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ARTÍCULO DE REVISIÓN

Marine and coastal organisms: a source of biomedically relevant dipeptidyl peptidase IV inhibitors

Los organismos marinos y costeros: fuente de inhibidores de la enzima dipeptidil peptidasa IV de relevancia biomédica

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ABSTRACT

Dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5), also known as CD26, is a serine aminopeptidase that preferentially cleaves Xaa-Pro or Xaa-Ala dipeptides from the N-terminus of peptides leading to their biological activation or inactivation. The enzyme is an homodimer and each subunit is formed by an αβ-hydrolase domain and a β-propeller domain. It has an important role in multiple physiological functions, including the regulation of glucose metabolism, being one of the current targets for the treatment of type 2 diabetes mellitus. It is also up-regulated in rheumatoid arthritis, psoriasis, colitis, multiple sclerosis and transplant rejection. This enzyme also regulates immune system responses mediated by CD4+ T lymphocytes; furthermore, DPP-IV activity is dysregulated in pathologies like thyroid, ovarian, lung, skin, prostate cancers and central nervous system tumors. In clinical practice, DPP-IV inhibitors have several beneficial effects such as antihyperglycemia and pancreatic islet protection, immune regulation, cardiovascular and renal protection, anticancer effects, and anti-inflammation. Thus, this enzyme evolved as a target of attention for the development of more efficient pathology diagnostics, and for the development of inhibitors to treat type 2 diabetes mellitus and cancer. Marine and coastal organisms are an abundant source of different bioactive molecules including peptidases and peptidases inhibitors of almost all mechanistic classes, being serine class the most studied. In the present contribution, we review the strategies used to identify and characterize DPP-IV inhibitors from marine and coastal organisms. We show that marine biodiversity is an important, promising, and still unexplored source of inhibitors of dipeptidyl peptidase IV, which may have biomedical applications in human diseases.

Keywords: serine peptidases, dipeptidyl peptidase IV, marine organisms, peptides, inhibitors

RESUMEN

La dipeptidil peptidasa IV (DPP-IV, EC 3.4.14.5), también conocida como CD26, es una aminopeptidasa de tipo serino con preferencia de corte por la secuencia Xaa-Pro o Xaa-Ala del extremo amino de péptidos provocando su activación/inactivación. La enzima es un homodímero y cada subunidad consiste en dos dominios: un dominio αβ-hidrolasa y un dominio de propela-β, ambos implicados en su función enzimática y su interacción con otras proteínas. Esta enzima desempeña funciones importantes en múltiples procesos fisiológicos relacionados con el metabolismo de la glucosa, por lo que constituye hoy en día uno de los blancos para el tratamiento de la diabetes mellitus tipo 2. Esta enzima se encuentra también sobre-expresada en la artritis reumatoidea, la psoriasis, la colitis, la esclerosis múltiple y el rechazo a los trasplantes. DPP-IV participa además, en la regulación de la respuesta inmune mediada por linfocitos CD4+. Recientemente, se ha observado que su actividad se altera en diferentes tipos de cáncer tales como: tiroides, ovario, pulmón, piel, próstata, tumores del sistema nervioso central, entre otros. En la clínica, los inhibidores de DPP-IV tienen efectos beneficiosos como anti-hiperglicémicos, protectores de los islotes pancreáticos, reguladores del sistema inmune, protectores de las funciones cardiovasculares y renales, y efectos anticancerígenos y anti-inflamatorios. Por estas razones, esta enzima constituye actualmente un importante foco de atención para el diagnóstico, al ser considerada como un potencial marcador molecular de determinados tipos de patologías, así como para el desarrollo de inhibidores para combatir la diabetes mellitus tipo 2 y el cáncer. Los organismos marinos y costeros son una fuente abundante de compuestos bioactivos que incluyen peptidasas e inhibidores de peptidasas de todas las clases mecanísticas, siendo los inhibidores de tipo serino los más estudiados. En la presente contribución centramos la atención en las diferentes estrategias desarrolladas para la identificación y caracterización de inhibidores de DPP-IV a partir de organismos marinos y costeros. La misma demuestra que la biodiversidad marina es una fuente importante, promisoria y aun poco explorada de inhibidores de DPP-IV con potenciales aplicaciones biomédicas en enfermedades humanas.

Palabras clave*: proteasas serino, dipeptidil peptidasa IV, organismos marinos, péptidos, inhibidores*

INTRODUCTION

Proteolysis is an essential process closely related to protein turnover. Proteases are also directly or indirectly involved in key physiological processes such as growth, cell differentiation, apoptosis, nutrition, and cell migration (Leung *et al.,* 2000; Abbenante and Fairlie, 2005). Given their versatility and ubiquity, they are also directly related to pathophysiological events, such as the development of cancer, neurodegenerative, respiratory and cardiovascular disorders, as well as parasitic, viral and fungal infections (Leung *et al.,* 2000; Abbenante and Fairlie, 2005; Turk, 2006; Kaman *et al.,* 2014; de Veer *et al.,* 2014).

Functions of such importance require highly effective regulatory counterparts: protease inhibitors. The physiological function of these inhibitors lies in their ability to prevent proteolysis where it is not required, or to regulate it when limited proteolysis is required (Leung *et al.,* 2000; Abbenante and Fairlie, 2005; Turk, 2006). One of the major incentives to discover regulatory networks of proteases lies in the fact that controlling their enzymatic activity is a valid principle from a pharmacological point of view. In general, when the underlying biochemical mechanisms in disease are elucidated, it is possible to identify at least one protease as a potential therapeutic target. Thus, protease inhibitors have emerged with indisputable practical utility. Proteases inhibitors are currently used in the treatment of hypertension, cardiovascular diseases, cancers, inflammation, immunological and respiratory disorders and metabolic disorders such as type 2 diabetes mellitus (Drag and Salvesen, 2010; Deu *et al*., 2012; Deu *et al*., 2017; Amin *et al*., 2018; Li *et al*., 2018).

Dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5, also known as CD26) is a highly glycosylated cell surface membrane serine aminopeptidase that preferentially cleaves Xaa-Pro or Xaa-Ala dipeptides from the N-terminus of oligopeptides with approximately 30 or fewer aminoacids. This enzyme belongs to the Clan SC, family S9, of serine peptidases (Rawlings *et al.,* 2018). The amino acid sequence and three-dimensional structure of DPP-IV are well known (Thoma *et al.,* 2003; Engel *et al.,* 2003) (Figure 1A). As for other serine peptidases, DPP-IV has a catalytic triad formed by Ser630, Asp708 and His740 (residues number correspond to the porcine enzyme) (Figure 1B).

DPP-IV has a peculiar substrate specificity. This explains its key role in the catabolism of a number of chemo- and cyto-kines, neuropeptides, immunopeptides and peptide hormones containing a X-Pro or X-Ala amino terminal sequence, e.g. C-X-C motif chemokine ligand 12, substance P, neuropeptide Y, peptide YY, enterostatin, glucose-dependent insulinotropic polypeptide (GIP), and glucagon-like peptides-1 and 2 (GLP-1, GLP-2) (Yu *et al.,* 2010). This variety of substrates also explains the many functions of DPP-IV. As a cell surface protease, DPP-IV/CD26 plays a relevant role in tumor progression and glucose metabolism (Zhao *et al.,* 2014). It is also implicated in pathologies such as rheumatoid arthritis, psoriasis, colitis, multiple sclerosis and the rejection of transplants (Sedo *et al.,* 2005; Jung *et al.,* 2006). Therefore, inhibitors of the activity of this enzyme are of therapeutic value. Information about structure-function relationships contributed to the identification of new potent and selective inhibitors of DPP-IV with potential biomedical applications (Patel and Ghate, 2014; Sharma *et al.,* 2019; Kęska and Stadnik, 2020). In clinical practice, DPP-IV inhibitors have several beneficial effects such as antihyperglycemia and pancreatic islet protection (Trzaskalski *et al.,* 2020), immune regulation (Dowarah and Singh, 2020), cardiovascular and renal functions protection (Lei *et al.,* 2017; Kanasaki, 2018) and anti-inflammation (Vliegen and De Meester, 2018).

Marine and coastal organismos are an abundant source of different bioactive molecules including peptidases (Alonso del Rivero *et al.,* 2009; Pascual *et al.,* 2020) and peptidases inhibitors of almost all mechanistic classes, being serine class the most studied (Chávez *et al.,* 1988; Delfín *et al.,* 1994, 1996; González *et al.,* 2004; Sue *et al.,* 2009; Reytor *et al.,* 2011; Gonzalez *et al.,* 2016; Hong *et al.,* 2018; Cabrera *et al.,* 2019). These serine peptidases inhibitors have a wide diversity of chemical structures, potency and specificity (Figure 2) (Nakao and Fusetani, 2007). Since most invertebrates (e.g., sponges, bryozoans, tunicates, …) lack morphological defense structures, peptidases inhibitors contribute to protection against predators, infection, and competition (Sue *et al.,* 2009; Hussain *et al.,* 2012). In the present contribution we review and summarize the status of serine proteases inhibitors active vs DPP-IV derived from marine and coastal organisms; we focus on their potential biomedical applications, mainly as antidiabetics.

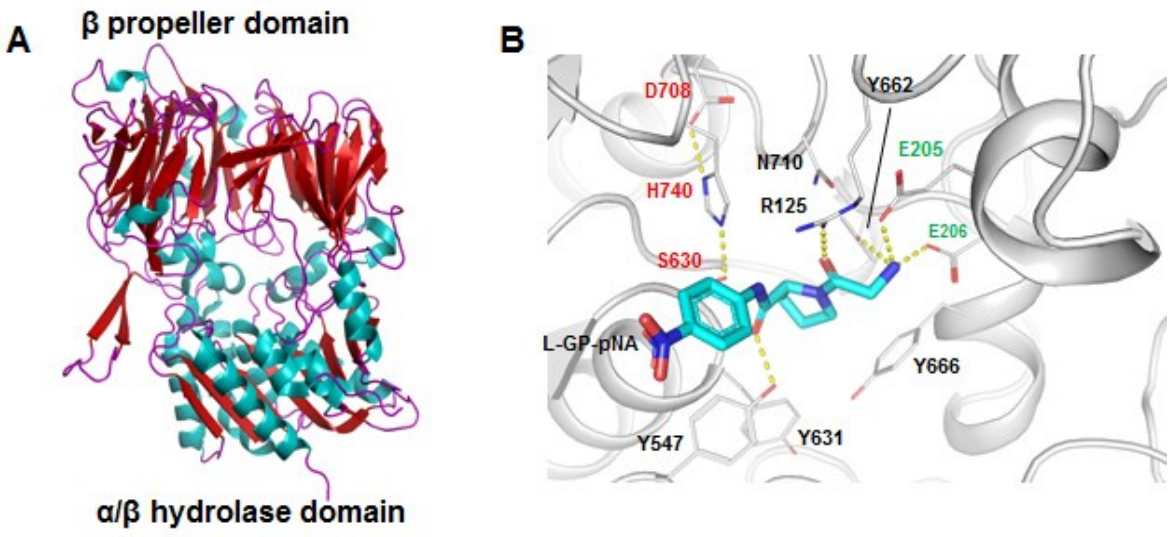

Figure 1. 3D structure of DPP-IV. A) Ribbon representation of porcine DPP-IV (PDB ID: 1j2e). Colors: alpha-helices (light blue), beta sheets (red) and loops (violet). B) Porcine DPP-IV active site. In red are highlighted the catalytic triad residues, and in green the residues involved in substrate binding.

Figura 1. *Estructura 3D de la DPP-IV. A) Representación en cintas de DPP-IV porcina (PDB ID: 1j2e). Colores: alfa-hélices (azul celeste), hojas beta (rojo) y lazos (violeta). B) Centro activo de DPP-IV porcina. En rojo se resaltan los residuos de la triada catalítica y en verde los involucrados en la fijación del sustrato.*

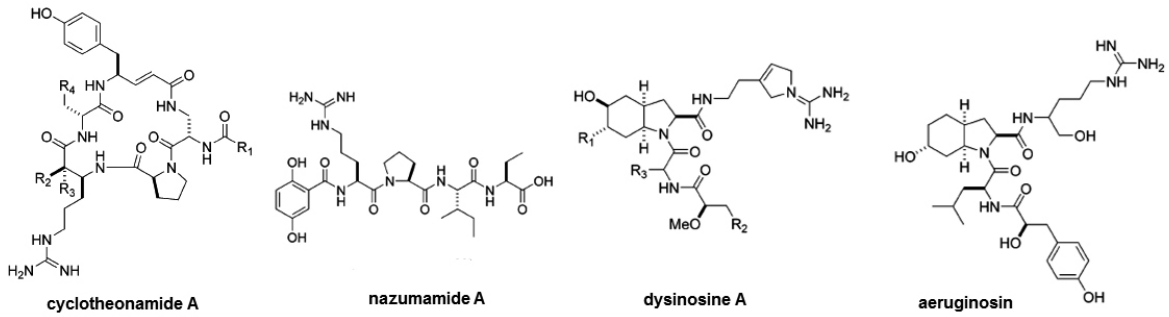

Figure 2. Structures of low molecular weight non-peptidic serine protease inhibitors isolated from marine organisms (Adapted from Nakao and Fusetani, 2007).

Figura 2*. Estructuras de inhibidores no peptídicos de bajo peso molecular de proteasas de tipo serino aislados de organismos marinos (Adaptado de Nakao y Fusetani, 2007).*

Serine peptidases. General characteristics and classification

The serine proteases comprise the best characterized clan of proteases, due to exhaustive studies conducted in the last 50 years with kinetic, chemical, physical, and genetic techniques (Rawlings *et al.,* 2018). In serine-like proteases, a hydroxyl group of a catalytic site serine residue performs a nucleophilic attack on the substrate peptide bond, which leads to its cleavage. This process constitutes the molecular basis for catalysis, and explains its various physiological functions, among which are intra and extracellular proteolytic digestion including activation of zymogens, release of hormones from precursors or biologically active peptides, displacement through membranes, processing of assembled proteins, activation of receptors (Neurath, 1984), regulation of inflammation, tissue repair, defense against infection, and synthesis of extracellular matrix (Hiemstra, 2002).

Currently, 13 clans of serine peptidases, have been described, comprising 44 families: SA (twelve families), SB (two families), SC (seven families), SE (three families), SF (two families), SH (four families), SJ (two families), SK (two families), SP, SR, SS and ST (one family each). At least three of them (clans SC, SE and SK) include exopeptidases (Rawlings *et al.,* 2018). Additionally, six families are currently unassigned to any clan. Families and clans were formed based on differences in the three-dimensional structure of the enzymes, as well as the order of the catalytic residues in their primary sequence. Clans represented by chymotrypsin (SA clan), Carlsberg subtilisin (SB clan), carboxypeptidase Y (SC clan) and Clp protease (SK clan) present the catalytic triad Asp-His-Ser located in different structural contexts. Thus, for example, the catalytic triad is ordered His-Asp-Ser in the serine proteases of the SA clan, Asp-His-Ser in the SB clan, Ser-Asp-His in the SC clan and Ser-His-Asp in the SK clan (Hedstrom, 2002; Tyndall *et al.,* 2005). Regardless of the order of the catalytic residues, the serine residue always acts as a nucleophile during catalysis, aspartate acts as an electrophile, and histidine as a general base (Rawlings *et al.,* 2018). Other catalytic triads and dyads are described in other clans; for example, His-Ser-His in the SH clan, Ser-Lys / His in the SE and SF clans, His-Ser in the SP clan and Lys-Ser in the SR clan.

Structurally, serine peptidases are characterized by the presence of two β-barrels each consisting of six antiparallel β-sheets. The active center of these enzymes is located between the two β-barrels. Three critical regions are recognized in the structure (3D) of the serine peptidases: that of the catalytic residues, that of the substrate recognition sites, and the zymogen activation domain. Although these regions are separated in the amino acid sequence, they are spatially close in the 3D structure (Kraut, 1971).

Clan SC of serine peptidases

In the SC clan of the serine proteases the order of the catalytic triad in the primary structure is: serine, aspartate, histidine. The peptidases belonging to this clan in turn share their 3D structure with many other hydrolases, among which are acetylcholine esterases, lipases, and halo-alkanes dehalogenases. The SC clan includes both endopeptidases and exopeptidases; the former are oligopeptidases, while exopeptidases are either amino or carboxypeptidases. Examples of these proteases are known in bacteria, archae, and eukaryotes, but not in viruses (Rawlings *et al.,* 2018). The SC clan includes the families S9, S10, S15, S28, S33, S37 and S82, in which we can find representatives at all levels of organization in the living world (Rawlings *et al.,* 2018) (Table 1).

Table 1. Representative members of the families of the clan SC of serine peptidases.

Tabla 1. *Miembros representativos de las familias del clan SC de serino peptidasas.*

Family and representative enzyme	IUBMB* Nomenclature	Sources
S9: Prolyl oligopeptidase	EC 3.4.21.26	*Sus scrofa*
S10: Carboxypeptidase Y	EC 3.4.16.5	*Saccharomyces cerevisiae*
S15: Xaa-Pro dipeptidyl-peptidase	EC 3.4.14.11	*Lactococcus lactis*
S28: Lysosomal Pro-Xaa carboxypeptidase	EC 3.4.16.2	*Homo sapiens*
S33: Prolyl aminopeptidase	EC 3.4.11.5	*Neisseria gonorrhoeae*
S37: PS-10 peptidase	EC 3.4-	*Streptomyces lividans*
S82: Autocrine proliferation repressor protein A	EC 3.4-	*Dictyostelium discoideum*

*IUBMB: International Union of Biochemistry and Molecular Biology

The 3D structure of the proteases that make up this clan can be described as parallel β-sheets comprised of β-α-β units. This folding has been described as "the α-β hydrolase fold" (Thoma *et al.,* 2003) (Figure 1A), although not all members are hydrolases and it is different from that of any other serine protease. Not all proteins with this folding have serine as a nucleophile during enzyme catalysis; for example, dienelactone hydrolase has a cysteine, and haloalkane dehalogenase an aspartate (Ollis *et al.,* 1992; Cygler *et al.,* 1993).

Family S9 of serine peptidases

The S9 family is also known as the prolyl-oligopeptidase family. This family groups together a series of aminopeptidases and endopeptidases capable of hydrolyzing the post-proline peptide link. The similarity in the amino acid sequence of these proteases shows that prolyl-oligopeptidase (the prototype protease of this family), oligopeptidase B, acylaminoacyl peptidase and DPP-IV are all members of the prolyl-oligopeptidase family (Barrett and Rawlings, 1992).

The amino acid sequence of prolyl oligopeptidases is more conserved towards the C-terminus, which includes the catalytic triad, indicating that these proteases may be made up of at least two domains. The substrate binding mode and catalytic mechanism of peptidases in this family differ from those of classical serine proteases (Rawlings *et al.,* 2018). The determination of the structure of prolyl oligopeptidase (80 kDa) by X-ray crystallography twenty years ago confirmed that the enzyme contains a folding domain of α -β hydrolase, and its catalytic triad is covered by the central tunnel of an unusual motif of β-propeller consisting of seven β-sheets (Figure 1A).

This domain works as a kind of filter that excludes large peptides from the active site, so that this peptidase cannot hydrolyze peptides with more than 30 residues (Polgar, 2000). DPP-IV, a membrane protein known in bacteria and eukaryotes, is the representative enzyme for the S9B subfamily.

The DPP-IV subfamily is a subgroup of the S9 prolyl oligopeptidase family of enzymes (Table 2), which are specialized in the cleavage of post-prolyl bonds, if proline is the second N-terminal aminoacid. Since most peptide hormones and neuropeptides include one or more proline residue, this family of enzymes process and degrades such peptides (Mentlein, 1988; Cunningham and O'Connor, 1997). DPP-IV and fibroblast activation protein (FAP, also known as Seprase) are closely related cell-surface enzymes, with DPP-IV-like enzyme activity. DP8 and DP9 are dimers with DPP-IV-like enzyme activity (Abbott *et al.,* 2000; Ajami *et al.,* 2004; Lee *et al.,* 2006; Bjelke *et al.,* 2006; Yu *et al.,* 2010). Although DP8 and DP9 are very closely related to each other and share similar distribution patterns, there are some differences in their cell biological effects, perhaps related to their cytoplasmic localization (Yu *et al.,* 2009). The non-enzymatic members of the family – DP6 (DPL1 ⁄DPX) and DP10 (DPL2 ⁄DPY) – are modulators of voltage-gated potassium channels in neurons and are primarily expressed in brain (Yu *et al.,* 2010). Although structurally similar to DPP-IV (Strop *et al.,* 2004; McNicholas *et al.,* 2009), they lack the catalytic serine and other residues necessary for enzyme activity. Thus, they are likely to exert effects via protein–protein interactions (McNicholas *et al.,* 2009), similarly to the enzyme dipeptidyl peptidase sub-family members that also have extra-enzymatic abilities.

Table 2. Classification criteria and subfamilies of the prolyl oligopeptidase family of enzymes (MEROPS –the Peptidase Database: merops.sanger.ac.uk; Rawlings *et al*., 2018; Adapted from Yu *et al*., 2010).

Tabla 2. *Criterios de clasificación y subfamilias de la familia de las prolil oligopeptidasas (MEROPS –la base de datos de peptidasas: merops.sanger.ac.uk; Rawlings* et al*., 2018; Adaptado de Yu et al., 2010).*

Criteria for S9 (prolyl oligopeptidase - POP) family members
DNA sequence homology to prolyl endopeptidase (PEP / POP)
Sub-families of the POP family
S9A Prolyl endopeptidase (PEP / POP) (EC 3.4.21.26)
S9B Dipeptidyl peptidase IV (DPIV)
S9C Acylaminoacyl peptidase
S9D Glutamyl endopeptidase (plant)
S9B (DPP-IV) sub-family
DPP-IV
Fibroblast activation protein (FAP)
DP8
DP9
Nonenzyme DPP-IV-related POP family members
DP6 (DPX, DPL1)
DP10 (DPY, DPL2)

Dipeptidyl peptidase IV

Dipeptidyl peptidase IV is a highly glycosylated type II integral membrane protein (Rawlings *et al.,* 2018). Also known as CD26, DPP-IV is an amino-peptidase that liberates dipeptides from its substrates. It preferentially cleaves peptides or small proteins (below 30 residues) with proline or (less often) alanine as the penultimate N-terminal residue, although some substrates with glycine, serine, valine, or leucine can be cleaved at a slower rate (Deacon, 2019). Several cytokines, growth factors, neuropeptides and peptide hormones have the Xaa-Pro sequence at their amino terminus; therefore, they are potential physiological substrates regulated through the proteolytic activity of this enzyme (Augustynus *et al.,* 1999). DPP-IV is an ubiquitous protein in mammalian tissues. In humans, DPP-IV is constitutively expressed in epithelial cells of the liver, small intestine, and kidney; in addition, a dimeric and soluble isoform circulates in extracellular fluids such as in serum, semen, saliva, and bile. DPP-IV expression is highly regulated in T and B cells (Bauvois *et al.,* 2000). The richest natural sources of DPP-IV are the seminal fluid (Wilson *et al.,* 1998) and kidney (Engel *et al.,* 2003). Porcine DPP-IV (pDPP-IV) and human DPP-IV (hDPP-IV) are 766 amino acids in length. The amino acid sequences of both molecules have 88% identity; both are functionally remarkably similar. For this reason, pDPP-IV is a useful surrogate model when, for economic or ethical reasons, hDPP-IV is not available (Engel *et al.,* 2003; Pascual *et al.,* 2011). The susceptibility of porcine and rat DAP-IV to divalent ions present in natural sources, including zinc and calcium, is likely relevant for the physiological regulation of this enzyme; furthermore, these data suggested the potential to design new powerful inhibitors (Pascual *et al.,* 2011; Gómez *et al.,* 2013).

Depending on cell type, DPP-IV can act as (1) a serine protease, (2) a receptor, (3) a signal transducer, (4) an adhesion molecule to collagen and fibronectin, or (5) an apoptosis actor (Figure 3). Among its natural substrates are at least nine chymosins, NPY, peptide YY, GLP-1 and GLP-2, and GIP. DPP-IV ligands include adenosine deaminase (ADA) (Gorrell *et al.,* 2001), the Na+ / H+ exchanger (Girardi et al., 2001), and fibronectin (Cheng *et al.,* 2003). DPP-IV is upregulated in diseases such as psoriasis, acne, keloid formation (Thielitz *et al.,* 2008), HIV infection (Ploquin *et al.,* 2018), diabetes, obesity, and tumor progression (Trzaskalski *et al.,* 2020).

The role of DPP-IV in both metabolic pathologies and tumor progression has been extensively studied in recent years (Carl-McGrath *et al.,* 2006; Yu *et al.,* 2010; Deacon, 2019; Trzaskalski *et al.,* 2020).

Regarding metabolic effects, the best characterized substrates of DPP-IV are the above-mentioned incretin hormones: GLP-1 and GIP. When DPP-IV hydrolyses endogenous GLP-1, it loses its insulinotropic activity; reducing this degradation results in increased intact GLP-1 levels, improved pancreatic islet responses (enhanced insulin and suppressed glucagon levels), and beneficial effects on glucose homeostasis. Similarly, the other incretin hormone, GIP is efficiently cleaved by DPP-IV in vivo, and DPP-IV inhibition increases intact GIP levels and enhances its effects.

Accordingly, it is well established that DPP-IV does play a pivotal role in the initial inactivation of both incretin hormones (Deacon, 2019; Trzaskalski *et al.,* 2020). Since DPP-IV contributes to 95% of the proteolytic degradation of GLP-1, the specific inhibition of the enzyme is a treatment option against type 2 diabetes mellitus (Thoma *et al.,* 2003; Deacon, 2019).

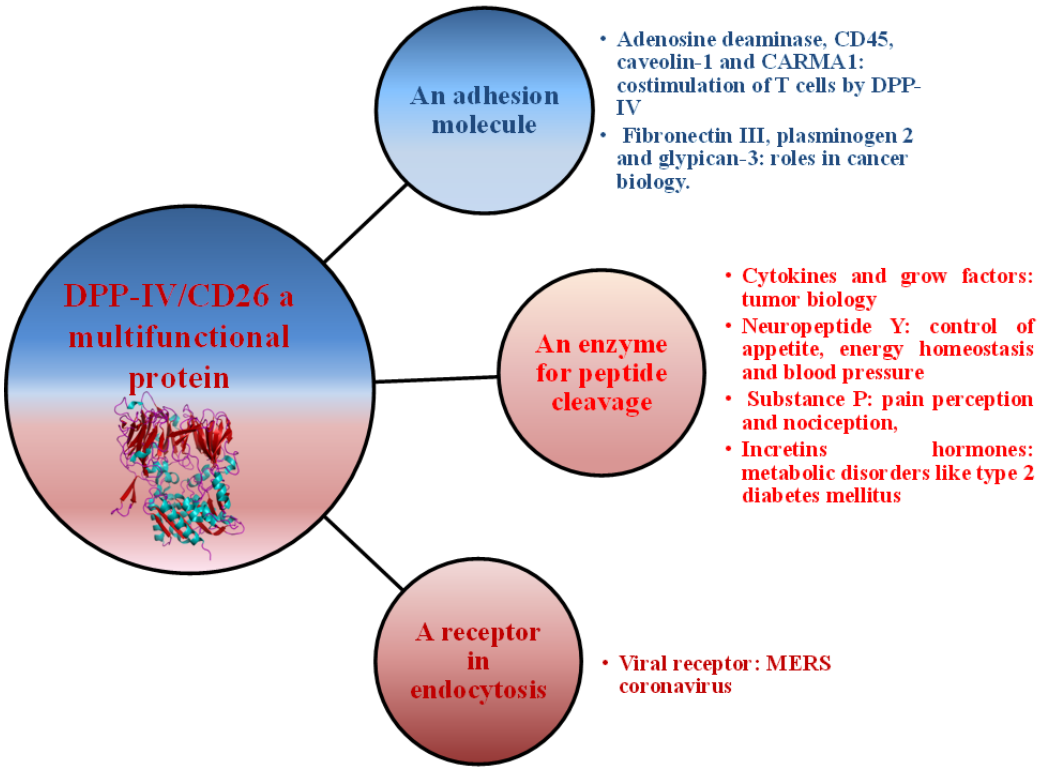

Figure 3. Functions of human DPP-IV/CD26.
Figura 3. *Funciones de la DPP-IV/CD26 humana.*

However, the antidiabetic drugs developed until now, including various DPP-IV inhibitors, help diabetes mellitus patients to maintain their blood glucose levels with various adverse effects, such as urine-tract infection, lactoacidosis, hypoglycemia, and obesity (Lin *et al.,* 2019). These drug-related adverse effects can deteriorate the quality of life of diabetes mellitus patients and create unsurmountable difficulties for proper dosing regimens in a clinical setting. As such, there is a clinical demand for novel DPP-IV inhibitors from various sources including natural sources (Lin *et al.,* 2019).

DPP-IV expression has also been linked to tumor evolution, since DPP-IV is upregulated in both solid and liquid tumors. T-lymphoblastic lymphoma, acute T-lymphoblastic leukemia, and T-anaplastic long cell lymphoma are blood pathologies in which overexpression of DPP-IV has been reported (Cro *et al*., 2009).

On the other hand, DPP-IV expression also increases in a wide range of solid tumors such as: thyroid carcinoma (Aratake *et al*., 1991; Kim *et al*., 2018), some ovarian tumors (Kikkawa *et al*., 2003), skin basal cell carcinoma (Pro and Dang, 2004), esophageal adenocarcinoma (Goscinski *et al*., 2008), astrocytoma (Balaisova *et al*., 2011), glioma (Stremenova *et al.,* 2007), meningioma (Stremenova *et al.,* 2010), mesothelioma (Amatya *et al*., 2011), some lung tumors (Karandikar *et al*., 2018), some prostate tumors (De *et al*., 2019), and hepatocarcinoma (Yamamoto *et al*., 2012; Fasolato *et al*., 2018; De *et al*., 2019). In tumors like glioma and thyroid carcinoma, the expression of DPP-IV increases in such a way during malignancy that it is often used as a classification criteria to define the stages of the tumor (Aratake *et al*., 1991; Stremenova *et al.,* 2007) (Figure 4).

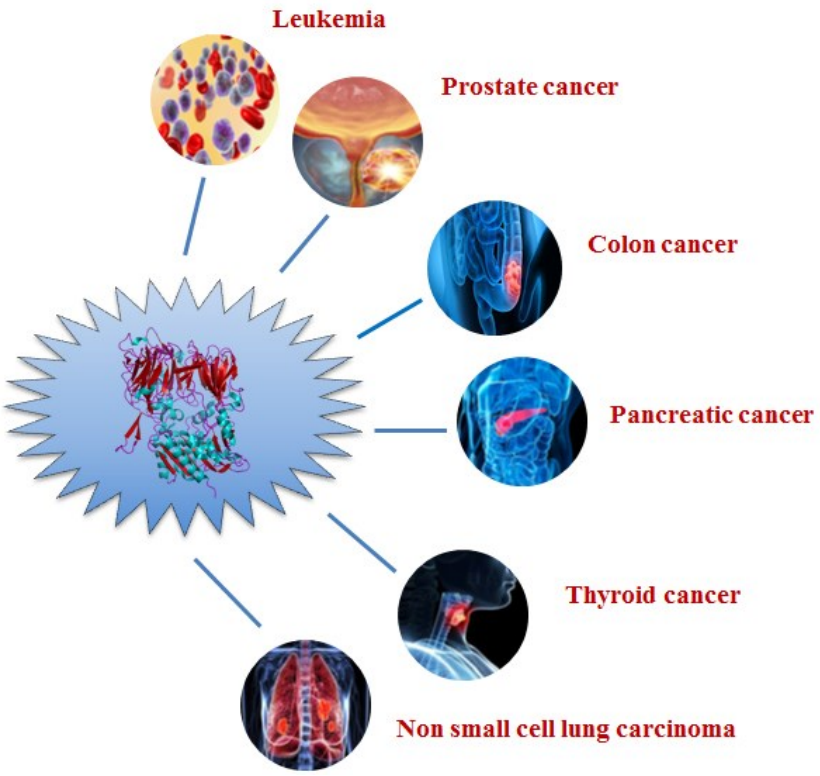

Figure 4. Up-regulation of DPP-IV in different cancers.
Figure 4. *Regulación positiva de DPP-IV en diferentes tipos de cáncer.*

Metastasis and tumor invasion are dependent upon matrix degradation, which allows cell mobility and tissue penetration. DPP-IV has been shown to have a pivotal role in both tumor cell metastasis and invasion. Various mechanisms may explain DPP-IV involvement in cancer metastasis. DPP-IV active site faces the extracellular space (it is an ectopeptidase) and, as we earlier mentioned, modulates chemokines, which can mobilize cancer cells. Moreover, DPP-IV binds matrix metalloproteinases, proteases that allow cell migration by removing abundant structural proteins such as collagens, laminins, and proteoglycans (Deacon, 2019).

Inhibitors of DPP-IV isolated from marine organisms

DPP-IV is thus an important target for pharmaceutical research. Potent, stable, and selective inhibitors of DPP-IV could be valuable tools for therapeutics (Carl-McGrath *et al*., 2006; Yu *et al*., 2010; Yamamoto *et al*., 2012; Deacon, 2019; De *et al*., 2019). Many DPP-IV inhibitors of different chemical kind have been identified (Aertgeerts *et al*., 2004; Patel and Ghate, 2014; Shao *et al.,* 2020); several of them are marketed (Reviewed in Turdu, 2018) (Figure 5). The search for inhibitory activity from different natural sources has been included within the strategies for the development of DPP-IV inhibitors. Among these sources are plant extracts (Lu *et al.,* 2019; Lin *et al.,* 2019), and marine organisms (Pascual *et al.,* 2007). Several inhibitors are derived from plants (Turdu *et al.,* 2018), like quercetin and coumarin (Singh *et al.,* 2020), while low molecular weight compounds such as bestatin and bacitracin, from microorganisms (Rivera *et al.,* 2020) (Figure 6). Additionally, some enzymatic hydrolysates from food are characterized by an hypoglycemic activity and DPP-IV inhibitory activity (Liu *et al.,* 2019; Gomez *et al*., 2019; Chakraborty *et al.,* 2020).

Among natural sources, marine organisms are an abundant and promissory source of DPP-IV inhibitors, especially for the development of new antidiabetics. During the last fifteen years, two strategies have prevailed to search for DPP-IV inhibitors from marine organisms. In the next sections, we discuss these strategies, their advantages and disadvantages.

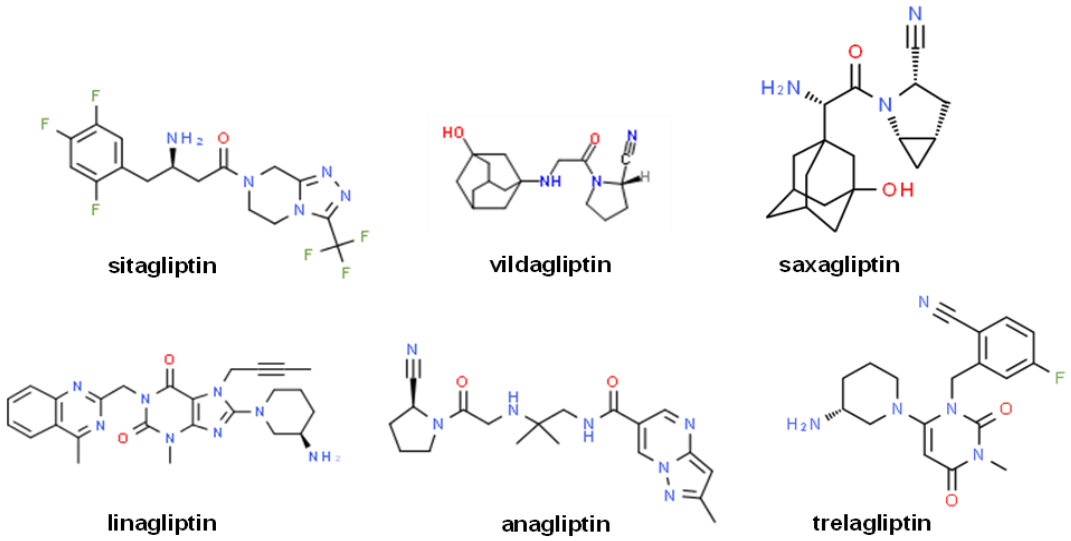

Figure 5. Examples of some of marketed DPP-IV inhibitors.
Figure 5. *Ejemplos de algunos de los inhibidores de DPP-IV que se comercializan actualmente.*

Figure 6. Examples of natural inhibitors of DPP-IV.
Figure 6. *Ejemplos de inhibidores naturales de DPP-IV.*

Screening of inhibitory activity of DPP-IV in extracts from marine and coastal organisms

Obtaining clarified extracts from different marine and coastal organisms to screen for inhibitory activity vs DPP-IV is one of the most common strategies. In general, obtaining inhibitors via clarified extracts has the limitations expected in any purification that starts from natural sources: the amount of the possible inhibitor(s) will depend strongly on the physiological status of the biological source used for purification.

Once the extract is obtained, researchers can achieve the purification (partial or total) of the molecule (s) responsible for the inhibition by testing the effects of heating at different temperatures, treatment with trichloroacetic acid (TCA) at distinct percentages, followed by various chromatographic separations.

It is a complex process due to the variability of chemical structures and molecular weights commonly found in those extracts. Perhaps for this reason, even the most in-depth studies are usually limited to report inhibitory activity and partial kinetic characterization in terms of time required to achieve inhibition, as well as IC_{50} or (less frequently) IC_{90}.

The results from three kinds of chemical extracts have been described: aqueous, alcoholic, and phenolic. Aqueous extracts have the enormous advantage of maintaining a similarity with the physiological conditions of both the original marine organism and the human organism, where they would be eventually used. This guarantees the stability of any putative inhibitor in a wide range of purification and/or use contexts.

On the other hand, alcoholic extracts (and mainly phenolic ones) have the disadvantage that the medium could modify the chemical nature of the inhibitor and/or the enzyme. Thus, inhibition might be produced by a side effect that substantially modifies the nature of the enzyme-inhibitor interaction.

So far, scientists have preferred to use these screening strategies to find DPP-IV inhibitors in marine invertebrates. They seem to be particularly attracted by the wide diversity of chemical compounds that those organisms usually contain. In fact, marine sponges have been considered as an excellent source of marine natural products since the 1950s, with about 4851 compounds described until 2016, contributing to nearly 30% of all marine natural products discovered until then (Lauritano and Ianora, 2016).

An inhibitory activity of pDPP-IV has been found in extracts of three species belonging to phyla Porifera and Cnidaria: the sponge *Xetospongia muta* and the sea anemones *Bunodosoma granulifera* and *Bartholomea annulata* (Table 3, Figure 7). In each case, the effect on pDPPIV activity is dose-dependent, the inhibition is slow, and the inhibitor has a low molecular weight. Pre incubation times in the order of minutes are required to attain the maximum inhibitory effect; the inhibition is sustained for at least one hour (Pascual *et al*., 2007). Treatments with 2.5% final concentration of TCA or with heat (60°C, 10min) increase inhibitory activity in *X. muta* but fail in the case of *B. granulifera* and *B. annulata* extracts. These data suggest that the inhibitory molecule from *X. muta* is resistant to both treatments wich induce its dissociation from endogenous inhibitor-target complexes or its activation (Pascual *et al*., 2007).

The slow and time-sustained inhibitions suggest that Pascual *et al* (2007) detected tight binding inhibitors, features are mainly characteristic of this class of inhibitors (Bieth, 1995). In particular, the titration behavior of the dose-response curve of *B. annulata* extract strongly suggests the existence of an irreversible, or pseudo-irreversible tight binding inhibitor in conditions of large E_o/K_i values. Therefore, because of its kinetic behavior and IC_{50} value, which is the lowest of the three extracts (Table 3), *B. annulata* extract is a promising source to identify an effective DPPIV inhibitor (Pascual *et al*., 2007). Interestingly, *B. granulifera* contains an inhibitory activity not only against porcine DPP-IV, but also against human DPP-IV (González *et al*., 2016).

In 2014, Unnikrishnan *et al.* prepared crude extracts of marine seaweed, *Turbinaria ornata*, to test their antidiabetic potential using enzyme inhibitory assays vs targets like α-amylase, α-glucosidase, and DPP-IV. Methanol and acetone extracts show significant inhibitory effects on α-amylase (IC_{50} 250.9 µg/mL), α-glucosidase (535.6 µg/mL), and DPP-IV activities (55.2µg/mL) (Table 3), respectively. Extracts were tested for *in vitro* toxicity using DNA fragmentation assay, haemolytic assay, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. None of the extracts shows toxicity, indicating that *Turbinaria ornata* is a potential source of compounds controlling hyperglycemia.

More recently, Chakraborty and Joy (2017) found an inhibitory activity against pDPP-IV in ethyl acetate-methanol extracts of five commonly available cephalopods: *Amphioctopus marginatus, Urothethis duvaucelli, Sepia pharaonis, Sepiella inermis, and Cistopus indicus*. Unfortunately, these extracts have not been characterized beyond their IC_{90} values (Table 3).

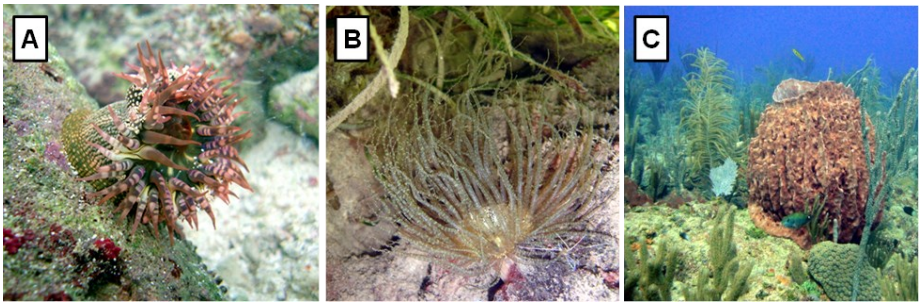

Figure 7. Some marine invertebrates sources of molecules with inhibitory activity of DPP-IV. A: *Budonosoma granulifera*, B: *Bartholomea annulata*, C: *Xetospongia mut*a. Picture courtesy of Professor José Espinosa, PhD, Instituto de Ciencias del Mar, CITMA, Cuba.

Figura 7. *Algunos de los invertebrados marinos fuente de moléculas con actividad inhibidora de DPP-IV. . A:* Budonosoma granulifera*, B:* Bartholomea annulata*, C:* Xetospongia muta*. Foto cortesía del Profesor José Espinosa, PhD, Instituto de Ciencias del Mar, CITMA, Cuba.*

Table 3. Preliminary characterization of the inhibitory activity of DPP-IV. in extracts from marine organisms. (Authors of the species in www.marinespecies.org).

Tabla 3. *Caracterización preliminar de la actividad inhibidora en extractos de organismos marinos con actividad inhibidora de DPP-IV. (Autor de la especie según www.marinespecies.org).*

Source	Phylum	Nature of the extract	Tested vs hDPP-IV	Tested vs pDPP-IV	Pre-incubation time (min)	IC_{50} (mg/mL)	IC_{90} (mg/mL)	References
Xetospongia muta (Schmidt, 1870)	Porifera	Aqueous	–	✓	10	0.800	–	Pascual *et al.* (2007)
Bunodosoma granulifera (Le Seur, 1817)	Cnidaria	Aqueous	*	✓	10	1.200	–	Pascual *et al.* (2007)
Bartholomea annulata (Le Seur, 1817)	Cnidaria	Aqueous	–	✓	3	0.138	–	Pascual *et al.* (2007)
Turbinaria ornata (Turner, 1848)	Ochrop-hyta	Methanol		✓	-	0.055	-	Unnikrishnan *et al.* (2014)
Amphioctopus marginatus (Taki, 1964)	Mollusca	Ethyl acetate-methanol	–	✓	–	–	3.60	Chakraborty and Joy (2017)
Uroteuthis duvaucelii (d'Orbigny, 1835)	Mollusca	Ethyl acetate-methanol	–	✓	–	–	4.54	Chakraborty and Joy (2017)
Sepia pharaonis (Ehrenberg, 1831)	Mollusca	Ethyl acetate-methanol	–	✓	–	–	5.37	Chakraborty and Joy (2017)
Sepiella inermis (Van Hasselt, 1835)	Mollusca	Ethyl acetate-methanol	–	✓	–	–	3.35	Chakraborty and Joy (2017)
Cistopus indicus (Rapp, 1835)	Mollusca	Ethyl acetate-methanol	–	✓	–	–	2.51	Chakraborty and Joy (2017)

* In 2016, González *et al.* proved that aqueous extracts from *B. granulifera* inhibit hDPP-IV, but did not determine IC_{50}.

Identification of DPP-IV peptidic inhibitors by partial digestion of tissues

Partial digestion of tissues (muscle, skin, connective tissue, etc.) from a marine organism via homogenization methods, followed by enzymatic digestion with commercial kits of digestive enzymes, is the second strategy commonly used for the identification and development of DPP-IV inhibitors. The inhibitory activity of the hydrolysate against the enzyme is then tested. If there is inhibition, purification is carried out using different chromatographic systems; it is common for the first separation to use size exclusion chromatography. In general, it is possible to complete the purification and sequence the peptides inhibiting DPP-IV activity. This is a contrived method, since the inhibitor is not found endogenously, but arises because of the tissue hydrolysis process.

It has the advantage to generate a high number of short peptides, which inhibitory potentials can be quickly verified or discarded; furthermore, purification of the possible inhibitor is a relatively easy process. However, this strategy has the considerable disadvantage of narrowing the search spectrum; the non-peptide structures that could inhibit the enzyme (aromatic rings, lactones, phosphorous or sulfur compounds, etc.) are left out of the analysis.

Peptides obtained through this strategy show multiple DPP-IV inhibitory modes, including competitive, uncompetitive, noncompetitive and mixed-type modes, which means they might exert DPP-IV inhibitory activity by binding either at the active site and/or outside the catalytic site of DPP-IV (Lacroix and Li-Chan, 2016).

It has been suggested that those natural food- or herb-derived constituents should be safer than synthetic forms, and could be used for glycemic management.

Among the sources of DPP-IV inhibitors, food protein -derived DPP-IV inhibitory peptides have attracted the attention of more and more researchers, owing to their high efficacy and safety (Lacroix and Li-Chan, 2014).

On the other hand, tissue hydrolysates that inhibit DPP-IV have produced variable results, since scientists have assayed a great variety of chromogenic and fluorogenic substrates (Harnedy *et al*., 2015; Neves *et al*., 2016; Xia *et al*., 2017; Gomez *et al.,* 2019). This wide variability means that, in certain cases, the results are not completely comparable between studies (note that, if inhibition is competitive, the nature of the substrate is a key factor).

Table 4 summarizes the sequences and IC_{50} of peptides inhibiting DPP-IV obtained via hydrolysates from marine and coastal organisms. To identify the preferential amino acids involved in DPP-IV inhibition, the amount of each amino acid occurring within these DPP -IV inhibitory peptides was estimated based on Table 4. The preferential amino acids were, in decreasing order: proline, leucine, glycine, alanine, tryptophan. Indeed, the existence of exclusion volumes in the S1 pocket of DPP-IV may restrict the access of bulky amino acids and allow access to smaller residues such as proline, alanine and glycine (Engel *et al.,* 2003; Lu *et al*., 2008).

These results are in perfect agreement with many studies that reported that di-, tri- and oligo-peptides, especially dipeptide and tripeptide containing proline, derived from food protein sources exhibit a strong inhibitory activity against DPP-IV (Lacroix and Li-Chan, 2012). Proline-containing di- or tripeptides can be resistant to gastrointestinal enzymes, an advantage for exerting their physiological effects in the body. Moreover, the di- or tri- peptides are expected to be of suitable size for transepithelial transport (Satake *et al.,* 2002).

These DPP-IV inhibitory peptides, exert their effect by binding either at the active site and/or outside the catalytic center of the enzyme. *In silico* studies predicted that the active site of DPP-IV comprises a hydrophobic S1 (Tyr662 and Tyr666) pocket and a charged S2 (Phe357 and Arg125) pocket with an overall negative charge (Engel *et al.,* 2003). Hydrogen bonds and hydrophobic interactions were involved between N-terminal amino acids of DPP-IV inhibitory peptides and the catalytic active site of DPP-IV.

A novel strategy integrating nano-liquid chromatography tandem mass spectrometry (nano-LC-MS/MS) and *in silico* analysis is more efficient to identify DPP-IV inhibitory peptides from tissue hydrolysates. This strategy consists of three steps: first, nano-LC-MS/MS is used for peptide identification in complex mixtures like protein hydrolysates; second, molecular docking or quantitative structure activity relationship (QSAR) models are used to identify those peptides targeting DPP-IV; third, potentially active peptides from virtual screening are synthesized and their efficacy determined *in vitro* or *in vivo* (Liu *et al.,* 2019).

Following this approach, Harnedy-Rothwell *et al.,* (2020) identified and characterized twenty-two novel dipeptidyl peptidase-IV (DPP-IV) inhibitory peptides (with IC_{50} values < 200 μM) in a boarfish protein hydrolysate generated at semi-pilot scale. The study was achieved by bioassay-driven semi-preparative reverse phase-high performance liquid chromatography fractionation, liquid chromatography-mass spectrometry and confirmatory studies with synthetic peptides. The most potent DPP-IV inhibitory peptide (IPVDM) had a DPP-IV half maximal inhibitory concentration (IC_{50}) value of 21.72 ± 1.08 μM (Table 4) in a conventional enzymatic assay *in vitro* and 44.26 ± 0.65 μM in an *in situ* cell-based (Caco-2) DPP-IV inhibition assay.

This indicates that IPVDM is potentially resistant to degradation by membrane associated peptidases and was efficiently transported across the cell membrane. Furthermore, IPVDM stimulates insulin secretion (1.6-fold increase compared to control) from pancreatic BRIN-BD11 cells grown in culture. The results indicate that boarfish proteins contain peptide sequences with potential to play a role in glycaemic management *in vivo*.

Thus, various of the DPP-IV inhibitory peptides purified from marine organisms merit further pre-clinical studies, and may lead to new therapeutics for type 2 diabetes mellitus. Additionally, these peptides also have potentialities as anticancer agents.

Table 4. Peptides with inhibitory activity of DPP-IV isolated from proteolytic hydrolysates from marine and coastal organisms (Authors of species in www.marinespecies.org). Peptide sequence and preliminary characterization of their inhibitory activity.
Tabla 4. *Péptidos con actividad inhibidora de DPP-IV aislados a partir de hidrolizados proteicos obtenidos de organismos marinos y costeros (Autores de especies según www.marinespecies.org). Secuencia primaria y caracterización preliminar de su actividad inhibidora.*

Source	Phylum	Hydrolyzed tissue	Peptide sequence obtained	IC_{50} (mg/mL)	References
Mytilus edulis (Linnaeus, 1758)	Rhodophyta	All tissues	I-P-A L-L-A-P M-A-G-V-D=H-I	0.04340 0.05367 0.15937	Harnedy *et al.* (2015)
Euphausia superba (Dana, 1850)	Mollusca	Muscle	–	1.14	Neves *et al.* (2016)
Palmaria palmata (F. Weber & D.Mohr, 1805)	Arthropoda	All tissues	A-P I-P-A	0.0530 0.0370	Ji *et al.* (2017)
Hippoglossus hippoglossus (Linneus, 1758)	Chordata	Skin	S-P-G-S-S-G-P-Q-G-F-T-G G-P-V-G-P-A-G-N-P-G-A-N-G-L-N P-P-G-P-T-G-P-R-G-Q-P-G-N-I-G-F	0.1016 0.0813 0.1467	Xia *et al.* (2017)
Oreochromis aureus (Steindachner, 1864)	Chordata	Skin	I-P-G-D-P-G-P-P-G-P-P-G-P L-P-G-E-R-G-R-P-G-A-P-G-P G-P-K-G-D-R-G-L-P-G-P-P-G-R-D-G	0.0654 0.0768 0.0896	Xia *et al.* (2017)
Thunnus orientalis (Temminck & Schlegel, 1844)	Chordata	Internal fluids	P-A-C-G-G-F-W-I-S-G-R-P-G C-A-Y-Q-W-Q-R-P-V-D-R-I-R P-G-V-G-G-P-L-G-P-I-G-P-C-Y-E	0.0964 0.0780 0.1161	Xia *et al.* (2017)
Salmo salar (Linnaeus, 1758)	Chordata	Cartilaginous connective tissue	G-P-A-E G-P-G-A	0.0496 0.0419	Xia *et al.* (2017)
Capros aper (Linnaeus, 1758)	Chordata	All tissues	I-P-V-D-M A-P-I-T L-P-V-D-M V-P-D-P-R G-P-G-I M-P-A-V-P	21.72 µM 34.73 µM 53.50 µM 79.19 µM 116.27 µM 115.00 µM	Harnedy-Rothwell *et al.* (2020)

CONCLUSIONS

Marine and coastal organisms contain biologically active compounds, most of them peptides with inhibitory activity of DPP-IV, with potential applications as anti-diabetic drugs, and also as a great source to explore new anticancer activities and treatments for pathologies associated to an up-regulation of DPP-IV activity.

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