuclein aggregates disappear upon treatment with a specific PO inhibitor, indicating that an interaction is lost [48]. At the moment it is not clear what kind of interactions play a role here, binding with monomeric forms/aggregates or conformational changes induced by the inhibitor. However, if these interactions also occur in the human brain, PO may be a target for the treatment or prevention of PD and other synucleinopathies.

## 5. Unexplored parallels in PO and α-synuclein research

### 5.1. Lithium, growth cone collapse, GAP-43 and inositol phosphates

One way of studying factors which interfere with lithium is to monitor the collapse of growth cones at the end of neurites of developing neurons in culture. Di Daniel and collaborators intended to confirm previous results obtained with PO inhibitors by studying lithium induced growth cone collapse using neurons isolated from PO knock-out mice [30]. Surprisingly they found that the morphology of growth cones in the neurons taken from PO null mice is aberrant. The abnormality can be reversed by transfecting cells with wild type PO, but also with a catalytically incompetent PO mutant. This is the first convincing report on a function of PO mediated by protein-protein interactions in animals. The observation could be explained by restoration of an interaction between PO and the growth cone associated protein GAP-43. Di Daniel and collaborators showed that GAP-43 is a binding partner of PO by yeast-two-hybrid experiments and co-immunoprecipitation. Whereas GAP-43 is certainly a plausible partner to explain the defects in growth cone morphology, it is still

[1] Pieter Van Der Veken, Vilmos Fülöp, Dean Rea, Melanie Gerard, Anne-Marie Lambeir, Koen Augustyns. Design, structure and biological activity of new prolyl oligopeptidase inhibitors. In preparation.
[2] Timo T. Myöhänen, Roos Van Elzen, Melanie Gerard, Pieter Van Den Veken, Veerle Baekelandt, Pekka T. Männistö, Anne-Marie Lambeir. The interaction between Prolyl oligopeptidase and alpha-synuclein is disturbed and aggregation is reduced by a selective inhibitor. In preparation.

possible that other interactions, e.g. with α-synuclein, are also restored in this experiment. It is noteworthy that Di Daniel *et al*. besides GAP-43 also identified α-synuclein as a binding partner of PO by yeast-two-hybrid experiments [30]. After the initial results with *Dictyostelium discoidum* [18], several investigators studied PO in relation to inositol phosphate signaling. PO knock-down or inhibition in an astroglioma cell line causes an approximately 30% increase in the $IP_3$ concentration [28]. In mouse brain PO colocalizes with the $IP_3$ receptor-1 [9]. On the other hand, in conditions where a PO inhibitor improves spacial memory in young rats, there is no change in $IP_3$ concentration in the brain [49]. In conclusion, there is no convincing evidence in the literature that PO inhibition causes an increase in $IP_3$ concentration in neurons. Nevertheless, the involvement of PO in $IP_3$ regulated functions of α-synuclein would be an interesting new area of research. $IP_3$, on account of its $Ca^{2+}$ mobilizing action, may indirectly affect neurite outgrowth and synaptogenesis, synaptic transmission and plasticity, as well as cell survival [50]. Binding of α-synuclein with phospholipase Cβ2, the enzyme that releases $IP_3$ from $PIP_2$ in the membrane, has also been reported [51].

**5.2. Binding to tubulin**

Both PO and α-synuclein bind to tubulin [45, 52, 53]. Microtubule directed trafficking of α-synuclein has been extensively studied, both in view of its functions in neurotransmitter release and synaptic maintenance and considering the fate of α-synuclein aggregates [reviewed in 1, 47]. Recent studies also indicate that a direct interaction between α-synuclein and tubulin interferes with tubulin polymerization and dynamics, while promoting α-synuclein aggregation [52, 53]. An interaction between PO and α-tubulin was identified by yeast-two-hybrid screening. The interaction site localizes to a 130 amino acids C-terminal fragment where also tau and the microtubule associated proteins 1 and 2 have a binding site [45]. This observation feeds the believe that PO is involved in intracellular transport and protein secretion. Indeed, PO inhibition causes an increase in protein secretion in human U-343 glioma cells. The availability of a PO knock-out mouse will undoubtedly facilitate the elucidation of a role for PO in intracellular trafficking of synuclein and its role in neurotransmitter release and recycling.

**5.3. Subcellular localization of PO and α-synuclein**

The perinuclear localization of PO and α-synuclein aggregates certainly agrees with their respective interactions with microtubules. However, there are other observations where both PO and α-synuclein currently present a challenge for investigators.

The first observation is that, although both proteins are primarily cytoplasmic, a significant percentage of PO and α-synuclein is found associated with membranes [2, 54]. Quite a large body of work describes the interaction of α-synuclein with phospholipid vesicles and how this interaction induces conformational changes in α-synuclein and curvature or pores in the membranes [reviewed in 3]. Membrane-bound PO was reported already quite some time ago in tissue homogenates [55] and, notably, purified from synaptosomal membranes [56, 57], the exact location where α-synuclein functions. More recently an association of PO with ER and Golgi membranes was found [54,58], which might be related to ER-Golgi trafficking of vesicles, a process that is disrupted by α-synuclein overexpression in yeast [59].

A second observation that puzzles scientists is the fact that PO and α-synuclein sometimes locate to the cell nucleus. The nuclei of α-synuclein expressing cells stain positive for α-synuclein, dependent

upon the type of antibody used, but do not contain α-synuclein fibrils [60]. An antibody that fails to stain α-synuclein in the nucleus recognizes an epitope that is located at the C-terminus of the protein, QDYxP, coincidentally the very same sequence that was discovered in brain homogenates by Brandt and coworkers (Figure 1). There is no firm evidence for a regulatory role of α-synuclein in transcription, but it is an area of intensive research. Nuclear PO immunoreactivity has not been observed in mature brain, but appears sometimes in somatic cells [10]. The current hypothesis is that PO migrates to the nucleus at certain stages during cell growth or differentiation.

Finally α-synuclein and PO have the common property to be secreted without a signal peptide or an ER targeting sequence [1, 7]. PO and α-synuclein are observed in body fluids, CSF or blood plasma, but the origin of these extracellular pools and the mechanism of secretion remain largely obscure. α-synuclein levels in CSF and PO activity in plasma have been proposed to be useful for diagnostic/prognostic purposes, to monitor disease progression or response to treatment [13,61]. Further investigation of this phenomenon is warranted, especially in the case of PO, where the published methods for activity measurements and their relevance have been seriously criticized [e.g. 62, 63].

**6. Conclusion**

This review of recent literature shows that there is good evidence for a molecular interaction between α-synuclein and PO. In addition there are several scarcely studied instances where PO intertwines with functions attributed to α-synuclein.

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**Figure legends**

**Figure 1**: Proteolytic cleavage sites in α-synuclein.
The sequence of α-synuclein is divided in several domains based upon the amino acid composition. The NAC domain corresponds to the fragment originally discovered in amyloid placques [32]. The PEST sequence is part of the acidic tail that stabilizes against aggregation. The C-terminal peptide identified as a PO substrate in porcine brain extract [29] is underlined. Two horizontal lines under the sequence locate documented cleavage sites for matrix metalloproteinases (|), m-calpain (▲) and the 20S proteasome (▼) [33-35].

**Figure 2**: The effect of PO on α-synuclein aggregation *in vitro*.
Panel A shows the effect of 10 nM PO (gray curve) on the aggregation of 200 µM α-synuclein in vitro [38]. Analysis of such turbidity traces indicates that PO accelerates the nucleation rate, causing a decreased lag period (arrow) while the propagation rate is little affected (slope of the dotted lines)[39, 40]. Panel B and C show an electron microscope picture of α-synuclein aggregates in absence and presence of PO, respectively.

**Figure 3**: Effect of PO inhibitors on α-synuclein deposition in a cellular model of synucleinopathy.
Oxidative stress induces the deposition of α-synuclein aggregates in α-synuclein overexpressing neuroblastoma cells. Addition of 10 µM PO inhibitors UAMC-21[36] and *Z*-Pro-Prolinal (ZPP) reduce the percentage of cells with deposits. Experiments were performed and analyzed as described [43].

**Figure 1**

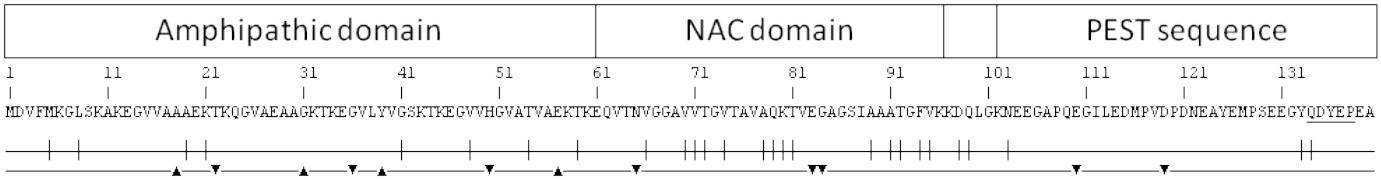



**Figure 2**

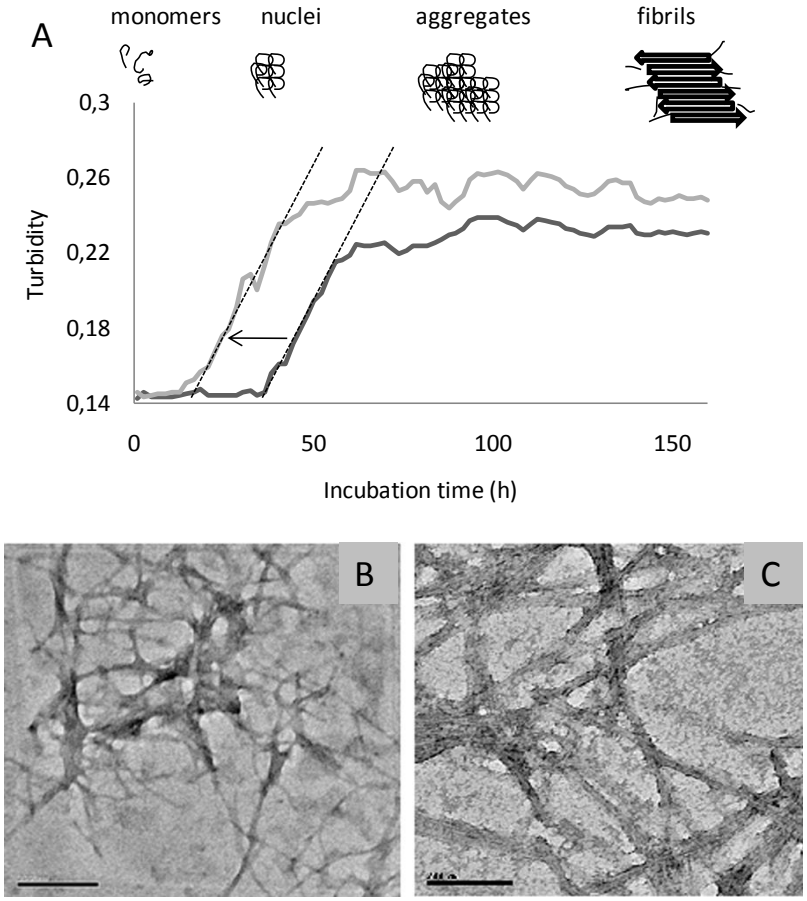
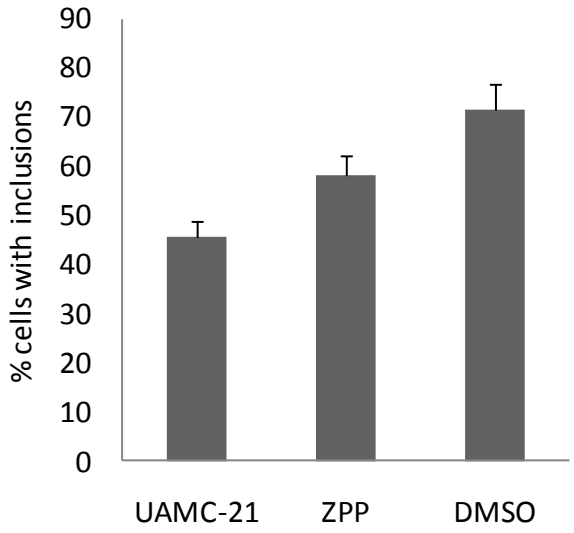


**Figure 3**


90
80
70
60
50
40
30
20
10
0
% cells with inclusions
UAMC-21
ZPP
DMSO