UNIVERSIDADE DA BEIRA INTERIOR
Ciências

Biosynthesis and isolation of STEAP1 protein from *Escherichia coli* cells lysates

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Resumo

O cancro da próstata é um dos cancros mais frequentemente diagnosticado nos últimos anos em todo o mundo e o mais comum na Europa, principalmente na região norte e ocidental, onde afeta mais de duzentos homens por 100.000. Em Portugal, a Associação Nacional de Urologia refere-se a este tipo de cancro como o mais comum em homens adultos, mas também refere que uma deteção precoce incrementa a taxa de sucesso no tratamento poderia atingir 85%. É a segunda causa de morte por cancro e é mais frequente em homens com mais de cinquenta anos. As terapias usadas atualmente são ineficientes e limitadas e não permitem o correto tratamento eficaz desta doença. Assim, novas abordagens ter devem ser avaliadas e novas terapias devem ser desenvolvidas de modo a aumentar a taxa de cura. De modo a alcançar esse objetivo, novas moléculas têm surgido com elevado potencial na patogénese do cancro da próstata.

A proteína *six transmembrane epithelial antigen of the prostate 1* (STEAP1) foi identificada como estando sobrexpressa em tecidos cancerígenos, designadamente em casos de cancro da próstata. A localização desta proteína na membrana celular e os seis domínios transmembranares sugerem uma participação na comunicação celular, mediando o transporte de iões e pequenas moléculas. Adicionalmente, a sobrexpressão em linhas celulares tumorais e a sua topologia tornam esta proteína um promissor alvo terapêutico para imunoterapia no cancro da próstata. Assim, torna-se essencial o desenvolvimento de estudos estruturais e de interação, de forma a obter uma elevada quantidade de proteína altamente purificada.

Assim, o objetivo principal deste trabalho é desenvolver uma plataforma laboratorial para a biossíntese, solubilização e purificação da proteína STEAP1. Os resultados obtidos demostraram que o uso de meio de cultura SOB, com IPTG a 1,25 mM como indutor após 5h de fermentação e com DMSO em 1,5% (v/v) são as condições ideais para a biossíntese da proteína STEAP1, em pequena escala. Adicionalmente, a aplicação de lactose como indutor influencia a compartimentalização da proteína alvo e promove a sua solubilidade. Na etapa de solubilização, os resultados obtidos demonstraram que a proteína STEAP1 é eficazmente solubilizada com uma concentração de 1% de Triton X-100.

Relativamente à etapa de purificação por IMAC, foram testadas colunas carregadas com níquel e cobalto, com diferentes concentrações de imidazol, nomeadamente 5, 7, 8.5 e 10 mM no passo de ligação, de modo a promover a retenção completa da proteína alvo. Dos perfis obtidos consta-se que várias proteínas do hospedeiro *E.coli* eluem juntamente com a proteína STEAP1, sendo assim necessárias otimizações adicionais por forma de obter uma amostra purificada da proteína STEAP1.

Palavras-chave

Cancro da próstata, STEAP1, Proteínas membranares, Biossíntese, *Escherichia coli*, IMAC.

Resumo Alargado

O cancro da próstata é um dos cancros mais frequentemente diagnosticados nos últimos anos em todo o mundo e o mais comum na Europa, principalmente na região norte e ocidental, onde afeta mais de duzentos homens por 100.000. Em Portugal, a Associação Nacional de Urologia refere-se a este tipo de cancro como o mais comum em homens adultos, mas também refere que uma deteção precoce incrementa a taxa de sucesso no tratamento poderia atingir 85%. É, no entanto, a segunda causa de morte por cancro e é mais frequente em homens com mais de cinquenta anos. As terapias usadas atualmente são ineficientes e limitadas, além do conforto do paciente ser posto em causa, e não permitem o correto tratamento desta doença. Existe ainda a possibilidade da ocorrência de infeções causadas pelas biópsias necessárias para o diagnóstico. Assim, novas abordagens terapêuticas devem ser avaliadas e desenvolvidas com o intuito de aumentar a taxa de cura.

A fim de alcançar esse objetivo, novas moléculas têm surgido com um elevado potencial na patogénese do cancro da próstata.

A proteína *six transmembrane epithelial antigen of the prostate 1* (STEAP1) foi identificada como estando sobrexpressa em tecidos cancerígenos, designadamente em casos de cancro da próstata. A localização desta proteína na membrana celular e os seis domínios transmembranares sugerem uma participação na comunicação celular, mediando o transporte de iões e pequenas moléculas, como é o caso do sódio, potássio e cálcio, bem como citocinas e quimiocinas. Adicionalmente, a sobrexpressão em linhas celulares tumorais, tais como LNCaP e PC3, a sua topologia e a ausência em órgãos vitais fazem desta proteína um promissor alvo terapêutico para imunoterapia no cancro da próstata. Assim, estudos estruturais e de biointeração são necessários, de forma a ter um conhecimento mais aprofundado sobre a sua estrutura tridimensional e de que modo esta proteína influencia a tumorogénese do cancro da próstata. Para tal, é necessário obter a proteína de interesse em quantidades bastante elevadas e com um grau de pureza sustentável que permita a sua cristalização.

O objetivo principal deste trabalho é desenvolver uma plataforma laboratorial para a biossíntese, solubilização e purificação da proteína STEAP1 e para a sua purificação. De forma a atingir esses objetivos, várias tarefas foram delineadas: construir o vetor pET101-STEAP1 e fazer a transformação de *E.coli*, avaliar vários meios de fermentação complexos (TB, SOB, SOC e 2YT), estudar o processo de indução (tipo e concentração de indutor, tempo de indução), avaliar a estabilização da proteína STEAP1 com *chaperones* (DMSO, arginina, sorbitol), bem com testar a solubilização da proteína alvo com detergentes e purificar a biomolécula de interesse.

Os meios TB, SOC e 2YT apresentaram densidades óticas mais reduzidas e níveis inferiores de expressão da proteína STEAP1. Assim, o uso de meio de cultura SOB, com IPTG a 1,25 mM como indutor após 5h de fermentação e com DMSO em 1,5% (v/v) são as condições ideais para a biossíntese da proteína STEAP1. Além disso, os resultados demonstraram que a

aplicação de lactose como indutor poderia influenciar a compartimentalização da proteína alvo e aumentar a sua solubilidade. A proteína foi recuperada com 1% do detergente não iónico Triton X-100.

Relativamente à etapa de purificação por IMAC, foram testadas colunas carregadas com níquel e cobalto, com diferentes concentrações de imidazol no passo de ligação, nomeadamente 5, 7, 8.5 e 10 mM no passo de ligação, de modo a promover a retenção completa da proteína alvo. Utilizando 5 mM de imidazol na fase móvel a proteína alvo não ligou à coluna. Foram então testadas concentrações superiores de forma a promover uma retenção da mesma, sendo que os melhores resultados foram obtidos com uma concentração de 7 Mm no passo de ligação, apesar da retenção não ser completa. A análise por Western blot demonstra que apesar de ser eliminada uma grande fração de proteínas do hospedeiro que eluem com a STEAP1.

Deste modo, é necessário considerar um ou mais passos adicionais de isolamento da biomolécula alvo de modo a remover todos os contaminantes da matriz.

Palavras-chave

Cancro da próstata, STEAP1, Proteínas membranares, Biossíntese, *Escherichia coli*, IMAC.

Abstract

Prostate cancer is one of the most frequent cancers diagnosed in last years, around the world, and the most common in Europe, mainly in northern and western Europe, where affects more than 200 men in 100.000. In Portugal, the Urology National Association refers to this type of cancer as the most common in adult men but also refers that a precocious detection, increase the treatment success rate to 85%. It is the second cause of death by cancer, and is the most frequent in men over 50 years. The therapies used nowadays are inefficient and limited and do not provide the efficient treatment of this disease. So, new approaches must be assessed and new therapies must be developed to increase the rate of cure. In order to achieve this goal, new molecules arise with a potential role in prostate cancer pathogenesis.

The six transmembrane epithelial antigen of the prostate 1 (STEAP1) was found overexpressed in cancer tissues, namely, in prostate cancer cases. The location of this proteins in cell membrane and the six transmembrane domains suggests a role in cell-cell communication, mediating the transport of ions and small molecules. Additionally, its overexpression in cancer cell lines, associated with its topology, makes this protein a promising therapeutic target for immunotherapy. Thus, structural and interaction studies are required, and to achieve that, large amount of highly purified proteins are required.

Therefore, the main goal of this work is to develop a laboratorial platform for the STEAP1 protein biosynthesis, solubilization and purification. The obtained results showed that the use of SOB medium, with IPTG at 1.25 mM as inducer after 5h of fermentation and using DMSO at 1.5% (v/v) are the ideal conditions for the biosynthesis of recombinant STEAP1 protein, in small scale. Moreover, applying lactose as inducer may influence the compartmentalization of the target membrane protein and promote its solubility. Concerning the IMAC purification step, a nickel and a cobalt charged columns were used, with different imidazole concentrations in binding step, in order to promote the compete retention of the target biomolecule. Although, several host proteins from *E.coli* elute along the STEAP1 protein, being necessary additional optimizations in order to obtain a purified STEAP1 sample.

Keywords

Prostate cancer, STEAP1, Membrane proteins, Biosynthesis, *Escherichia coli*, IMAC.

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ACRATA	Apoptosis, cancer and redox associated transmembrane
ADC	Antibody-drug conjugate
APC	Antigen-presenting cell
AR	Androgen receptor
AS	Active surveillance
ATPS	Aqueous two-phase system
BPH	Bening prostatic hyperplasia
CBX	Chromobox homolog 7
CgA	Chromogranin A
CMC	Critical micelle concentration
COX	Cyclooxygenase
CTL	Cytotoxic T-lymphocyte
CV	Column volume
Cx	Connexin
DHT	Dihydrotestosterone
EAU	European Association of Urology
EGFR	Epidermal growth factor receptor
EMEA	European Medicine Agency
ER	Estrogen receptor
FDA	Food and Drug Administration
FNO	$F_{420}H_2$:$NADP^+$ oxidoreductase
GSTP1	Glutathione s-transferase P1
HIFU	High-intensity focused ultrasound
HRPC	High risk prostate cancer
IGFR	Insuline-like growth factor receptor
IMAC	Immobilized Metal Affinity Chromatography
IPTG	Isopropyl-ß -D-thiogalactopyranoiside
LH	Luteinizing hormone
MRI	Magnetic resonance imaging
MW	Molecular weight
NRF	Nuclear receptor family
NSAIDs	Nonsteroidal anti-inflammatory drugs
OD	Optical density
ORF	Open reading frame
OS	Overall survival
PCa	Prostate cancer

PCR	Polymerase chain reaction
PIA	Proliferative inflammatory atrophy
PIN	Prostate intraepithelial neoplasia
PR	Progesterone receptor
PRC1	Polycomb repressive complex 1
PSA	Prostate-specific antigen
PTM	Post translational modifications
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
RP	Radical prostatectomy
RT	Radiotherapy
SNP	Single nucleotide polymorphisms
STAMP1	Six transmembrane protein of prostate
STEAP1	Six transmembrane epithelial antigen of the prostate 1
TAA	Tumour-associated antigen
TAE	Tris-acetic acid
TCTP	Translationally controlled tumour protein
Tf	Transferrin
TfR1	Transferrin receptor 1
TMPRSS2	Type II transmembrane serine protease

1. Introduction

1.1 Overview of prostate anatomy-physiology

The human prostate is an accessory gland of the male reproductive system and together with the seminal vesicles, secrete a nutritive medium called seminal fluid, which forms the bulk of semen (30-50%) and contributes to sperm motility and also neutralize the acidic conditions of vaginal tract [1,2].

It is localized frontal to the rectum and below the bladder and surrounds the initial part of the urethra, the prostatic urethra. The urethra trough the prostate and at its apex is designated by membranous urethra. It is about 3 cm thick and consists of branched tubulo-acinar glands enclosed by stroma [3]. The anterior and apical surfaces are bounded by the fibromuscular stroma, namely collagenous stroma and muscle fibres, while the posterior and lateral parts are enclosed by a fibrous capsule [2]. The stroma that gives support to prostate gland is composed by a mixture of collagenous fibrous tissue and smooth muscle fibres [2,4].

Nowadays, it is assumed that the prostate is divided in three glandular zones: the transition zone, the central zone and the peripheral zone (Figure 1).

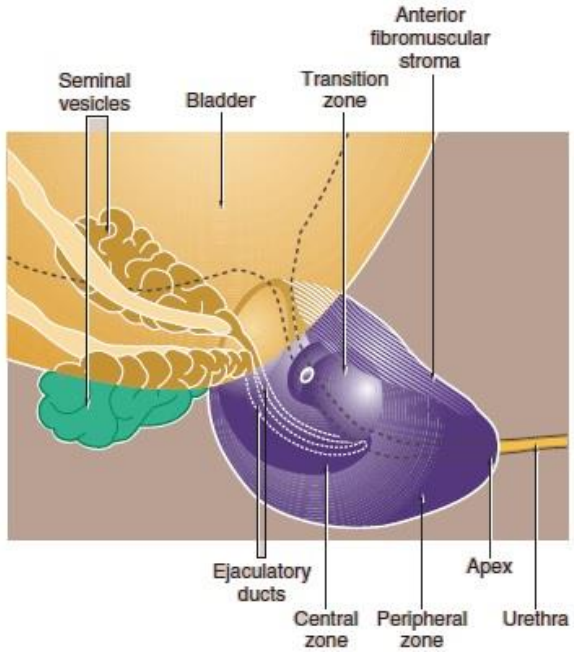

Figure 1 – Schematic representation of the male human prostate and its different zones (Adapted from [1]).

The transition zone surrounds the prostatic urethra, while the central zone surrounds the ejaculatory ducts [5]. The peripheral zone is the major region of the prostate gland, being responsible for 70% of the total glandular volume. The prostate also has a non-glandular section, the anterior fibromuscular zone. The different zones of the gland are more implicated in different disease scenarios: while benign prostatic hyperplasia (BPH) is more likely to develop in transition zone, the prostate cancer (PCa) tends to develop in peripheral section [1,5].

Due to the epithelium folds, the prostate presents an irregular shape [2]. The prostate epithelium is formed by two different glandular layers: the secretory luminal layer and the basal cells [6]. The first one is constituted by columnar cells and are responsible by prostatic secretions, which contains growth factors and the prostate specific antigen (PSA), and also where the androgen receptor (AR), the CK8 and 18 are expressed. Typically, cuboidal form cells which have the ability to produce keratin, cytokeratin 5 and 14 and p63 [7]. Also, they could express the insulin-like growth factor receptor (IGFR), epidermal growth factor receptors (EGFR), the estrogen receptor (ER), and progesterone receptor (PR) [1,3].

Several features are attributed to the basal layer, specifically this cellular layer may act like a stem cell reservoir that can differentiate into the secretory luminal layer cells, and has an important role in molecular exchange between luminal cells and extracellular space. These basal cells are separated from the stroma by a basement membrane [1,7,8]. There is another rare type of cells present in prostate gland, neuroendocrine cells. These cells are located between the epithelial cells and typically express chromogranin A (CgA).

The prostate growth and its secretory activity of is regulated by that cells, and occur during the fetal state and also in puberty. The growth of this gland is reinitiated around the 40 years of life [9,10]. At this age, the balance between the cell proliferation and death is unbalanced, favouring the first phenomena. Also, the ratio of cell proliferation and death is maintained mainly by androgens, being testosterone the dominant molecule that reach peripheral tissues [1].

The prostate is the preferential tissue in which testosterone is metabolized to 5α-dihydrotestosterone (DHT) by action of the enzyme 5α-reductase type II, after binding to the gland through the AR, localized in the luminal layer [11,12]. Indeed, androgens, are the mainly regulation factors that regulate and control prostate cells proliferation and differentiation [13-15]. With aging, it is possible to identify the formation of *corpora amylacea*, small hyaline masses also present in glial cells and pulmonary alveoli. These masses are formed by dense secretions that accumulate in some glands, which can develop into spherical concretions [16,17].

The DHT prostatic concentration decrease with aging, while concentration of testosterone does not suffer any variation due to the age. Thus, the ratio of serum sex steroid concentration is unbalanced in favor of estrogens, suggesting a putative role in BPH and PCa [1,3].

1.2 Prostate cancer

1.2.1 Epidemiology

The PCa is one of the most frequent cancers diagnosed in last years, around the world, and the most common in Europe, mainly in northern and western Europe, where affects more than 200 men in 100.000 [18,19]. The differences in the use of PSA testing in different countries and regions help to explain the incidents rate variations [20]. This type of cancer increased 28%, in 20 years, in European population with age between 35 and 64 years old, and is estimated an increase of 1% per year, in worldwide population [18,21]. While this data is increasing in developed countries, the inverse is noticed in developing countries. The typical lifestyle of modern societies leads to these statistics. Also, the increase of PSA test, mostly in developed countries, contributes to this values [22]. In all Europe, in 2008, were detected almost 382 thousand cases of PCa. In 2012, this number increased to almost 420.000. In the same year, the number of deaths caused by PCa was 92.000. The increase in detection cases of PCa, is mainly due to PSA test [23,24]. In 2015, only in United States of America, PCa accounting about one quarter of total cancers diagnosed, about 220.000 [25].

In Portugal, the Urology National Association refers to this type of cancer as the most common in adult men but also refers that, with a precocious detection, the treatment success rate could reach 85%. Is the second cause of death by cancer, and is the most frequent in men over 50 years. In fact, in Portugal, 82 in 100.000 men were affected by PCa, being the rate of fatalities of 33% [26]. In the future, new serum or urine markers will make possible new detection procedures and contribute to an even superior rate of PCa cases early detected and consequently, a decreasing rate of fatalities [24].

1.2.2 Risk factors

Several factors could contribute to PCa developing. As in other cancers, PCa can arise due to several factors, but that doesn´t mean that the occurrence of these factors are crucial, by itself, to tumour developing.

The risk factors for PCa could be distinguished in two groups, depending on whether they are inherent to the individual, or not. Thus, the described endogenous risk factors are age, hormones, family history, ethnicity, prostate volume, PSA velocity, oxidative stress, BPH and prostatic intraepithelial neoplasia (PIN) [27,28]. Exogenous factors includes diet, environmental exposure, occupation, smoking, obesity and physical activity [5,29]. Age, family history and also ethnicity are considered the main risk factors, once is described in the literature a strongest relation between them and cancer cases (Gann P., 2002; Adjakly M., 2015).

Relation between aging and PCa is very marked, since the incidence rate increases harshly after age 50, has its peak around 70 [30]. Before the age of 50, only about 0.6% are diagnosed. Between 50 and 70 years, are diagnosed about 30% of all cases of PCa. After the 65-70 years, the number of occurrences increases to 60-80% [28,31,32]. These data show that there is, indeed, an association with PCa diagnosed and aging, since aging is associated with an increase of oxidative stress. The detoxification processes occur by action of the enzyme glutathione S-transferase P1 (GSTP1), which gene is silenced in 90% of all PCa cases [33]. Also, DNA damage and mutation are more probable to occur in older population, once the mechanisms that usually control and avoid such occurrences are ineffective. Vitamin E, which inhibit the formation of reactive oxygen species (ROS), and the tumour suppressor gene p53, are some of the organism defence lines that are reduced with aging [34,35]. Once p53 has a crucial role in tumour prevention, the declining in is activity with aging contributes to an increase of PCa cases in older age ranges [36]. Also, type II transmembrane serine protease (TMPRSS2), an androgen regulated gene that is related with epithelial cell growth, could suffer mutation in PCa cases [37].

PCa has also a hereditary condition and could occur by two modes: as an autosomal dominant syndrome or as a sex-linked syndrome [28]. Several predisposing genes for PCa disease have been described. HPC1, RNASEL and PCaP was the two first genes identified as susceptible to PCa condition [38-40]. After that, the genes HPCX, HPC20 and PG1 was also identified. The genes BRCA2 and CAPB are not only susceptible to PCa but also to cancer and brain [28].

The hereditary condition is strongly supported by the numbers of several studies of family cases of PCa. The risk increase 2,3-folf if the individual have both father and one or more brother with PCa cancer. If the father or the brother has been diagnosed before 60 years of age, the risk is near 2,16-fold [29].

The genetic component of PCa is supported not only by the above described, but also by the fact that five single nucleotide polymorphisms that presents an association with the disease have been identified [28,38]. There is single nucleotide polymorphisms (SNP) in chromosome 8q24, 17q12 and 17q24.3, 2p15, 10q11, 11q13 and Zp11 that are associated with the risk of PCa. The fact that these SNP varies with race and ethnicity justify the differences between rates in the diagnosis of PCa [41,42]. Also, these differences could be related to the different access to health care and diagnose tests [27].

Among the age and family history, PCa has a strong correlation with the race or ethnicity [27]. Several reports show that PCa cases are lower in Africa and Asia in comparison with Europe and the United States. Indeed, the incidence rate not only varies geographically, but also between ethnic groups once the Afro-American male population has a risk of 60% higher than white Americans [28]. In fact, the high risk to develop the disease are among Afro-Americans men [43].

1.2.3 Molecular pathways of carcinogenesis

As many tumours, PCa is originated from a single cell that differentiates to multiple heterogeneous cells [5]. However, how this process occurs in not yet entirely known. Since the tumour cells express cytokine 8 and 18 and secrete PSA, it was assumed that the tumour has it origin in luminal cells of prostate epithelium [45,46]. Still, nowadays, several studies show that most cases of PCa express basal cell features, such the expression of the proto-oncogene Bcl-2, which contributes for apoptotic-resistant phenomenon in this cells [44,47]. Indeed, in prostate adenocarcinoma, the predominant histological subtype of PCa, it was reported that the tumor has it origin in $CD49f^{hi}$ basal cell lines and is then propagated by $CD49f^{do}$ luminal-like cell [8].

The prostate is an androgen-regulated organ, therefore is extremely relevant to understand the role of androgens in the tumour genesis and development. Testosterone and the derivate DHT are the most abundant androgens in males, being testosterone the principal circulating form in blood [48,49]. DHT is the primary effector androgen and is converted from testosterone by the enzyme 5α-reductase type II in peripheral tissues of prostate [49]. Testosterone secretion occurs in testis, regulated by the luteinizing hormone (LH), present in Leydig cells. Huggins and Hodges, were the first researchers to suggest a correlation between testosterone and PCa growth [50]. In that study, it was demonstrated that testosterone plays an important role in both progression and regression of PCa. However, the role of testosterone levels in prostate carcinogenesis is not clear since there are several contradictories studies reporting that high levels of testosterone are related with increased risk of PCa. Nevertheless, some studies reported opposite correlations, and other research groups described no association at all [51-53].

The actions of testosterone and DHT are mediated by the AR, to which bind with equal affinity. The AR is a member of the nuclear receptor family (NRF), that also mediate the action of steroids, retinoids, vitamin D3 and several thyroid hormones [54]. This receptor contains domains that modulate the interactions between the complex receptor-ligand and specific target DNA sequences, and thus mediates the biological responses to androgens [54,55]. Several experimental data suggest that the AR plays an important role in the biology of PCa, namely the amplification of the receptor and also its enhanced expression in PCa [55-57]. Such evidences suggest the AR as a central point in PCa carcinogenesis.

Prostate tumors, initially, presents androgen-dependent characteristics. Then, with androgen ablation therapy, the tumor seems to adapt to that new condition and become androgen-independent [44]. The majority of patients with PCa initially respond to androgen-ablation therapy [58,59]. However, at some stage of the disease, the tumor assumes an androgen-independent stage, in order to assure the progression. It is known that the AR is expressed in both androgen-dependent and independent PCa. At this point, two different hypotheses are considered. The first one considers that at the point of androgen ablation, tumorous cells gradually assume anti-apoptotic features of basal cells of the prostate. This

adaptation is associated with increasing levels of Bcl-2. The second hypotheses suggest that in the first stage of the tumor (androgen-dependent phase), are present both androgen-responsive and non-responsive cells (these last in minor proportion). Then, when androgen ablation therapy is provided, all tumorous cells dies except the androgen-dependents ones, allowing the tumor progression [44,48]. However, some of the androgen-dependent cells express PSA, what may be due to inappropriate differentiation [44].

The molecular mechanism of that dependence to non-dependence transition is not yet well understood but is presumed that the AR is a key point [54,60,61]. The selection of cancer clones, cells adaptation to a non-androgen environment and an alternative molecular pathway are other known causes to trigger that modification in the tumor [54].

Oxidative stress has also an important role in tumor development. ROS can directly attack DNA and form promutagenic oxidized DNA base [62]. An increase in the oxidative stress could have exogenous sources, such environmental toxins and dietary factors, or endogenous, such hypoxia, mitochondrial dysfunction and one in particularly that suggest a link between inflammation and PCa: the release of oxidative species in order to eradicate infectious identities [63]. Although the data that support this relation is not definitive, there are a main aspect of molecular pathogenesis of PCa that lead to a role of prostatic inflammation in prostate carcinogenesis, the inactivation of the enzyme GSTP1. The enzyme GSTP1 is a detoxification enzyme responsible for reduce oxidative stress catalyzing the conjugation between oxidants and neutrophils with the chemical scavenger glutathione [64]. In almost the totality of PCa cases, GSTP1 is found absent or reduced due to hypermethylation of deoxycytidine residues of the promoter CpG Island [65-67]. Also, it is well known that the ingestion of nonsteroidal anti-inflammatory drugs (NSAIDs) reduced PCa risk [68]. The potential role of prostatitis in PCa raise the possibility that cyclooxygenase (COX), particularly the inducible isoform COX-2 may play a relevant role in the development of the tumor [62].

The prostate inflammation scenario usually leads to a proliferative inflammatory atrophy (PIA) [69]. This pathology is characterized by atrophic, still proliferative, prostatic epithelial cells that are often located near to inflammatory cells [70]. There are evidences that suggests PIA as a precursor of PCa [62,71,72]. In some cases, PIN arises from this inflammation condition, that could promote and develop to PCa [66].

Tumor progressing is a dynamic process that cannot be attributed to single causes or events. The transition to androgen independence phase is a multi-factorial process that involves both selection (clonal selection) or adaptive up regulation of genes that allow tumor to progress. This transition is a key point in the tumor growth, and consequently, a key point to better understanding PCa carcinogenesis.

While the tumorous microenvironment is particularly important in tumor progression, blocking several biomolecules involved in the uptake of nutrients by cells to promote tumour growth could be an efficient strategy in treatment [73]. The six transmembrane epithelial antigen of the prostate 1 (STEAP1) is one of these biomolecules [74]. Since its discovery and overexpression in several tumors, with strongly incidence in PCa, STEAP1 has been pointed as

a target protein in PCa development and progression. In fact, overexpression in PCa, the six transmembrane domains and its location in cell-cell junctions are considered as relevant biochemical and structural evidences so as to postulate that STEAP1 can have an important role in PCa [75].

1.2.4 Diagnostic, treatment and limitations

The PSA screening is the main cause of the low rate of deaths associated with PCa. In the last decades, this methodology has been introduced and the consequence is that PCa is a leading cancer diagnosed in men of the United States of America [25, 76].

The relevance of PSA screen is based in the early diagnostic of PCa. This is an important factor since earlier the tumour is detected, and consequently, better outcomes are possible [77]. The overall survival (OS) rate at 5 and 10 years follow-up is around 75% and 60%, respectively [78].

In 2004, WHO develop a consultation in order to define new markers and conclude that PSA is still the most adequate marker of PCa [79]. High serum PSA levels are associated with an increased risk of tumour metastasis. Locally advanced PCa and a poorly differentiated tumour are also indications of such condition [80].

Nowadays, diagnosis is based on examination of several histopathological or cytological specimens from the prostate and the local staging of PCa (T staging) is done mainly by digital rectal examination. The samples are acquired by systematic transrectal core biopsies, which are carried out by ultrasound. The samples obtained are then graded using the Gleason score [81,82]. This method is the most common to classify biopsy specimens from prostate and indicates the aggressiveness of PCa. The output is a score indicating the aggressiveness of the tumour. Patients with a PSA level ≤ 10ng/mL and Gleason score ≤ 6 are considered the tumour is considered less aggressive. Intermediate level is attributed to individuals who have a PSA level between 10 and 20 ng/mL and a Gleason score of 7, while the most aggressive cases are declared when the PSA level is greater than 20 ng/mL and the Gleason score is ≥ 8 [82,83]. However, more accurate information is lacking in order to complete the PCa patient profile.

Depending on the stage, PCa is initially treated with radiotherapy (RT), radical prostatectomy (RP), transperineal brachytherapy, cryotherapy, high-intensity focused ultrasound (HIFU) and androgen ablation therapy [84].

In the past, radical prostatectomy (RP) was the standard procedure for localized PCa, even in patients with low and intermediate risk levels. Nowadays, these treatments are considered overtreatment, being surgery only applied in some cases of high-risk prostate cancer (HRPC). However, RP is the most effective treatment in order to assure cancer survival [84]. Usually, HRPC is treated with androgen ablation therapy or combined radiohormonetherapy. Once RP monotherapy is not effective in most cases, salvage radiation or hormone treatment should be taken in consideration [85]. The European Association of Urology (EAU) proposed RP

as best therapy in cases of locally advanced cancer cases with a PSA level ≤ 20 and a Gleason score ≤ 8 [86].

In the United States of America around one million prostate biopsies are executed [87,88]. There are approximately 20% of false negative diagnostics in transrectal ultrasound-guided biopsy [88]. Therefore, several samples are needed to assure a correct diagnose. The problem here is that biopsies are painful and not comfortable for the patients. Moreover, there are a certain possibility of infection in this procedure [89]. In order to improve the biopsy procedure and then reduce the number of samples needed, a 3D statistical model of the prostate, divided in 48 zones and 6000 subzones, was created in 2003 [90]. This model allows to detect the zone were the samples must be taken to assure the tumour detection.

Nowadays, advance diagnosis methods are available, such as magnetic resonance imaging (MRI) and brachytherapy. MRI have a lower false negative rate, allow the identification of low risk tumours and 51% of men exposed to that therapy have no need to be exposed to needle biopsies [91]. Brachytherapy represents an extension of radiotherapy. Radioactive seeds are placed inside the prostate in order to potentiate the therapy [92]. This technique has some risks associated such urinary problems and erectile dysfunction [77]. Among this, active surveillance (AS) has become an important alternative in order to prevent and treat PCa. The AS may involve close observation of patients, with regular PSA tests, and even biopsies [77].

PCa could grow slowly and a large number of patients can live many years after the diagnostic. There are numerous secondary effects associated with some of the current treatments. Bladder inflammation is a common scenario in many treatments such as transperineal brachytherapy, RT and RP [93]. Bleeding erectile dysfunction and incontinence are also common side effects of such treatments. Finally, hormone ablation therapy is associated with anaemia, hot flashes, lipid abnormalities and weight gain [84,94].

1.3 Six Transmembrane Epithelial Antigen of the Prostate

1.3.1 Overview of STEAP protein family

It is predicted that 20-30% of all open reading frames (ORF) encode human membrane proteins, what correspond to 5539 genes encoding almost 8000 membrane proteins. Membrane proteins are key elements in cell functions and could be important therapeutic targets [95-97]. STEAP protein family is one of that cases. This protein family includes four different proteins, STEAP1, STEAP2, STEAP3 and STEAP4, in accordance with discovery order. All the STEAP proteins have in common six transmembrane domains and the same COOH and N-terminal location, both intracellular. The COOH-terminal is shows high homology with the yeast FRE family of b-type cytochrome metalloreductases, and the N-terminal is highly homologous with the archaeal and bacterial $F_{420}H_2$:$NADP^+$ oxidoreductase (FNO) and to human NADPH-oxidoreductases [98,99]. STEAP proteins also have, at least, one intramembrane heme group binded to the protein by two conserved histidine residues. This heme domain is also present in

Nox and YedZ family and was named apoptosis, cancer and redox associated transmembrane (ACRATA) [100,101].

Typically, the majority of STEAP proteins share the YXXØ consensus sequence, where the Ø stands for a large hydrophobic amino acid. That sequence is responsible for the targeting of proteins to lysosomes and endosomes. They also present the Rossman fold (GXGXXG/A), a motif that enables proteins to have oxidoreductase and dehydrogenase functions. It is common the designation of the STEAP protein family as metalloreductases. However, such activity is not present in the case of STEAP1 [99].

STEAP2, or six transmembrane protein of the prostate 1 (STAMP1), is a shuttle between Golgi complex and plasma membrane [98]. It may act like a receptor for both endogenous and exogenous ligands and a regulator of protein delivery, since moves in both directions in endocytic and exocytic pathways. Also, it is predicted, also, that STEAP2 may have a role in endosomal Tf cycle of erythroid cells. Its specific role is linked with the uptake of iron and copper, reducing Fe^{3+} and Cu^{2+}, and also regulate its availability in several sites like the choroid plexus, and in the gastrointestinal tract [102].

STEAP3 is known as tumor-supressor activated pathway-6 (TSAP6) or dudulin-2. It is upregulated upon p53 activation in LTR6 and MCF7 cell lines and contributes to cell death by apoptosis by affecting cell progression, namely the G2-M transition [98,103]. STEAP3 also interacts with the translationally controlled tumour protein (TCTP) [104]. TCTP have a role in inflammatory responses caused by some parasites. STEAP3 and TCTP are both present near the cellular nucleus and the first one, in fact, mediates the secretion of the tumour protein [105-107].

STEAP4, also denominated as STAMP2, colocalizes with the early endosome protein EEA1 in cell periphery, suggesting an involvement in secretory pathway or endocytic pathway. STEAP4 is responsible for the uptake of vesicular tubular structures from the cytoplasm to cell periphery [98,102]. In the last years, this protein has been associated, also, with obesity, insulin resistance, inflammation and PCa progression [102,108,109].

Although the expression and function of STEAP1 will be described with more detail in the next section. Its gene is located in chromosome 7q21.13 spanning 10.4 kb, and it is constituted by 5 exons and 4 introns [98] (Table 1).

Table 1 – Chromosome location and biochemical characteristics of the STEAP proteins (Adapted from [98]).

STEAP gene	Chromosome location	Gene size (kb)	Amino acids	MW (kDa)
STEAP 1	7q21.13	10.4	339	39
STEAP 2	7q21.13	26	490	56
STEAP 3	2q14.2	42	488	50
STEAP 4	7q21.12	26	459	52

The gene transcription gives rise to two mRNA transcripts having 1.4 and 4.0 Kb but only the smaller one encodes the STEAP1. The protein contains 339 amino acid with a molecular weight (MW) of 39.72 kDa having six transmembrane domains with both N- and C- terminus on cytoplasm side [98,110,111]. This topology suggests that STEAP1 folds in a "serpentine" form, giving three extracellular and two intracellular domains [112].

In order to acquire more detailed information about structure and functions of STEAP1 protein, allowing the design of drugs that could interact with STEAP1 with higher efficacy, its production and isolation is assumed like a matter of interest [113].

1.3.2 STEAP1 expression, regulation and function in prostate cancer

In 1999, STEAP1 was identified as overexpressed in several human tumours such as bladder, colon, pancreas, ovary, testis, breast, cervix, Ewing sarcoma and with particularly relevance in prostate [98,110,114] (Table 2).

Table 2 – Expression levels of STEAP1 protein in different tissues (Adapted from [98]).

Cancer Tissue	***Protein***
Lungs	++
Colon	+
Kidney	++
Bladder	++
Breast	++
Prostate	+++
Prostate lymph node metastasis	+++
Prostate bone metastasis	+++

+: low expression level; ++: intermediate expression level; +++: high expression level

In normal tissues, STEAP1 is present in major abundance in prostate gland, but also in ureter, fallopian tubes, uterus, pituitary, pancreas, stomach, colon and breast [98,102,111]. STEAP1 messenger RNA (mRNA) is detected in peripheral blood of cancer patients and its detection in bone marrow could indicate occult residual cancer cells in Ewing sarcoma patients [115,116].

The fact that STEAP1 is overexpressed in several tumours may be related with a higher stability of its mRNA and protein in neoplastic cells when compared to non-neoplastic [110,111,113,114]. Post-translational modifications (PTM) are an important mechanism for regulation of the proteome, influencing the stability and also protein transcription, apoptosis and degradation [117-119]. *In silico* analysis identified several potential PTM sites, namely N-glycosylation site in Asp143, glycation sites in positions 5, 15, 17, 30, 108, 148, 149, 156 and 162 in amino-acid (aa) chain, all corresponding to lysine aa. Phosphorylation sites are localized in Ser3, 187, 240 and 244; Tre160 and 246; Tyr27, 147, 219 and 252. Probably occurring

simultaneously with phosphorilation, GlcNAcylation may happen in the following O-ß-GlcNAc anchor sites: Tre236 and 333, and Ser237 and 242 [111].

STEAP1 is the only protein of the family that does not have the ability to reduce metals. This lack of activity is possibly due to the absence of some native characteristics presents in all others STEAP's: the Rossman fold and the FNO-like domain (Figure 2).

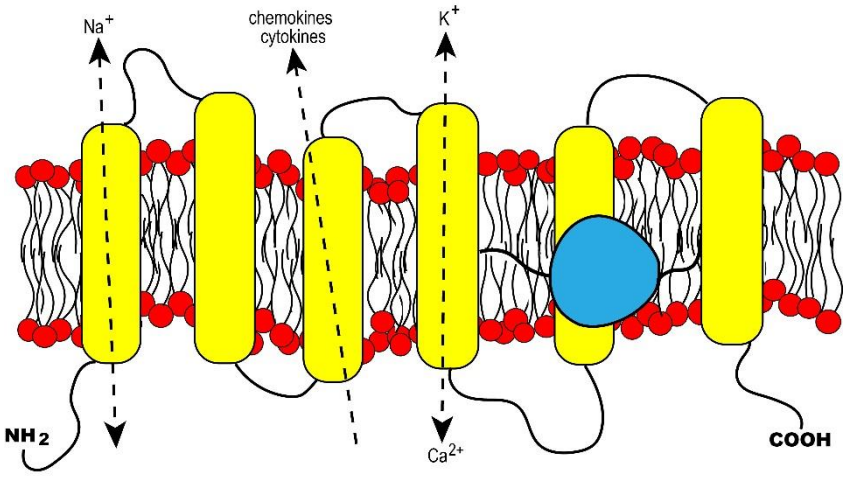

Figure 2 – Schematic representation of STEAP1, cellular location and functions. With the six-transmembrane domains and both N- and C- terminus on cytoplasm side, STEAP1 folds in a "serpentine" form, giving three extracellular and two intracellular domains. This topology and location at cell-cell junctions of the secretory epithelium of prostate indicates a putative role in intercellular communication, modulating the transport of small molecules and ions such as Na^+, Ca^{2+} and K^+, and releasing soluble cytokines and chemokines to extracellular medium. In blue is represented the heme group, homologous to the present in Nox and YedZ family, the ACRATA domain.

Nevertheless, it is possible that STEAP1 have a role in iron metabolism due to its location in the same zones of transferrin (Tf), transferrin receptor 1 (TfR1) and endosomes specialized in iron metabolism are some evidences that support that thesis [99]. Also, STEAP1 protein presents an intramembranar heme-binding domain [120]. This domain, in which only two conserved histidines are present, is homologous to a domain present in Nox and YedZ family and was named ACRATA [99; 101]. Therefore, this may indicate that STEAP proteins have the ability to uptake iron and copper. They have the ability to use intracellular flavin adenine dinucleotide derivate flavins as electron donors for reduction of that metals, due to the FNO-like domain [121].

Although the cellular role is not yet well known, it seems that STEAP1 acts like an ion channel or transporter protein in tight junctions and/or gap junctions. The secondary structure of STEAP1 associated to its location at cell-cell junctions of the secretory epithelium of prostate indicates a putative role in intercellular communication, modulating the transport of small molecules and ions such as Na^+, Ca^{2+} and K^+, and the releasing of soluble cytokines and

chemokines [120,122]. In addition, high levels of Na^+ leads to a loss of the AR expression and the progression from androgen-dependent to the androgen independent stage of PCa. Thus, Na^+ channels seems to confer a more invasive phenotype to PCa tumour cells [123,124]. STEAP1 seems to regulate intercellular communication and cellular proliferation, but also regulate cell fate and cancer cells invasiveness [125,126].

Connexin (Cx) is a protein present in gap junction channels that mediates the exchange of ions and small molecules between cells [127]. The transcription of Cx gene and protein expression is reduced along the cancer development [128,129]. In a tumorous environment, cellular communication assumes a more important role in order to maintain the appropriate conditions to tumour development [73]. If so, there must be other entity acting as a transporter, besides Cx proteins in order to assure tumour growth. STEAP1 is seen as an excellent candidate, since its ability to transport small molecules has been reported [130]. In the same study, it was shown that blocking STEAP1 with monoclonal antibodies against STEAP1, intercellular communication was reduced, leading to an antitumor effect.

Complementary reports demonstrated the relevance of STEAP1 in tumor growth *in vivo*, and its role to promote cell-cell interaction between tumor cells and its microenvironment *in vitro* [122].

Besides its contribution to intercellular communication, STEAP1 may have a relevant role in modulation of intracellular pathways. Studies have shown that STEAP1 appears to act as an intervenient on cell growth, raising the levels of ROS. In the same report it was demonstrated that there is a link between STEAP1 and the invasiveness of Ewing sarcoma, once STEAP1 promotes tumour cell proliferation. It is unclear the mechanism by which the overexpression of STEAP1 leads to increased levels of ROS, but is predicted that MMP1 and ADIPOR1 acts as regulators of this process [120]. In PCa, STEAP1 expression varies with the metastatic potential of cell lines, being more expressed in those in earlier stages of the disease [110].

However, its regulation in prostate is not yet entirely explained nowadays. It is known that zoledronic acid, an inhibitor of bone resorption for PCa patients, decreases STEAP1 mRNA [115]. Also, it was demonstrated the down regulation of STEAP1 by action of 17β-estradiol (E2) and DHT in LNCaP prostate cancer cells. Such results may clarify the role of STEAP1 in PCa, since its inhibition by both hormones may indicate that STEAP1 participates in the androgen-dependent stage [98]. Recently, it was stated that chromobox homolog 7 (CBX) of the polycomb repressive complex 1 (PRC1) also regulates STEAP1 expression, by interacting with its promoter in human thyroid cancer cells [131].

1.3.3 STEAP1 as an immunotherapeutic target

Once STEAP1 is overexpressed in several tumour cell lines, namely in PCa, it is located in cell membrane, and is found absent in vital organs, this protein has been highlighted as a promising therapeutic not only with application on PCa but also in colon, pancreas and testicular cancers [110,132].

The identification of tumour-associated antigens (TAA) that are overexpressed in tumour cells comparative to normal cell lines, such as STEAP1, is a key point to the success of T-cell based immunotherapy [133]. The aim of this technique is the production of vaccines containing epitopes that stimulate $CD8^+$ and $CD4^+$ T-cell immune responses that will cause the regression of tumour [134].

There are some STEAP1 epitopes used to stimulate antitumor immune response, namely $STEAP_{292}$ (MIAVFLPIV) and its modified version $STEAP_{292.2L}$ (MLAVFLPIV), $STEAP_{86-94}$, $STEAP_{262-270}$, $STEAP_{192-206}$ (LLNWAYQQVQQNKED), $STEAP_{102-116}$ (HQQYFYKIPILVINK), $STEAP_{281-296}$ [98].

$STEAP_{292}$ and $STEAP_{292.2L}$, but particularly the last one, have shown a strong binding to HLA-A_0201 molecules and the capacity to trigger a cytotoxic T-lymphocyte (CTL) response in $CD8^+$ T cells. This CTL induced by the $STEAP_{292.2L}$ epitope is capable to recognize peptide-loaded cells and prostate, colon, bladder, Ewing sarcoma, melanoma and embryonic rhabdomyosarcoma tumour cells, expressing STEAP1 [98,135,136]. The nonameric STEAP1 epitopes $STEAP_{86-94}$ and $STEAP_{262-270}$ are HLA-A*0201 restricted epitopes. Both are found in mouse and human CD8*T cells, with some differences in constitution between them and were capable to recognize STEAP1 from tumour cells in an HLA-A*0201 restricted way [98].

Beyond STEAP1 trigger an immune response stimulating $CD8^+$ cells into CTL, it is also capable to induce $CD4^+$ T cells [98,135]. $STEAP_{192-206}$ and $STEAP_{102-116}$ are two synthetic STEAP1 peptides that strongly binds to different classes of HLA-DR. Both epitopes are processed endogenously, via direct presentation of STEAP1 peptides by HLA-DR molecules, and exogenously by antigen-presenting cells (APC) that process STEAP1 peptides from cell lysates [137].

Despite of these concerns, there is no commercial formulations of therapeutic vaccination yet and its development is in the early stages.

It is well known that the tumour microenvironment is a barrier to trigger an immune response. Antibody-drug conjugates (ADC) arise as a promising therapeutic approach, combining the specificity of the monoclonal antibody and the cytotoxic character of the chemotherapeutic drug to which the antibody is conjugated [138].

1.4 Recombinant protein biosynthesis in prokaryotic cells

1.4.1 Overview

Membrane protein expression is a challenging field, either by the difficulties of biosynthesis stage or the complexity of protocols required for that requires their stabilization and complete purification.

Currently, protein production via recombinant synthesis is widely used for several macromolecules. Recombinant production is used because it allows to produce large amounts of the target molecule in short periods of time. This is possible due to the "super strains" hosts development, capable of high rates of production, and to the optimization of the production procedures. There are several expression systems that have been used over the years: mammalian cells [139], yeast [140], bacterial cells [141], insects [142] and plants [143]. The selection of host is a very important step when we have to design and establish a proper platform for protein production. There are several aspects that must be considered such the viability of each expression system, the possible toxicity for the host cell, the characteristics and source of the protein of interest and also the integration with the downstream stage [144].

Prokaryotic cells are the most used expression system [145]. Despite the several factors that we have to consider, prokaryotic organisms appear as first choice for recombinant protein biosynthesis. Its popularity is due, mainly, to the economical and easier production, the ability to grow rapidly and the extremely well known genetic and metabolic pathways. In fact, *Escherichia coli* (*E.coli*) was the first organism used to produce a recombinant molecule, in 1982, and for a long period was the dominant organism used as host to produce pharmaceutic valuable molecules [146]. Indeed, 90% of the structures available in the Protein Data Bank were determined by producing the target protein in *E.coli* [147]. Moreover, *E.coli* recombinant production as boosted the biopharmaceutical industry, once 30% of the recombinant biopharmaceuticals licensed up to 2011 by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) was produced in this host cell [148]. Also, the *E.coli* dominance as host system is due to the fact that the process to construct the prokaryotic expression system is also easier compared with a eukaryotic or mammalian system. The transformation and recovery of the target protein stages are also more easily achieved in that systems

In last years, the need to produce more complex recombinant proteins lead to a major problem associated with these organisms. The inability to do post-translational modifications, and consequently to obtain the native 3D structure is one of such problems. Therefore, eukaryotic and mammalian cells overcame the popularity achieved by the prokaryotic systems [146]. Due to the inability to form disulfide bonds and the lack of PTM, prokaryotic cells could be pointed as an inappropriate host for protein biosynthesis [149]. PTM are an important mechanism for regulation of the proteome, influencing the stability and also protein transcription, apoptosis and degradation [117,118].

Thus, protein modifications like glycosylation, glycation and phosphorylation could not occur in prokaryotic cells and in consequence, the protein do not acquire the correct native fold. Also, some prokaryotic organisms, such as *E.coli*, presents two complex membranes that difficult the secretion of the protein of interest [149,150]. However, most proteins are able to maintain their functions even without PTM, what gives liability to a process of biosynthesis in a prokaryotic system, such as *E.coli*. Moreover, in the case of the STEAP1, which its biotechnological synthesis has never been performed, and considering the advantages described above, *E.coli* arises as a very important organism to be a first approach to test in a laboratorial scale.

1.4.2 *E.coli* BL21 (DE3) as strain host

1.4.2.1 General characteristics

E.coli is a Gram-negative, anaerobic facultative bacteria with a cellular volume around 0.7 μm [151,152]. Despite the disadvantages mentioned, many proteins do not require post-translational modifications to acquire the proper function and structure, what makes *E.coli* an appreciated host for recombinant protein production [150]. The main advantages of using *E.coli* as host are the fast growth, may the duplication time be only 20 minutes, the ease to reach high cell density, adequate fermentation media could be obtained from accessible and cheap components, and the ease and fast transformation process [153].

There are several *E.coli* strains, being the most common for recombinant proteins the *E.coli* B, K-12, BL21, DH5α and AD494 [154]. Among these strains, *E.coli* BL21 has the main advantage of lacking two proteases: *lon* and *ompT*, thus avoiding protein degradation. A particular derivation from BL21, the BL21 (DE3), is especially designed for overexpression of membrane proteins [155]. Therefore, it may be a good option to develop the present work.

The bioprocess design to produce recombinant human proteins, the plasmid choice is another important parameter. Nowadays, there are several types of plasmids compositions available (size, conjugative or non-conjugative, high or low replication, expression or cloning) with different features, and that have emerged from derivations of some already commercialized vectors [156].

One example are the pET series, which includes the pET 101, used in this work and that will be described in the section "Materials and Methods". The application of this plasmid in the host BL21 (DE3) is a favorable conjugation, due to an additional stabilization promoted by the strain [157]. Also, BL21 (DE3) contains the *rne131* mutation, which encodes an endonuclease that participates is rRNA stabilization but also in mRNA degradation. The C-terminal portion of the enzyme is responsible for mRNA degradation and the N-terminal portion interacts with rRNA. This mutation encodes an RNase which lacks the C-terminal [158].

Once chosen the host for recombinant protein synthesis, the selection of a proper promoter and the mechanism of expression control is important to ensure a straightforward process. In the case of *E.coli*, several options are available. The most common promoter is the

T7 promoter DNA sequence, being the expression controlled by the *lac* operon. A T7 RNA polymerase assure an elongation chain faster than *E.coli* RNA polymerase, improving the expression [154]. In addition, the *lac* promoter is strongly activated by lactose or isopropyl-b-D-thiogalactopyranoside (IPTG), a galactose analogue. Also, lactose may be used as a carbon source, interfering with fermentation [157].

1.4.2.2 Biosynthesis of membrane proteins: optimization strategies

The production, in a laboratorial scale, of recombinant proteins, and namely membrane proteins, comprises several stages. Firstly, the bacterial inoculum should present a correct initial OD, near 0.2, so the fermentation may be controlled. After the process of culture, cells must be harvested and lysed, if the target molecule is a membrane protein or if it is not excreted to the extracellular media. Since membrane proteins requires a stabilization structure mimicking the cell membrane, detergents or some advanced structures like liposomes should be used [159].

It is widely known that are several parameters affecting bacterial growth, such as the fermentation media, temperature, pH, agitation rate and the presence of toxic nutrients and metabolits in the medium [153]. All these parameters must be adjusted in order to allow an optimal growth rate of the strain and an efficient massic and volumetric productivity of the target bioproduct. In a batch culture, a regular growth profile has 4 different phases: lag, exponential growth, stationary and dead phase [160]. In the case of *E.coli,* this typical profile can be achieved for several different media, for instance: SOC, SOB, TB and 2YD. In this work, the fermentation aim is to synthetize recombinant proteins, its expression must be induced, if necessary, in the exponential phase. At this stage the culture will be established, adapted to the medium and therefore it is in optimal conditions to biosynthesize the target protein [161].

In the field of fermentation, over the years, the development of super strains and plasmids, and optimizations of the methods applied allow the production of a wide range of recombinant products in small to large scale [162]. For the development of this master thesis, the plasmid used was the pET 101, with 5753 bp since is one of the most widely used for production of recombinant proteins (Figure 3).

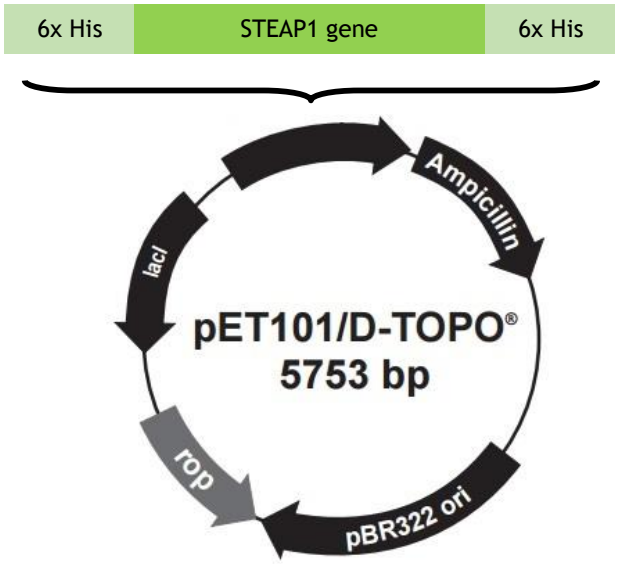

Figure 3 –pET101 vector map (ThermoFisher.com, consulted at 08/28/2016)

The promoter is based in the T7 RNA polymerase, and the expression of the protein of interest is regulated by the *lac* operon. The selection marker added to the plasmid is an ampicillin resistance gene [141]. The commercial form of this vector have a particular specificity that facilitates the purification of recombinant human proteins, an affinity tag, specifically a polyhistidine region (His-tag). In the present work, the His-tag was added through the primers design.

1.5 Recombinant protein purification

1.5.1 Overview of membrane proteins isolation and solubilization

Purification and isolation is a requisite of all macromolecules production process, either the aim be protein crystallization, biochemical or enzymatic assays. For all that cases, large amounts of protein are required. So, protein purification is a fundamental step in order to obtain purified fractions of the proteins for further bio-interaction and structural studies. During the last decades, the development of purification techniques has been a growing field, that followed the advances in biotechnological procedures for recombinant molecules synthesis [163,164].

In the case of membrane proteins, there are some additional difficulties associated with its biosynthesis and stabilization. Membrane proteins are located, naturally, in a complex, heterogeneous and dynamic mosaic lipid bilayer. As consequence, most of that proteins are not soluble in aqueous solutions [165]. The need to study membrane proteins lead to development of strategies that makes possible to mimic the lipid bilayer *in vitro*. Therefore, the use of

detergents as a tool for studying membrane proteins became very popular [166]. Detergents are amphipathic molecules, having a polar head group and a hydrophobic chain, allowing the formation of spherical micellar structures [167]. Membrane proteins are solubilized due to that micelles that mimic the natural lipid bilayer [165]. Currently, numerous detergents, classified as ionic, nonionic, bile acid salts and zwitterionic are been used for proteins solubilization (Figure 4), being the most commons SDS, Triton X-100, Tween 20, Tween 80, CHAPS and digitonine, among others [168].

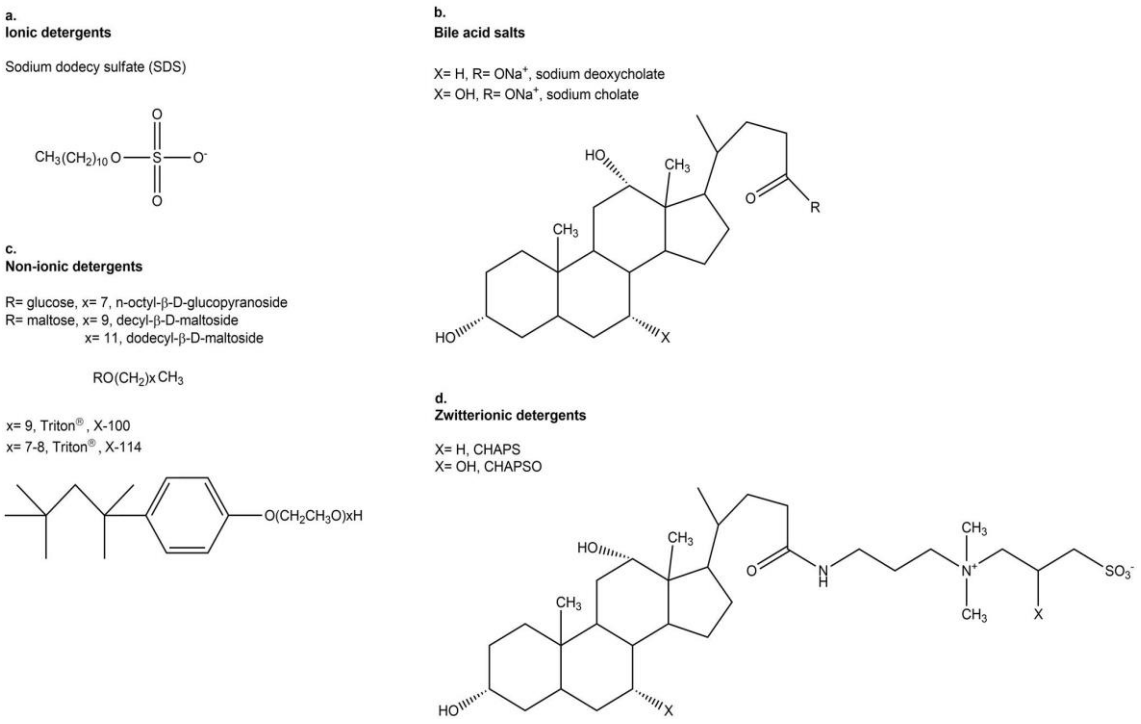

Figure 4 – Structure of different types of detergents [165].

The application of detergents as a solubilize agent may lead to a major problem caused by the fact that some detergents could promote denaturation, due to the excessive amount applied in the solubilization process [169]. Every detergent has a critical micellar concentration (CMC), a minimal concentration that allows the formation of micellar structures [165]. Typically, CMC decreases with the length of the alkyl chain of the detergent and increases with introduction of double bonds.

So, it is imperative the use of one or more detergents to promote the solubilization of membrane proteins [166]. Nowadays, aqueous two-phase system consisted by a detergent/polymer (ATPS) mix is used to overcome the denaturation caused by using detergents [170]. The addition of detergents to solubilize membrane proteins, or any other compound, means that such compound must be removed from the target sample. Currently, there are

several methods currently used to remove detergents, taking advantage from its natural properties, such CMS and charge [165].

Chromatography is the primordial used method for protein purification [61]. This strategy is due to its high resolving power and variety of chromatography methods with different selectivity available [171]. Ion change, reverse phase, hydrophobic interaction and affinity chromatography are widely used to collect purified protein fractions from complex biological matrices [165,170]. Immobilized metal-ion affinity chromatography (IMAC), specifically, is very used in bioprocesses procedures, aiming a maximum isolation of the target protein.

1.5.2 Immobilized metal-ion affinity chromatography

Biospecific affinity separation methods became popular in 1960s, and until today have been continuously improved. IMAC of proteins, specifically, was introduced in 1975, under the nomination of "metal chelate chromatography" [172].

IMAC is based on the interactions between a metal ion, such Zn^{2+}, Cu^{2+}, Ni^{2+} or Co^{2+}, immobilized on a matrix and amino acid side chain, namely, histidine and cysteine [173]. Nowadays, this chromatography technique has been improved and the is performed to separate fractions of proteins containing more or less histidine and cysteine residues [174]. This development was accelerated by the increasing and consolidation of recombinant techniques and the generation of fusion tags that improved the separation effectiveness of IMAC. That fusion tags consist in a polypeptide chain, namely, a polyhistidine chain (His-tag), containing six or more histidine residues, attached to the protein of interest, by either they N- or C-terminal. Histidine is an amino acid encoded by CAU and CAC codons that presents a side chain imidazole ring, an aromatic heterocycle. The key of IMAC purification is the strong interaction between this imidazole ring and transition metal ions [175].

Typically, there are five stages in a chromatography process: equilibration, loading of sample, washing, elution and regeneration [176]. The column equilibration ensure that the stationary phase is totally in the optimal conditions to allow the binding of the target biomolecule. After that, the appropriate volume of sample is loaded and it goes through the column by continuously adding mobile phase. After the sample was loaded, it is necessary to remove all the impurities present in the mixture that do not interact with the stationary phase. After that, the desired protein must be collected. During the elution, proteins strongly attached to the column will elute later than the proteins with less affinity. In the case of IMAC, the proteins with His-tag can be eluted by soft pH adjustment or adding free imidazole in the mobile phase. Finally, the matrix must be cleaned by a regeneration step in order to maintain its binding capacity and selectivity, using NaCl, NaOH, ethanol or isopropanol [172].

So, the addition of an affinity tag to the N- or C- terminal of the target protein allows the strong interaction between the macromolecule and the immobilized metal ion in the column, and consequently its partial or full purification. However, it should be noted that the

addition of the His-tag may compromises the overexpression of the protein, contributing to its proteolytic degradation and protein-protein aggregation [177].

Despite that, chromatography techniques have been used as single purification step or combined with other methods [178,179]. IMAC, in particularly, is a versatile and fast method that allows the separation of purified fractions until 95% purity at high yield rate [180]. In fact, there are several published papers that refer this chromatography technique as a valuable method for recombinant protein isolation. For instance, the TGF-ß1, a Polyhistidine-bearing Amphipol and the calcium-binding soybean protein are isolated using IMAC [181].

2. Aims

Although the evidences demonstrated in several studies that STEAP1 may have an important role in the development and invasiveness of PCa, its specific contribution in this disease is not yet entirely known. More detailed information and knowledge about the protein structure and function is required, and for that, platforms from integrative production to complete isolation of STEAP1 are extremely needed. Until now, the literature lacks from an exhaustive study concerning the recombinant biosynthesis of this protein.

So, the main goal of this work is to develop a laboratorial-scale platform able to produce effectively the STEAP1 protein in *E.coli* BL21 (DE3) strain and obtain it with high levels of purity. To achieve that, several intermediate goals were proposed:

- Design a plasmid that allows the STEAP1 expression;
- Screen of induction parameters (type, time, concentration);
- Evaluate protein stabilization using chaperones;
- Study the solubilization of the target protein with detergents;
- Isolation the STEAP1 from the cell matrix.

After that, STEAP1 3D structure may be resolved, its function in PCa may be better known and new molecules with high binding capacity can be obtained.

3. Materials and Methods

3.1 Materials

Ultrapure reagent-grade water was obtained from a Merck Milipore Mili-Q system (Darmstadt, Germany). Agar and European Bacteriological Agar were obtained from Laboratorios CONDA (Madrid, Spain). Tryptone and yeast extract were obtained from BIOKAR Diagnostics (Allonne, France). Lactose was obtained from Merck Milipore (Darmstadt, Germany). SDS and lysozyme were acquired from AppliChem (Darmstadt, Germany). Magnesium chloride, magnesium sulfate, glass beads, ampicilin (sodium salt), IPTG and DNase I were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride, glucose, tris base, Triton X-100 and methanol were acquired from Fisher Scientific (Loughborough UK). Potassium chloride was obtained from Panreac (Barcelona, Spain). Glycerol was obtained from HiMedia Group (Mumbai, India). NZY ladder VI, NZYColour protein marker II and GreenSafe Premium were purchased from NZYTech (Lisbon, Portugal).

3.2 Methods

3.2.1 Strains, plasmids and media

Two different plasmids were manipulated to obtain the pET101-STEAP1 expression vector. The pET101-hCOMT and the pIRES-STEAP1 were obtain from previous works developed by our research group. The vector pET101-STEAP1 was used as the expression system in *E.coli* BL21 (DE3) cells (Invitrogen, Carlsbad, USA). *E.coli* TOP10 cells (Invitrogen, Carlsbad, USA) were used to contrusct the pET-STEAP1 plasmid.

E.coli BL21 (DE3) were selected on LB plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 35 g/L agar, pH 7.5), complemented with ampicillin 100 µg/mL at 37°C. The cells selected were then transferred to several typical complex mediums: LB (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCL, pH 7.5), TB (12 g/L tryptone, 24 g/L yeast extract, 4 mL glycerol, 100 mL of 0.17M KH_2PO_4 and 0.72M K_2HPO_4), SOB (20 g/L tryptone, 5 g/L yeast extract, 0.25 g/L NaCl, 10 mL $MgCl_2$ 1M, 10 mL $MgSO_4$ 1M), SOC (20 g/L tryptone, 5 g/L yeast extract, 2 mL of 5M NaCl, 2.5 mL of 1M KCl, 20 mL/L glucose 1M, 10 mL/L $MgCl_2$, 10 mL/L $MgSO_4$) and 2YT (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7). Ampicillin 100 µg/mL was added to all the media used in this work in order to avoid parallel contaminations.

3.2.2 Construction of the pET101-STEAP1 expression vector

Briefly, STEAP1 gene and pET101 vector were collected and amplified from different sources. STEAP1 gene was amplified from the pIRES-STEAP1 template by polymerase chain reaction (PCR) in a Thermal Cycler (Bio-Rad, Hercules, USA) using specific primers, containing restriction sites for EcoRI (Takara Bio Inc, Shiga, Japan) and SacI (New England BioLabs Inc, MA, USA), and optimized PCR conditions (Tables 3 and 4).

Table 3 - STEAP1 specific primers sequence applied in the development of this work.

	Sequence
STEAP1_Fw	5'- AAGG AATT CAGG AGCC CTTC ACCA TGGA AAGC AGAA AAGA CATC – 3'
STEAP1_Rv	5'- AATG AGCT CCTA ATGG TGAT GGTG ATGG TGCA ACTG GGAA CATA TCTC AGT – 3'

Table 4 - PCR profile for STEAP1 amplification.

PCR Step	Temperature (°C)	Time
Denaturation	95	5 minute
	95	30 seconds
30 cycles	60	30 seconds
	72	1 minute
Final Extension	72	5 minutes

After PCR procedure, DNA fragment amplification was confirmed by agarose gel electrophoresis and purified using NucleoSpin Extract II kit (Macherrey-Nager). The fragment was then digested using EcoRI and SacI restriction enzymes, and the digestion confirmed by agarose gel electrophoresis.

The pET101 vector was amplified in a *E.coli* TOP10 culture in LB medium, recovered and purified using NZYMiniprep kit (NZYTech). After that, the vector was digested with the same restriction enzymes as the STEAP1 gene, and the ligation was performed using T4 DNA ligase (NZYTech) for 4h at room temperature. In order to confirm the ligation, the pET101-STEAP1 construct was introduced into *E.coli* TOP10 cells, which grown overnight at 37°C in LB plate supplemented with ampicillin 100 µg/mL. Fragments from positive colonies were used to confirm the integrity of pET101-STEAP1 after cell transformation. Subsequently, the positive colonies were inoculated in 5 mL of LB medium and grown overnight at 37°C, 250 rpm. From that culture, pET101-STEAP1 plasmid was recovered and purified using NZYMiniprep kit (NZYTech) to further DNA sequence analysis.

3.2.3 *E.coli* BL21 (DE3) transformation with pET101-STEAP1 construct

After DNA sequence of the pET101-STEAP1 construct was confirmed, a vector stock was prepared. *E.coli* BL21 (DE3) cells were transformed with the pET101-STEAP1 construct and

grown overnight, at 37°C, in LB plates. After the incubation, a partial colony was used to confirm the transformation by PCR, and after the positive result, the colony was transferred to a pre-inoculum flask containing 62,5 mL, respectively. The culture grown overnight at 37°C and after that period the OD_{600} was measured. According with equation 1, a proper volume was then then transferred to a new flask containing 62.5 mL of fresh LB medium, with ampicillin 100 µg/mL, in order to obtain an initial OD_{600} equal to 0.5.

$$OD_{pre-inoculum} \times Volume_{pre-inoculum} = OD_{inoculum} \times (Volume_{pre-inoculum} + Volume_{inoculum})$$

(Equation 1)

At that point, cryovials containing 30% of glycerol and 70% of inoculum were prepared and stored at -80°C.

3.2.4 STEAP1 recombinant biosynthesis in *E.coli* BL21 (DE3)

All the *E.coli* BL21 (DE3) cells transformed with the pET101-STEAP1 vector were grown in LB plates, with ampicillin 100 µg/mL, at 37°C. After that, cells were transferred to a pre-inoculum flasks containing 25 mL of complex media: LB, TB, SOB, SOC and 2YT, until reach an OD_{600} of 2.6. In this study, all the fermentations occur at 37°C and 250 rpm and all the mediums were supplemented with ampicillin 100 µg/mL.

After that, according with equation 1, a proper volume of the pre-inoculum was transferred to flasks containing 125 mL of each medium, being the initial OD_{600} set up to 0.2. When the culture reach OD_{600} values between 0.6 and 0.8, IPTG or lactose was added to a final concentration of 1mM. Molecular chaperones such as DMSO, sorbitol and arginine were added to the culture. The growth profile of the different cultures was screened by measuring the OD_{600} and the STEAP1 expression levels were analyzed by western blotting and SDS-PAGE.

Finally, the cells were harvested by centrifugation at 3900g, 4°C, over 20 minutes and stored at -80°C until further use.

3.2.5 Cell lysis and STEAP1 solubilization

The harvested cells were resuspended in a lysis buffer containing 500 mM NaCl, 50 mM Tris, 1 mM $MgCl_2$, pH 7.8**.** Since the pellet was properly solubilized, lyzosyme 0.5 mg/mL was added, at room temperature, and after 15 minutes the process of cell lysis was performed following the ratio 1 g cell mass : 2 g glass beads : 2 mL lysis buffer (500 mM NaCl, 50 mM Tris, 1 mM $MgCl_2$, pH 7.8), according to a protocol developed by our research group [185]. After the mixture was formed, it was submitted to 7 cycles of vortex (1 minute) and ice (1 minute) and DNase I 0.1 mg/mL was added. A centrifugation at 16000g, over 30 minutes at 4°C was performed. The membrane pellet from that centrifugation was then solubilized with lysis buffer supplemented Triton X-100 (1%) overnight at 4°C. After full solubilization, total protein concentration was measured in both pellet and supernatant, using Pierce BCA Protein Assay Kit (ThermoFisher Scientific, MA, USA), according to the manufacturer's instructions.

3.2.6 STEAP1 immobilized metal-ion affinity chromatography purification

The purification assays were performed using ÄKTA avant purifier system. All the buffers used were prepared with Mili-Q system water and then filtered through a 0.2 µm pore size membrane (Schleicher Schuell, Dassel, Germany) and sonicated. Two IMAC commercial HisTrap™ FF crude 5 mL columns (GE Healthcare) were used. Both columns were packed following manufactures's guidelines with different metals, namely nickel (Ni) and cobalt (Co).

Several assays were performed, in both Ni and Co columns, by varying imidazole (5, 7, 8.5 and 10mM) concentration in binding buffer (500 mM NacL, 50 mM Tris-base, 1 mM $MgCl_2$, pH 7.8). The sample containing STEAP1 protein was diluted 1:2 in binding buffer and injected in the system by a 2 mL capillary loop at a 0.5 mL/min flow rate along 5 column volumes (CV). The elution occurred by a linear gradient using a proper elution buffer (500 mM NaCl, 50 mM Tris-base, 1 mM $MgCl_2$, 500 mM imidazole, pH 7.8) from 0 to 100% elution buffer at 1 mL/min flow rate along 5 CV. A final elution step was carried out during 2 CV in order to perform a complete elution of the linked species.

In all experiments several parameters such as absorbance at 280 nm, pH profile, conductivity and system pressure were measured. The peaks of interest were collected in 1 mL fractions and then concentrated using Pierce™ Protein Concentrator PES (10000 MW) at 4°C. The samples were conserved at -80°C until further Western blot analysis. The total protein concentration was measured using Pierce BCA Protein Assay Kit (ThermoFisher Scientific) according with manufacturer's guidelines.

3.2.7 Agarose gel electrophoresis

All the STEAP1 DNA fragment and pET101 manipulations were confirmed on a 1% agarose gel (GRiSP, Porto, Portugal). It was used a Tris-acetic acid (TAE) buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8). The electrophoresis occurred ate 120 V for 30 minutes. The gels containing GreenSafe Premium reagent were analysed under UV light in UVITEC FireReader (UVItec, Cambridge, UK).

3.2.8 Total protein quantification

The total protein content in lysate, pellet and supernatant samples from cell culture was measured using Pierce BCA Protein Assay Kit (ThermoFisher Scientific). Standards of bovine serum albumin (BSA) from 0.125 to 2 mg/mL were used (Figure 5). Therefore, the total protein in samples was measured using a lysis buffer (500 mM NaCl, 50 mM Tris, 1 mM $MgCl_2$, pH 7.8) calibration curve, according to manufacturer's guidelines in xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). Another calibration curve was obtained, using standards of BSA from 0.125 to 2 mg/mL diluted in elution buffer (500 mM NaCl, 50 mM Tris-base, 1 mM $MgCl_2$, 500 mM imidazole, pH 7.8) for sample quantification after IMAC (Figure 6).

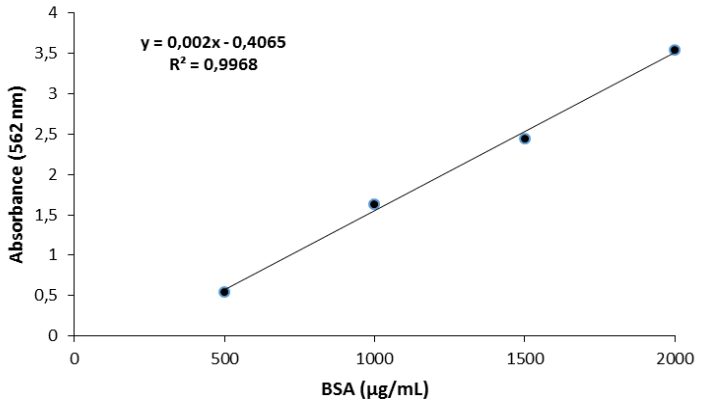

Figure 5 – Calibration curve of BSA solubilized in lysis buffer (500 mM NaCl, 50 mM Tris, 1 mM $MgCl_2$, pH 7.8)

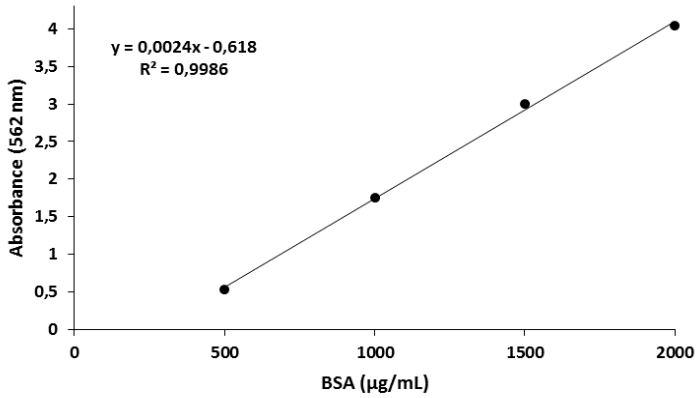

Figure 6 – Calibration curve of BSA solubilized in elution buffer (500 mM NaCl, 50 mM Tris-base, 1 mM $MgCl_2$, 500 mM imidazole, pH 7.8)

3.2.9 SDS-PAGE and Western Blot analysis

According with total protein quantification, 5 µg were set to SDS-PAGE and Western Blot analysis. A loading buffer (500 mM Tris-Cl at pH 6.8, 10% SDS (w/v), 0.02% bromophenol blue (w/v), 0.2% glycerol (v/v), 0.02% ß-mercaptoethanol (v/v)) and the mixture was heated to 100°C for 5 minutes. After that, the samples run on a 12.5% acrylamide gel (GRiSP) with 0.1% SDS, at 120 V for 90 minutes in a running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS (w/v)). After this step, two procedures were followed, depending if it was intended to stain the gel, or detect STEAP1 using antibodies. In the case of gel staining, it was used a solution of Coomassie brilliant blue (50% methanol, 10% glacial acetic acid, 40% H_2O) for 1 hour, over stirring. Then two discoloration solutions were applied, 30 minutes each. Discoloration solutions contains 320 mL/L methanol and 56 mL/L acetic acid glacial 99%, and discoloration solution II contains 50 mL/L methanol and 70 mL/L acetic acid glacial 99%.

In the case of Western blot analysis was required, the proteins from gel were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, UK) at 0.75 mA, during 45 minutes. The electrotransfer buffer contains 10 mM CAPS and 10% (v/v) methanol and was maintained cooled during the electrotransference procedure.

Consecutively, the membrane was blocked with a washing buffer (100 mL TBS, 1mL Tween20) with 5% milk (w/v), for 1 hour at room temperature. After blocking, the membrane was incubated overnight at 4°C with a rabbit anti-STEAP1 (Invitrogen) monoclonal primary antibody diluted 1:600, previously optimized, in washing buffer followed by incubation with an anti-rabbit IgG secondary antibody (Invitrogen) diluted 1:40000 in the washing buffer.

Finally, the membrane was incubated with Clarity™ Western ECL Blotting Substrate (Bio-Rad), for 3 minutes and was revealed by chemiluminescence detection using Molecular Imager FX Pro Plus Multiimager (Bio-Rad).

4. Results and Discussion

4.1 Construction of the pET101-STEAP1 expression vector

The first step of pET101-STEAP1 expression vector was the amplification by PCR of the STEAP1 gene from another vector, pIRES-STEAP1. Specific primers were used in such reaction (forward primer: 5'- AAGG AATT CAGG AGCC CTTC ACCA TGGA AAGC AGAA AAGA CATC – 3'; reverse primer: 5'- AATG AGCT CCTA ATGG TGAT GGTG ATGG TGCA ACTG GGAA CATA TCTC AGT – 3'). Three different PCR profiles, with three different annealing temperature, 60°, 62° and 65°were tested (Figure 7).

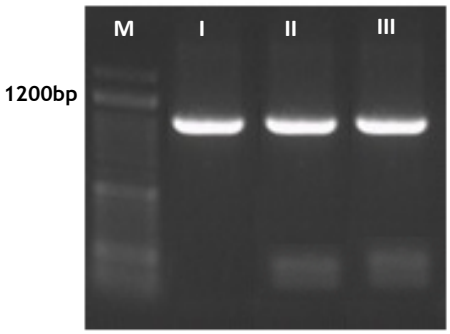

Figure 7 – Agarose gel electrophoresis of PCR products of STEAP1 gene amplification at different annealing temperatures: 60 °C (I), 62 °C (II) and 65 °C (III).

Since no differences were noticed in amplification levels, the lowest temperature was chosen to further experiments.

After amplification, the STEAP1 gene was purified using the NucleoSpin Extract II kit and digested with EcoRI and SacI restriction enzymes. Gel electrophoresis was performed in order to confirm fragment digestion.

The pET vector was amplified in *E.coli* cells in LB medium, extracted and purified with NZYMiniprep kit. The vector was also digested with EcoRI and SacI restriction enzymes. The digestion was confirmed by agarose gel electrophoresis and the vector was purified from the gel using the NucleoSpin Extract II kit

The STEAP1 was then cloned into the pET101 vector, using different vector: insert ratios (1:1, 1:4, 1:6). The ligation occurred using T4 DNA ligase, over 4h, at room temperature, with the ratio 1:4, followed by *E.coli* TOP10 competent cells transformation by heat shock (1h in ice, 1min at 42°C). The cells grown overnight in LB agar plates, at 37°C and the colonies were picked, lysed (95 °C for 10min) and inoculated overnight in 4 mL of LB medium, at 37°C and 250 rpm. Then, the DNA plasmid was extracted and purified using NZYMiniprep kit (Figure 8A).

DNA sequence of the pET101-STEAP1 vector was analyzed by sequencing with T7 primers and BLAST analysis allowed to confirm that DNA fragment encodes STEAP1 protein. In order to confirm the uptake level of the plasmid, *E.coli* BL21 (DE3) cells were transformed with

the pET101-STEAP1 plasmid, by heat shock, and inoculated in LB agar plates overnight at 37°C. The colonies were picked and lysed and a PCR was performed (Figure 8B).

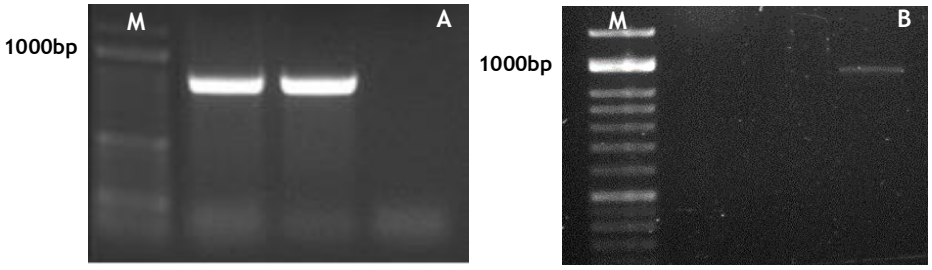

Figure 8 – (A) Agarose gel electrophoresis of purified pET101-STEAP1 from E.coli TOP10 competent cells. (B) Agarose gel electrophoresis of purified pET-STEAP1 from E.coli BL21 (DE3) cells.

The integrity of the plasmid was confirmed by PCR, since the obtained band match the correct MW of STEAP1. Also, figure 8B show a loss of the plasmid in the purification step.

4.2 Recombinant STEAP1 biosynthesis

E.coli is one of the most used hosts for macromolecules biosynthesis [148,150]. However, STEAP1 protein was never produced in a recombinant way, not even in this host. So, it is imperative in the early stage of this work to control and evaluate the behavior of the host transformed with the plasmid. In order to achieve that, *E.coli* BL21 (DE3) cells transformed with the pET101-STEAP1 vector were cultivated in four different media: TB, SOB, SOC and 2YT, at 37°C and 250 rpm, and the growth profile was traced by measuring OD_{600} every hour until stationary phase, without any induction. In every case the cultures presented a typical growth profile with a short lag phase due to the pre-inoculum in the same conditions (data not shown).

The next step was to induce the production of the protein of interest and evaluate the culture growth profile in different complex media. The induction was made with IPTG 1mM at 1h of fermentation, in the beginning of exponential growth phase, with an OD_{600} between 0.6 and 0.8 (Figure 9).

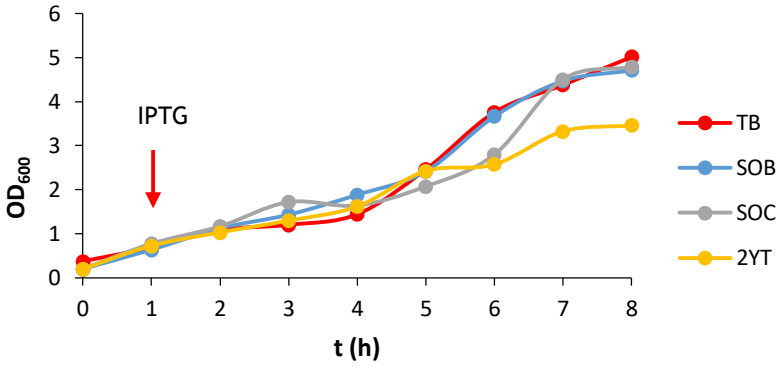

Figure 9 - Growth profile of E.coli BL21 (DE3) culture transformed with the pET-STEAP1 vector in four different media: 2YT, SOC, SOB and TB. IPTG at 1mM was added after 1h of fermentation.

The TB, SOB and SOC media present a similar profile, but the 2YT reach a lower OD in the stationary phase, probably because it is a less rich medium, due to the reduced amount of salts present.

Subsequently, it was important to evaluate in which media STEAP1 was produced with higher expression and where it gets compartmentalized in cell. To verify that, Western blot analysis was performed with both cell pellet and supernatant from cell lysis. The results indicated that STEAP1 was expressed only in TB and SOB media and accumulates with higher expression levels in SOB (Figure 10).

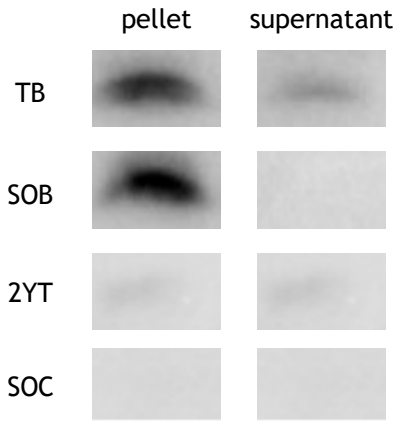

Figure 10 – Western blot analysis of pellet and supernatant samples after cell lysis d from four different media: TB, SOB, 2YT and SOC.

The protein accumulation in pellet is an expected result due to the macromolecule properties and characteristics. Its six transmembrane domains give to the protein a hydrophobicity level that prevents it to being solubilized in the supernatant, remaining attached to the fragmented cell membrane, as observed for another membrane proteins produced in *E.coli* [141, 148] . The fact that STEAP1 was expressed only in TB an SOB media

may be related with the fact that these media guarantee a better stabilization of culture allowing the expression of heterologous proteins.

Despite the Western blot analysis shown that STEAP1 was produced in two media, the following strategy was trying to improve the biosynthesis yield. In this first approach, production was induced after 1h of fermentation. Consequently, STEAP1 protein was harvested from the culture medium after 7 hours. Since STEAP1 is not naturally synthesized by *E.coli*, defense mechanisms from the host could be triggered to neutralize the high levels of this heterologous protein, what it is not beneficial to the process [182]. To overcome this problem, the growth profiles of the culture without STEAP1 production were performed and analyzed to find another strategy (Figure 11).

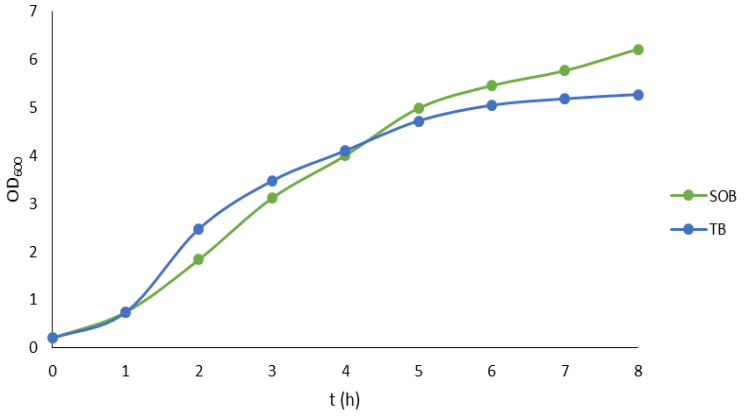

Figure 11 – Growth profile of E.coli BL21 (DE3) culture transformed with the pET-STEAP1 vector in TB and SOB media, without induction.

The ideal time point to induce STEAP1 protein production must be a compromise between high OD and the end of the exponential phase. So, in this case, IPTG 1mM was added at 5h of fermentation. Western blot analysis comparing both induction strategies reveals that higher expression levels were achieved inducing at 5h, once the band intensity increased (Figure 12). Also the growth profile from cultures induced at 1h and 5h of fermentation were compared (Figure 12). The lower OD reached when IPTG was added at 5h could be explained by the higher expression level of STEAP1 protein. The readjustment of *E.coli* metabolic pathways to protein expression implies a lower growth rate [183].

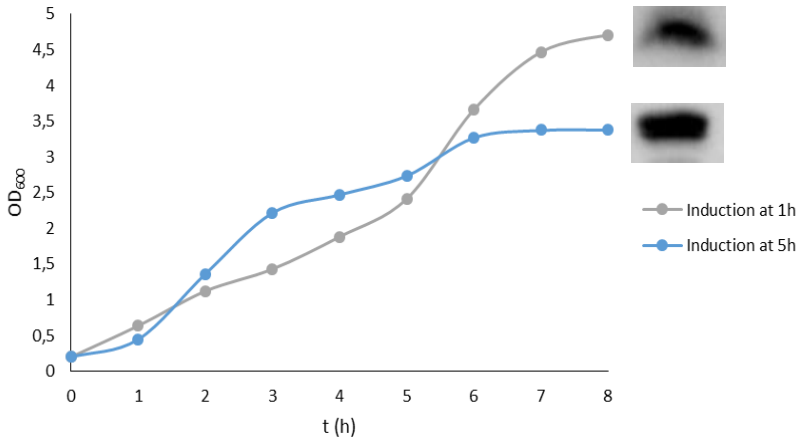

Figure 12 – Growth profile of E.coli BL21 (DE3) culture, with induction with IPTG 1mM at two different times: 1h and 5h after fermentation.

Additional factors that must be taken into account is the time of cell harvesting and the optimal IPTG concentration to be used. The harvesting time after induction is a relevant parameter in order to minimize the time that STEAP1 protein remains in the host, subject to its defense mechanisms and to undesirable structural modifications. IPTG concentration is extremely relevant because of its potential toxicity, so a sustainable strategy is to use a minimal concentration as possible, without affect the protein yields.

If so, three time points for cell harvesting (1, 2 and 3h after induction) and three IPTG concentrations (1, 1.25, 1.5mM) were tested (Figure 13).

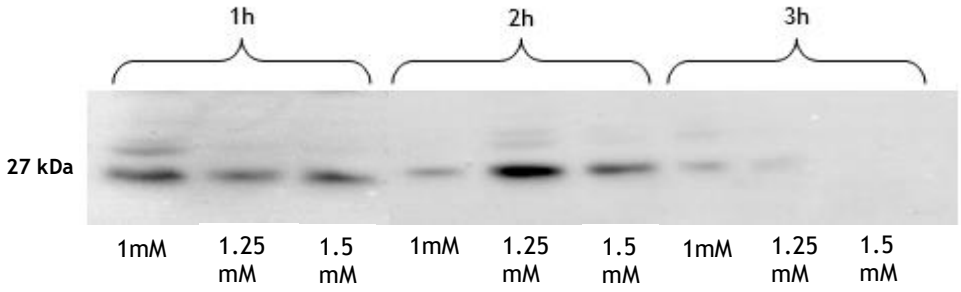

Figure 13 – Screening of cell harvesting time (1, 2 and 3h) and IPTG concentration (1, 1.25 and 1.5 mM).

The results obtained were analyzed by Western blot and it showed that a higher expression level of STEAP1 protein occurs using IPTG at 1.25 mM following cell harvesting 2h later. However, the band corresponding to STEAP1 protein obtained in these results have a MW of approximately 27 kDa and it is well known that native STEAP1 have a MW of 39 kDa [98]. The hypothesis of a STEAP1 with a modified structure or not fully synthesized was considered as a possible explanation to that. Since the host is a prokaryotic organism with inability to form disulfide bonds and the lack of post-translational mechanisms (PTM), such as glycosylation, glycation and phosphorylation, the protein may not acquire the correct native fold [111, 182]

To overcome that, a new approach was performed: since the expression of STEAP1 protein is controlled by *lac* operon, lactose could also be used as inducer [184]. The induction was made with lactose 0.5, 1 and 1.5 mM at 1 and 5h of fermentation, being the cells harvested 2h later. TB and SOB media was tested with this new conditions and both cell pellet and supernatant from lysis were analyzed by western blot (Figure 14). In the case of using lactose 1 mM at 5h, higher expression levels occur in supernatant, instead of cell pellet, as occurred using IPTG. However, the MW of the band was lower than the expected for STEAP1 protein.

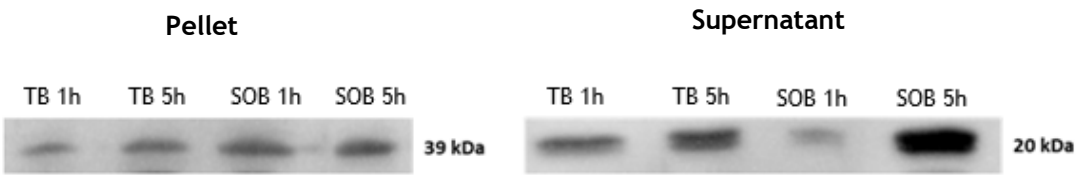

Figure 14 – Western blot analysis of pellet and supernatant samples from TB and SOB media E.coli BL21 (DE3) cells transformed with pET101-STEAP1 vector, induced with lactose 1 mM at 1 and 5h of fermentation.

In pellet samples, the MW of the band is accordant with the native STEAP1 but the inconsistency of these results makes the work proceed using IPTG at 1.25 mM and trying additional strategies to produce a STEAP1 with the correct MW.

Chemical chaperones are molecules used to avoid protein misfolding and aggregation, by up-regulate the expression of genes involved in membrane lipid components [185]. Therefore, this type of molecules could be applied to overcome structural modifications or aggregation of STEAP1 protein [186]. Three chemical chaperones were used: DMSO, sorbitol and arginine at different concentrations, added to the fermentation media before induction [187] (Figure 15).

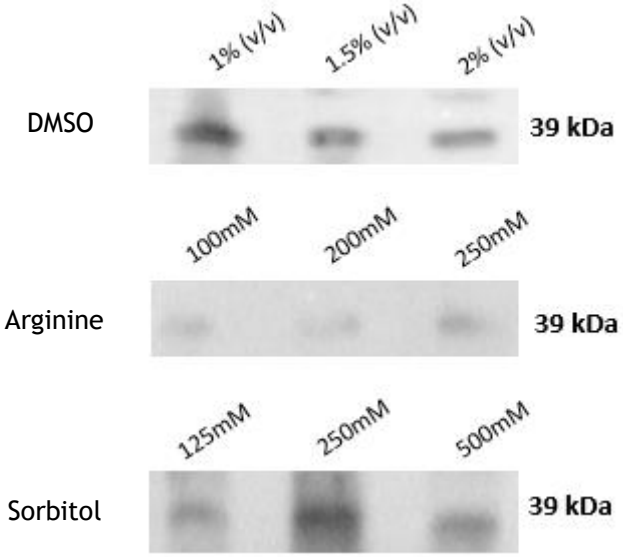

Figure 15 – Western blot analysis of samples with three chemical chaperones added to the fermentation media before induction. DMSO was tested at 1, 1.5 and 2%; Arginine at 100, 200 and 250 mM; Sorbitol at 125, 250 and 500 mM.

Summarily, DMSO and sorbitol had a positive effect in protein stabilization, once the STEAP1 band match the correct protein MW (39 kDa). Arginine shows no effect in STEAP1 protein stabilization, since the band obtained do not match the correct MW. This is possibly due to the fact that arginine could enter in the *E.coli* cell to be converted in succinate and participate in the tricarboxylic acid cycle, instead of binding the residues present in the surface of the protein [188].

Therefore, synergetic effect between DMSO and sorbitol was investigated. The Western blot analysis showed that using this two chaperones simultaneously have no positive effect in STEAP1 stabilization, since the band do not correspond to the correct MW (Figure 16).

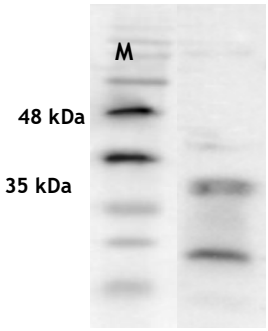

Figure 16 – Western blot analysis of a cell pellet sample, using DMSO (1%) and sorbitol (250mM), added to the fermentation media before induction.

This result could be explained by the fact that combined concentrations of both DMSO and sorbitol, these chaperones could lead to an increased stress of the culture, thus inhibiting some metabolic pathways, such as host internalization, secondary metabolism and expression of heterologous proteins, such STEAP1 [183].

So, further assays were developed using DMSO 1.5%, added to the fermentation medium before induction, since it was the chaperones that leads to a higher expression level of STEAP1, with the correct MW.

4.3 STEAP1 detergent solubilization

The solubilization of membrane proteins is a major concern and a bottleneck in a biosynthesis and isolation process. Such macromolecules are located in a dynamic mosaic lipid bilayer. As consequence, they are not soluble in aqueous solutions [165]. Therefore, the use of detergents as a tool for studying membrane proteins became very popular, namely the nonionic surfactant Triton X-100 [166,168].

A screening of several Triton X-100 concentrations (1 to 4%) was performed, being this added to the moisture after cell lysis by glass beads and heat shock procedure (Figure 17). The Triton X-100 was already referred as the best surfactant to solubilize the STEAP1 immunodomain [189].

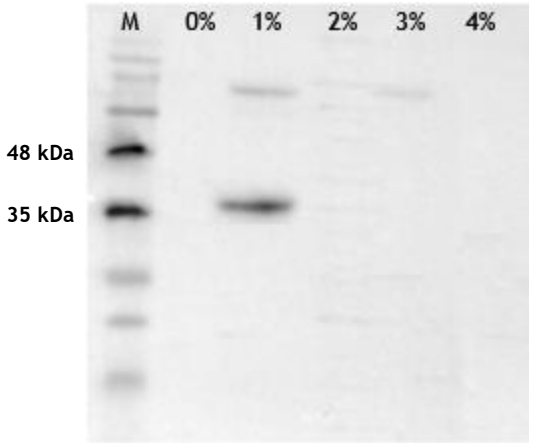

Figure 17 – Screening of Triton X-100 concentration.

The STEAP1 protein was easily recovered using the non-ionic detergent Triton X-100 in a concentration of 1% (v/v). High concentrations of Triton X-100 affected dramatically protein recovery, as well as in the absence of Triton X-100, which is expected due the considerable hydrophobicity level of STEAP1 protein. These results may be explained by the fact that having Triton X-100 in high concentration in samples, may lead to a disruption of the protein structure [165]. At concentrations above 1%, Triton X-100 seems to act as a denaturant agent, instead of provide a desirable protein stabilization [165,169].

4.4 Purification of STEAP1 protein by immobilized metal-ion affinity chromatography

The last step of a recombinant protein bioprocess comprises one or several purification stages. One of the most widely used methods is based in the presence of a fusion-tag with histidine that bio-recognize resins charged with transition metals ions [190]. So, in this work, a fusion tag (His-tag or 6xHis-tag) was added to the primary sequence of STEAP1 in order to allows its purification in Co^{2+} and Ni^{+} packed IMAC commercial HisTrap^{TM} FF with 5 mL of resin (GE Healthcare).

Initially, four assays were performed in each column, where different imidazole concentrations in binding buffer were tested (5, 7, 8.5 and 10 mM). The elution buffer, with 500 mM of imidazole was maintained in all the assays. It is well known that the presence of imidazole in the binding step prevents undesirable interactions (ionic and affinity) and avoid the binding of host proteins with a great percentage of histidine in its surface [191]. Therefore, sample contaminants can be eliminated straightforward in the injection step [191].

The first assay was realized in nickel and cobalt charged columns, with an imidazole concentration of 5 mM in mobile phase (Figure 18).

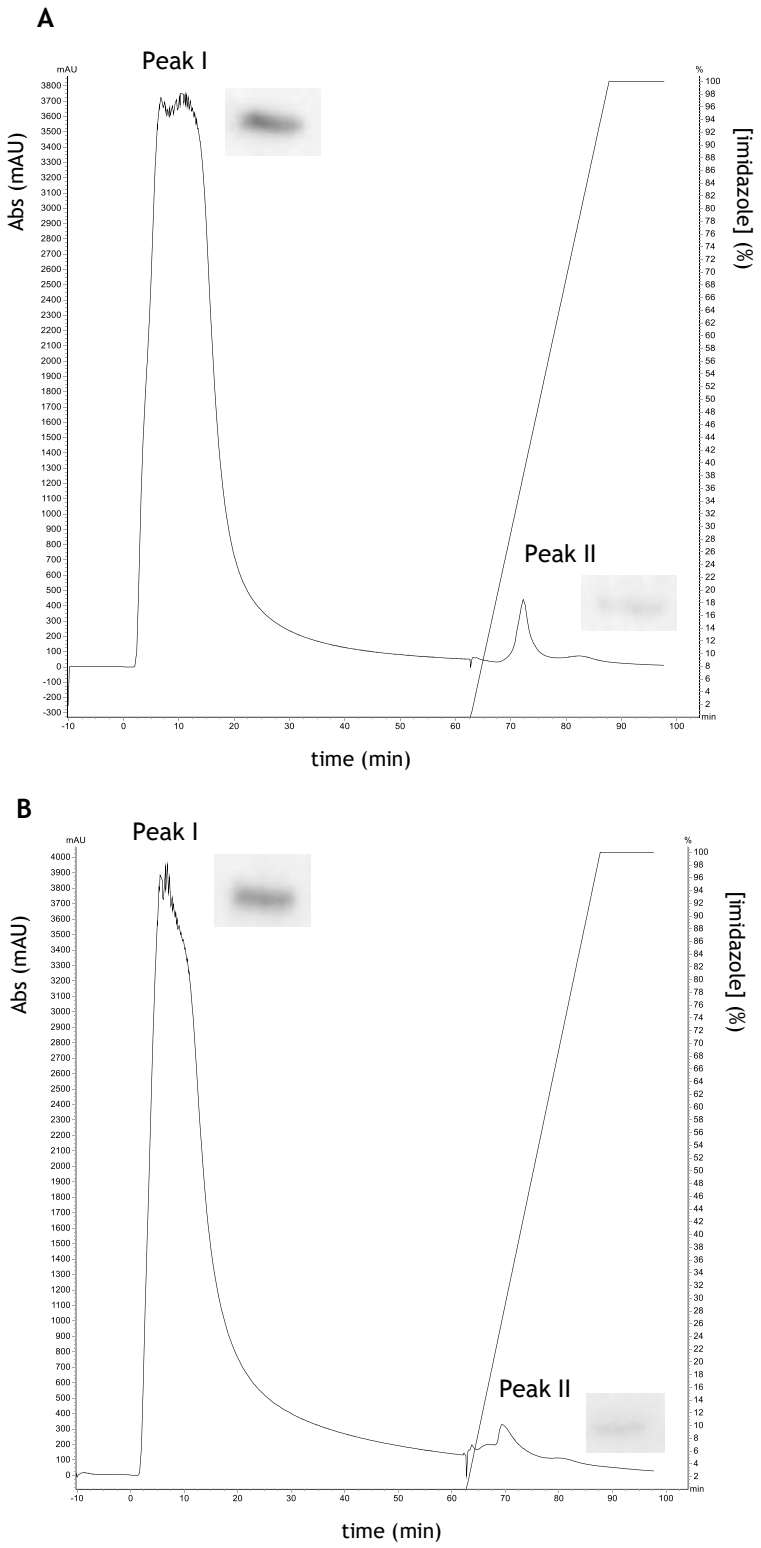

Figure 18 – Chromatogram of STEAP1 purification from E.coli BL21(DE3) lysates in a HisTrap FF crude with Ni^{+} (A) and Co^{2+} (B) ions immobilized and using an imidazole concentration of 5 mM in binding buffer and a linear gradient from 0 to 500 mM of imidazole in elution buffer. Western blot analysis of each peak can be observed in the chromatographic profile.

The analysis of both chromatograms showed two peaks of interest, one in the injection phase and second one in the elution phase. Both were analyzed by Western blot and reveals an incomplete retention of STEAP1 onto the matrices. Therefore, further assays were performed increasing imidazole concentration in mobile phase, aiming to reduce unspecific bindings and to promote a complete binding of the target protein.

From the three concentrations tested (7, 8.5 and 10 mM), the satisfactory results were obtained using a mobile phase with 7 mM of imidazole (Figure 19 and 20

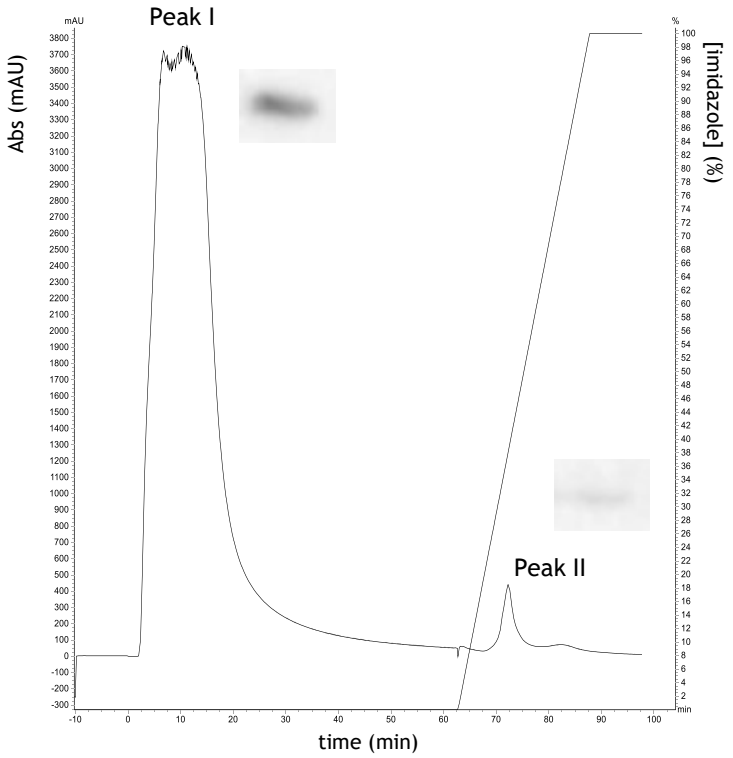

Figure 19 – Chromatogram of STEAP1 purification from E.coli BL21(DE3) lysates in a HisTrap FF crude with Ni^{+} ions and using an imidazole concentration of 7 mM in binding buffer and followed with a linear gradient from 0 to 500 mM of imidazole. Western blot analysis of each peak can be observed in the chromatographic profile.

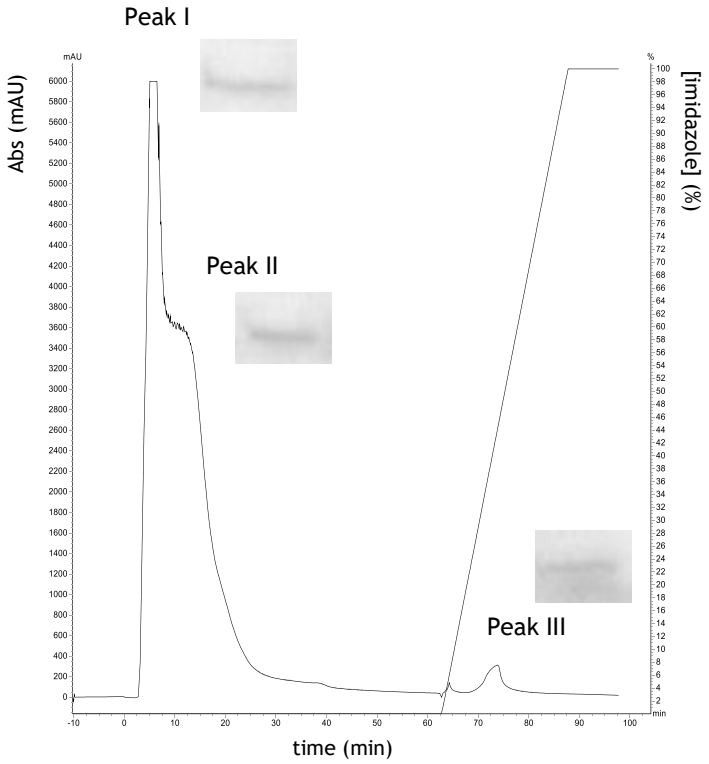

Figure 20 – Chromatogram of STEAP1 purification from E.coli BL21(DE3) lysates in a HisTrap FF crude with Co^{2+} ions and using an imidazole concentration of 7mM in binding buffer and followed with a linear gradient from 0 to 500 mM of imidazole. Western blot analysis of each peak can be observed in the chromatographic profile.

Despite the fact that the binding of the target protein onto the column do not occurs in a complete way and elution does not allow a total elimination of the host proteins, the application of 7 mM of imidazole in binding buffer lead to a satisfactory results, allowing a partial STEAP1-column binding. In addition, other concentrations tested (8.5 and 10 mM) were inefficient for protein binding. From the literature, it is known that cobalt presents a lower binding capacity but a higher specificity for His-tag [192]. However, the obtained results illustrate that the Co^{2+} charged column promote a higher binding capacity for STEAP1, since the Western blot analysis shows a lower level of STEAP1 in the fraction collected in the binding step. Furthermore, the chromatograms obtained lead to a typical profile tracing for STEAP1 elution for all the developed assays, in which the metal charged and the mobile phase were ranged.

Nevertheless, these results suggest that additional chromatographic steps are needed to promote a complete binding of STEAP1 in the IMAC matrices.

5. Conclusion and Future Perspectives

Actually, membrane proteins could be the key leading to a sustainable development of new therapeutic approaches in the treatment of several human diseases, such as oncological diseases. Also, the biotechnological field could play an extremely important role in this subject, enabling and enhancing the biosynthesis process, in order to obtain large amounts of the target biomolecule in highly purified fractions for further structural and biointeraction studies. One of that biomolecules is the STEAP1, whose its recombinant biosynthesis, solubilization and purification can open new paths for the development of innovative treatments in PCa.

In the developed work, *E.coli* BL21 (DE3) harboring the pET101-STEAP1 vector allowed an efficient expression of the STEAP1. The SOB culture medium was established as the most appropriate for STEAP1 expression, in a temperature of 37°C and orbital stirring of 250 rpm. It was stated that STEAP1 protein needs the presence of an inducer to be expressed in this strain. Indeed, the inducer IPTG, in a concentration of 1.25 mM, leads to the maximum expression levels. Also, this stage is improved with the induction performed at 5h of fermentation, using DMSO at 1.5% added to the complex media before induction, being the cells harvested 2h later. Alternatively, additional studies carried out with lactose as inducer, leads to a distinct STEAP1 compartmentalization, typically in supernatants but with an incorrect molecular weight or lower yields.

Once STEAP1 is a membrane protein, its recovery was performed efficiently with 1% (v/v) of a non-ionic surfactant, Triton X-100. However, the use of more efficient structures such as liposomes and micelles can be a good option to improve the recovery of this protein. Also, additional centrifugation steps could also be applied, in order to obtain a better lysate clarification.

The purification of STEAP1 using affinity chromatography, due to the presence of an additional His-tag, showed that Co^{2+} charged column promote a satisfactory binding of the target molecule, since columns charged with this metal also presents a higher specify for affinity tags in membrane proteins than the Ni^{+} matrices. In all the assays performed, the chromatographic profile is consistent, despite the binding stage is not totally efficient. This step needs to be improved, namely by adding a purification step before IMAC, performing a step elution IMAC and/or perform an ionic exchange chromatography in a final polishing stage.

Succinctly, this work has established several key points in the global process of biosynthesis and purification of a promising target molecule in PCa. The next approaches can be the optimization of several parameters, such as the fermentation conditions, the solubilization protocol and the optimization of the purification phase. Once a small scale platform is established, there is a need to evaluate the scale-up of it, aiming to establish a biosynthesis platform that allows the structural and biointeraction assays required to a better resolution of human STEAP1 and its potential in PCa therapy.

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Attachments

This master thesis originated two scientific communications:

Attachment I: Oral communication at the XI Annual CICS-UBI Symposium – July/2016, Covilhã, Portugal.
Attachment II: Poster presentation at the 11th YES Meeting – September/2016, Porto, Portugal.

Attachment I

BIOSYNTHESIS AND ISOLATION OF STEAP1:
A PROMISING THERAPEUTIC TARGET FOR PROSTATE CANCER.

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CICS-UBI – Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal

The six transmembrane epithelial antigen of the prostate 1 (STEAP1) is a member of STEAP protein family, which also includes STEAP2, STEAP3 and STEAP4. STEAP1 is overexpressed in several human cancers, including bladder, colon, pancreas and breast, and with a higher expression profile for prostate cancer (PCa) [1, 2].
Although the role of STEAP1 in human cells is not yet entirely understood, several studies have demonstrated that STEAP1 is involved in intercellular communication, allowing tumour growth [3]. In fact, some research groups have shown that blocking STEAP1 leads to a reduction of cell proliferation. Taking into account the localization of STEAP1 on cell membrane, associated to its overexpression in cancer cells, STEAP1 arises as an important therapeutic target for PCa [1, 3].
So, the aim of this study was to produce the recombinant human STEAP1 protein in *E.coli* cells and perform its isolation by IMAC, allowing the recovery of pure protein fractions in order to develop structural interaction assays and design specific molecules to inhibit the action of STEAP1 in cancer cells.
Globally, it was performed several experiments in order to optimize the fermentation, in which the medium composition, the inducer concentration, the time of induction and the use of chemical chaperones (DMSO, sorbitol and arginine) was tested. The results showed that the use of SOB medium, with IPTG at 1,25 mM as inducer after 5h of fermentation and using DMSO at 1,5% could be the ideal conditions for the biosynthesis of recombinant STEAP1 protein . Also, the results demonstrated that applying lactose as inducer could increase the solubility of the target protein.

Keywords: STEAP1, Prostate, Prostate Cancer, E.coli, IMAC.

References:
[1] Hubert R.S. et al., Proc Natl Acad Sci USA, 1999, Dec 7;96(25):14523-8. STEAP: a prostate-specific cell-surface antigen highly expressed in human prostate tumors.

[2] Gomes I.M. et al., Urol Oncol, 2014, 32(1): 53 e23-59. STEAP1 is overexpressed in prostate cancer and prostatic intraepithelial neoplasia lesions, and it is positively associated with Gleason score.

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Attachment II

CICS-UBI
Centro de Investigação em Ciências da Saúde
Health Sciences Research Centre

FACULDADE CIÊNCIAS DA SAÚDE

RECOMBINANT EXPRESSION OF STEAP1 IN *ESCHERICHIA COLI*: Screening of bioprocess parameters, compartmentalization and chaperones stabilization.

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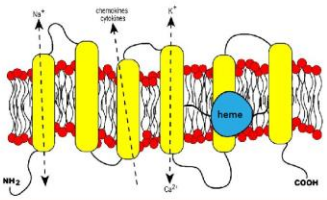
Introduction

The six transmembrane epithelial antigen of the prostate 1 (STEAP1, 39 kDa) is a member of STEAP protein family. STEAP1 was identified overexpressed in several human cancers, including bladder, colon, pancreas and breast, with a higher expression profile for prostate cancer (PCa) [1, 2], located in cell-cell junctions of the secretory epithelium of the prostate.

Although the role of STEAP1 in human cells is not yet entirely understood, several studies have demonstrated that STEAP1 is involved in intercellular communication, allowing tumour growth [3]. In fact, some research groups have shown that blocking STEAP1 leads to a reduction of cell proliferation.

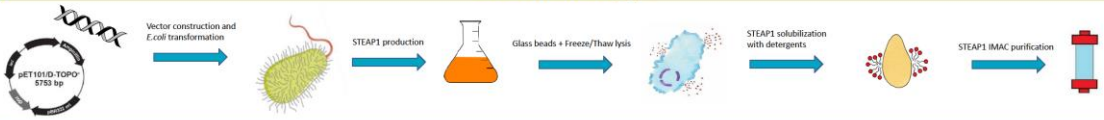
Taking into account the localization of STEAP1 on cell membrane, associated to its overexpression in cancer cells, STEAP1 arises as a relevant protein in PCa pathway and a promising therapeutic target for the disease [1, 3].



Global Aim

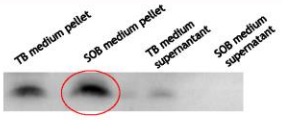
The aim of this study was to produce the recombinant human STEAP1 protein in *E.coli* cells and perform its isolation by IMAC, allowing the recovery of pure protein fractions in order to develop structural interaction assays and design specific molecules to inhibit the action of STEAP1 in specific cancer cells lines.

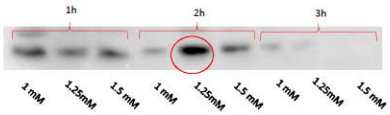
Methodology



Results

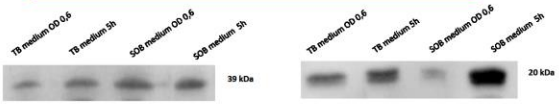
SOB and TB media are the most indicated to STEAP1 biosynthesis.



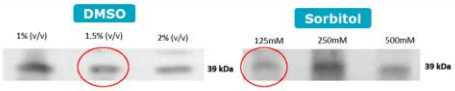


STEAP1 accumulates mainly in cell pellet and a maximum expression occurs with IPTG 1.25mM at 5h fermentation.

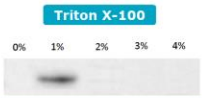
Using lactose as inducer, STEAP1 accumulates mainly in supernatant, but with an incorrect molecular weight.



Using DMSO and Sorbitol as chaperones, recombinant STEAP1 acquires the correct MW



STEAP1 solubilization was achieved with Triton X-100 1% (v/v).



Conclusions

The use of SOB medium, with IPTG at 1.25 mM as inducer after 5h of fermentation, using DMSO at 1.5% (v/v) combined with sorbitol 125-250 mM, could be the ideal conditions for the biosynthesis of recombinant STEAP1 protein. Moreover, the results demonstrated that applying lactose as inducer could influence the compartmentalization of the target membrane protein and increase its solubility. The solubilization is achieved with 1% Triton X-100.

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[1] Hubert R.S. et al., Proc Natl Acad Sci USA, 1999, Dec 7;96(25):14523-8. STEAP: a prostate-specific cell-surface antigen highly expressed in human prostate tumors.
[2] Gomes I.M. et al., Urol Oncol, 2014, 32(1): 53 e23-59. STEAP1 is overexpressed in prostate cancer and prostatic intraepithelial neoplasia lesions, and it is positively associated with Gleason score.
[3] Yakamoto T., et al, Exp Cell Res, 2013, 319(17): 2617-2626. Six-transmembrane epithelial antigen of the prostate-1 plays a role for in vivo tumor growth via intercellular communication.

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