Theoretical development for the discovery of a drug as a treatment of primary dysmenorrhea.

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Abstract

Primary dysmenorrhea is a gynaecological disease that causes pain at the beginning of menstruation without any identifiable factor that causes it. It is a very prevalent disease among the female population. However, it has not received the necessary attention to be able to alleviate it. Therefore, we propose a theoretical development whose main objective is to find a drug that specifically mitigates it. In this way, we propose both high-throughput screening and pre-clinical studies *in vitro* and *in vivo*. All these applying techniques established and recognized by the scientific community. By this way, we want to encourage the investigation of primary dysmenorrhea, a disease that is as underlying as it is neglected.

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Introduction

Primary dysmenorrhea is the presence of pain at the beginning of the menstrual cycle with no identifiable cause (Hong, Jones et al., 2014). It is one of the most prevalent gynecological conditions among the female population, and up to 90% on young women may be present. Between all of them, the 15% suffer from pain of such severity that it prevents them from developing a normal daily life (Ferries-Rowe, Corey et al., 2020). Despite its high prevalence, there are hardly any specific drugs to treat the pain occurs in this pathology, which leads to a very high rate of self-medication. First-line treatment is the administration of non-steroidal anti-inflammatory drugs (NSAIDs) (Ryan, 2016). However, it has been observed that the 10% of women manifest resistance to NSAIDs, making the development of a specific drug completely necessary so that to alleviates this pathology in a much more specific and effective way (Oladosu, Tu et al., 2018).

Etiology and pathogenesis are not completely elucidated, but the theory most accepted by experts suggests that the appearance of pain would have its origin in the overproduction of prostaglandin F2α (PGF2α) in the myometrium at the beginning of menstruation (Lacovides, Avidon *et al.,* 2015). The degeneration of the corpus luteum at the end of the menstrual cycle produces a sharp drop in progesterone Levels, which leads to an exacerbated activity of matrix metalloproteinases in the myometrium, generating free fatty acids. Afterwards, phospholipase A2 (PLA2) is activated to produce arachidonic acid, which is then processed by COX 1/2, whose activity originates a cyclic endoperoxidase H2 that is instantly transformed into PGF2α in myometrium (Dawood, 2006). The activity of PGF2α can be autocrine or paracrine through the FP receptor, a GPCR coupled to Gq protein, whereby Ca^{+2} is ultimately released from the endoplasmic reticulum into the cytosol. This calcium is responsible for triggering muscle contraction, which is what generates vasoconstriction, hypoxia, and pain (**Fig. 1)** (Altunyurt, Göl *et al.,* 2005; Lacovides, Avidon *et al.,* 2015; Ferries-Rowe, Corey *et al.,* 2020).

Between all the molecules that participate in the pathogenesis of primary dysmenorrhea, FP receptor has been selected as the most suitable target to be inhibited due to its specificity in this pathology and because, up to now, its inhibition is compatible with life. First of all, a bibliographic review has been carried out to establish what knowledge we start from. Thus, it has been assembled a chemical library with all drugs and compounds which are known for inhibiting FP receptor (**Table 1**). Currently, none of these drugs are marketed.

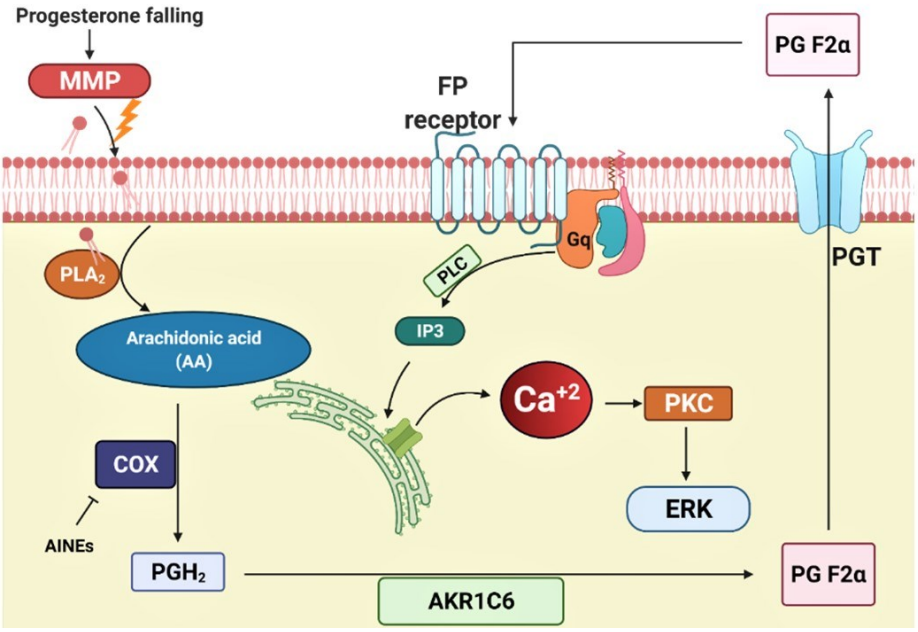


Fig. 1. Pathogenesis of primary dysmenorrhea.

HTS assay

Therefore, the purpose posed in this article is to avoid the effect of the PGF2α. It is worth mentioning that the most of women who suffered from dysmenorrhea choose anti-inflammatory drugs as the main treatment against the presence of pain. These therapies usually have the COX receptor as main therapeutic target triggering to many off target effects and the occurrence of resistances. Hence, what it is proposed here is to avoid or at least reduce the myometrium contractions downstream the COX receptor by blocking the FP receptor. Since the FP receptor has been observed to be completely specific for this prostaglandin, it seems to be likely a good therapeutic target for dysmenorrhea disease.

Name	Effect on fp receptor	Experimental model	Reference
BAY-6672	Antagonist	Mouse, rat and dog	Beck, Thaler *et al*., 2020
OBE022	Antagonist	Human	Pohl, Marchand *et al*., 2018
AL-8810	Antagonist	Mouse	Ahmad, Akoum *et al*., 2015
AS604872	Antagonist	Mouse	Sharif and Klimko, 2019
TGH-113	Antagonist	Mouse	Peri, Quiniou *et al*., 2002
AGN 211377	Antagonist	Human	Wang, Woodward *et al*., 2016
AL-3138	Antagonist	Mouse	Sharif, Crider *et al*., 2000
PDC31	Antagonist	Mouse and human	Sharif and Klimko, 2019
PDC113.824	Antagonist	Mouse	Goupil, Tassy *et al*., 2010

Table 1. Chemical library with antagonists (inhibitors) of FP receptor.

However, as all drug design and development projects, it is necessary to choose from the chemical library those molecules, which are found to provoke an effect on the target. Herein, a sensitive and ultrarapid method that permits the selection of a hit is suggested. Fluorescence Imaging Plate Reader or FLIPR is based on the fluorescence emission by a complex formed between the fluorescence indicator and free Ca^{2+} ions. Since the action of the FP receptor results in the release of calcium from the endoplasmatic reticulum to the cytosol, FLIPR becomes a useful and excellent technique for the selection of a hit compound. By this way, miles of possible molecules can be tested and therefore, a good hit can be selected so that to continue with in vitro and in vivo assays for validating the drug.

Based on the use of a fluorescence indicator, FLIPR is capable to determine with high sensitivity the production of calcium from cells (Arkin and Connor, 2012). Therefore, Isikawa cells were proposed for the development of the experiment since they have been seen to be able to mimic the myometrium cells in dysmenorrhea uterus (Dong and Yallampalli, 2000). Furthermore, different control samples were suggested in this article. On the one hand, as the negative control, the use of PGF2α itself is proposed, as

its effect is already known. On the other hand, based on the previous study referring to primary dysmenhorrea investigations (**Table 1**), BAY-6672 was suggested as the positive control considering that its positive effects against primary dysmenhorrea have been already demonstrated. Finally, for the detection of the release of calcium from Isikawa cells, a chelating agent, which traps the free ions triggering the formation of a fluorescent complex, was proposed. This was Fluo-4, a fluorescent indicator that permits to convert the presence of free calcium ions into a measurable signal, which shows the action of the FP receptor (Gee and Brown, 2000) (**Table 2**).

HTS assay		
Cellular line	Myometrium cells equivalent	Isikawa cells
Negative control	Agonist	PGF2α
Positive control	Usual treatment	Ibuprofen
Calcium indicator	Fluorescence chelating agent	Fluo-4

Table 2. Conditions proposed for the realization of the high throughput screening to determine the suitable molecules for the block of FP receptor.

In vitro assay

As previously it has been commented, *in vitro* assays were made with Ishikawa cell line, a characterized cell line that endogenously express the FP receptor and was established from an endometrial adenocarcinoma of a 39-year-old woman (Public Health England: Culture Collection, 2021). It is required to overexpress the FP membrane receptor in Ishikawa cells, so estradiol-17β was incorporated into cell culture (Dong and Yallampalli, 2000).

In this assay, untreated cells in Gibco™ DMEM/F-12 medium was used. It was also worked with a negative control, which corresponds to the addition of prostaglandin F2α to the cell medium. Furthermore, a positive control was used, whose medium contains the antagonist BAY-6672 (Beck *et al.*, 2020), as it has been mentioned in HTS assays. In addition, the HIT compound that have been previously validated in HTS assays was analyzed, which was incorporated into the DMEM/F-12 medium together with prostaglandin F2α. All these samples were studied at different concentrations.

These *in vitro* assays include an MTT cytotoxicity assay, a PCR assay, a Western-blot and a calcium monitoring assay using a genetically encoded calcium indicator (GECI).

MTT assays

Firstly, an MTT cytotoxicity assay was made to determine cell viability. MTT is a compound that, metabolized by the mitochondria of living cells, is reduced to its insoluble formazan form. In this way, the amount of reduced MTT is quantified by a colorimetric method, since a color change from yellow to blue occurs as a result of the reaction.

This ability of cells to reduce MTT constitutes an indicator of mitochondrial integrity, and the mitochondrial activity is interpreted as a measure of cell viability. In this way, there is more blue color the more living cells in a well. To detect staining, the 96-well plate is incorporated into a spectrophotometer, which measures absorbance of the plate.

Control cells, those that have not been treated, will show a certain color, which corresponds to 100% viability. From this data, the percentage of viability of a cell culture treated with the compound can be calculated knowing its optical density. The results are expressed as a percentage of living cells (**Fig. 3**) (Arencibia, 2009).

$$\%\, Viability = \frac{OD\ treated\ cells}{OD\ control\ cells}\, x\ 100$$

Fig. 3. Calculation of the percentage of cell viability (Arencibia, 2009).

The expected result was that several of concentrations of compounds analyzed had a low percentage of viability, so that they were rejected in the following tests.

PCR and Western blot assay

A PCR and a Western blot assay were performed to calculate the amount of DNA and protein, respectively, of inositol phosphate, phospholipase C and ERK 1 and ERK 2. Additionally, phosphorylated

ERK (p-ERK) and unphosphorylated ERK were analyzed by the Western-blot. All of these components are involved in the signaling pathway activated by the FP receptor.

No graphical results were presented, but the prediction is that the cells treated with the HIT compound reduce the protein expression of p-ERK, while in the other cases analyzed there were no significant differences between the different samples.

Genetically encoded calcium indicator (GECI)

To detect calcium levels, GECIs have been used, genetically encoded calcium indicators. The endoplasmic reticulum is the most important reservoir of calcium in the cell, and through the binding of prostaglandin F2 alpha to the FP receptor, the calcium channels of the endoplasmic reticulum open and it allows the exit of calcium into the cytosol. Specifically, to investigate the dynamics of calcium in the ER, the cyan-colored BP-GECIs with endoplasmic reticulum localization signal are used.

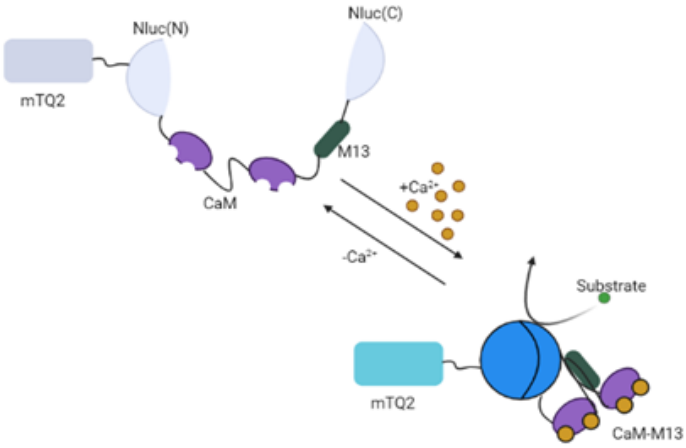


Fig. 4. Schematic description of the calcium detection mechanism by means of the BP-GECI construct. The components of BP-GECI named from the N to the C-terminus are: mTQ2, the N-terminus of Nluc (Nluc (N)), the CaM-M13 domain and the C-terminus of Nluc (Nluc (C)). Substrate and calcium are represented in green and yellow circles, respectively (Image modified from Hossain *et al*., 2018).

BP-GECI is composed of mTQ2, a protein that fluoresces in the presence of calcium, the N-terminal and C-terminal of Nluc, which in the presence of calcium these ends join and generate a bioluminescent protein, and the sensor domain of calcium calmodulin (CaM) -M13. Thanks to mTQ2 and Nluc, two types of detections are obtained: mTQ2 shows fluorescence by illumination of the sample and Nluc emits luminescence thanks to its substrate (**Fig. 4**).

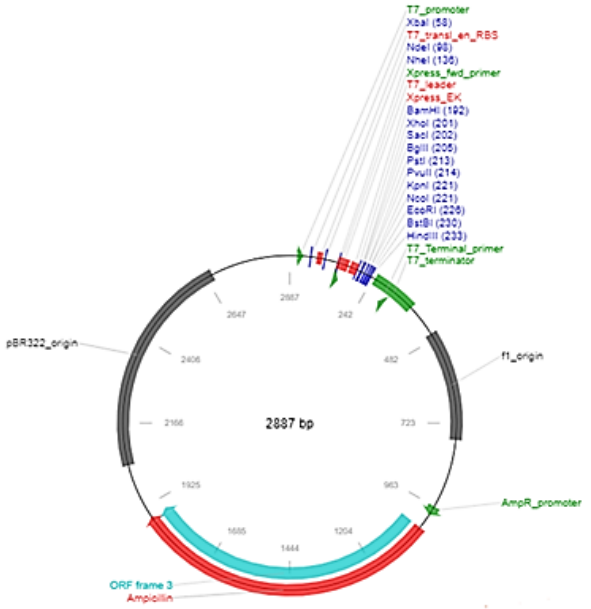


Fig. 5. DNA plasmid map (Addgene, 2021).

For the genetic construction of these GECIs, the cDNA of these 4 components (mTQ2, Nluc, CaM,M13) was ligated with a plasmid named CeNL_pRSETB (Hossain *et al.*, 2018). This plasmid has a size of 2,887 base pairs, with a polycloning site where restriction enzymes cut, an ampicillin resistance selection gene, a constitutive promoter of T7 RNA polymerase and a polyhistidine tag to purify recombinant proteins by an agarose affinity column (**Fig** transfected with lipofectamine into Ishikawa cells (Hossain *et al.*, 2018).

The expected results obtained from a spectrophotometer, thanks to the luminescence provided by the Nluc protein of the BP-GECI construction, are presented in figure 6. It is expected that in the positive control (prostaglandin F2α in the medium), calcium is dispersed throughout the cell because the prostaglandin opens the calcium channels of the reticulum, and the calcium bound to the BP-GECI construct exits the cytosol. On the other hand, in those cells treated with the compound together with the prostaglandin, the calcium would be located around the nucleus, in the endoplasmic reticulum, therefore, in this way, the action of the compound prevents the exit of calcium from the endoplasmic reticulum.

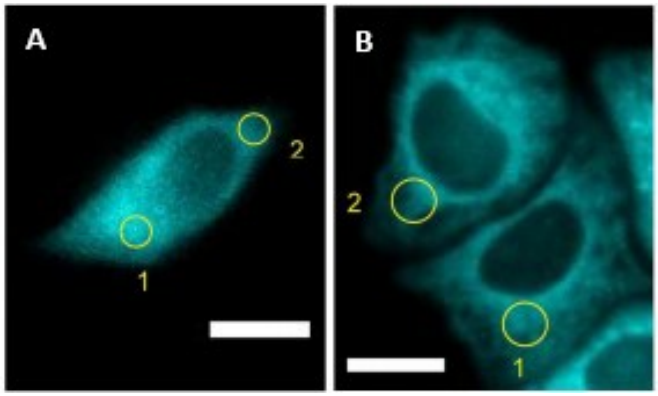


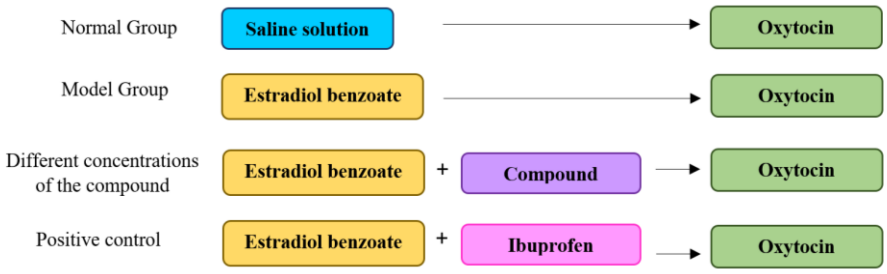
Fig. 6. Images at cellular level where calcium is found around the entire cytosol (A), and where calcium is in the reticulum (B) (Hossain *et al.*, 2018).

In vivo assay

In the vivo assay, it has been chosen the animal model proposed by *Yang et al*. (Yang *et al.,* 2015) which mimics the clinical effects of primary dysmenorrhea. For the welfare of the animals, this assay must be approved by the corresponding Ethics Committee in order to minimize animal suffering.

Female ICR mice (Imprinting Control Region) weighing 18-22g and 6-8 weeks were selected (Lu *et al.*, 2016). The animals were randomly divided into groups. They were treated with estradiol benzoate (1mg/kg/day) intraperitoneally (IP) for 3 days. As a control, a group of mice treated with saline solution (same volume as estradiol benzoate) also by IP injection. The rest of mice were divided into groups which different concentrations of the compound were administered, previously validated in the *in vitro* study. As a positive control, a group of mice were given ibuprofen instead of the compound. On the fourth day, estradiol benzoate and the compound were injected as usual and after 1 hour, 0.4 U of oxytocin was injected IP. During the next 30 minutes a contortion test was performed, where the number of abdominal contractions, pelvic rotation and stretching of the hind limbs were measured.

Subsequently, the animals were sacrificed, and plasma and uterus samples were collected for their biochemical analysis.



In this model, estradiol increases the expression of oxytocin receptors (OTR) in the uterus, so when oxytocin is administered, it binds to its receptors, triggering the exit of calcium from the endoplasmic reticulum and the increase in the expression of PGF2α that exits the cell and binds to its FP receptor. Thus, activating the signaling cascade that causes contraction of the uterus, reducing blood flow in the ovarian artery, ischemia in the myometrium, and spasms or pain which are symptoms of primary dysmenorrhea (**Fig. 7**) (Peng *et al.,* 2020).

Finally, regarding the expected results, uterine sections were analyzed histologically with a Hematoxylin-Eosin stain. It was observed that the edema presented by the control group was reduced both, treated at different concentrations of the compound and with ibuprofen. Oxytocin is also seen to increase the thickness of the myometrium. Mice treated with oxytocin showed increased calcium levels in uterine tissue samples. The compound significantly decreased these oxytocin-induced calcium levels. The concentration in the uterus sample of PGF2α was also observed to increase in the control group. The compound decreased PGF2α production gradually as the treatment dose increased.

By last, an organ bath test was proposed to measure uterine contractility. We used tissue from the upper part of the myometrium (region with contractile capacity), we added increasing concentrations of PGF2α, and we observed that the spikes increase and were more frequent, but with our compound, these spikes tended to be less frequent and intense (Ayar, 2007).

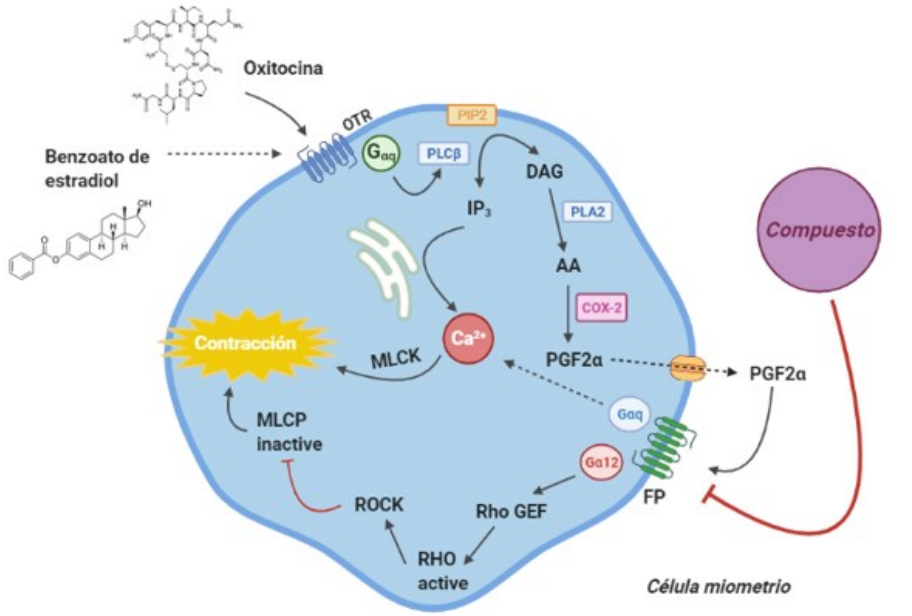


Fig. 7. Myometrial cell (uterus) pretreated with estradiol benzoate and subsequently with oxytocin.

Conclusions

Considering that science works for the progress of our society offering solutions to solve real problems that affects the population, it seems evident that primary dysmenorrhea has not obtained the attention that it deserves. Bearing in mind that primary dysmenorrhea affects more than the 90% of women in fertile age, many problems such as high levels of menstruation-related absenteeism surge as a consequence of this disease. Another problem derived from the lack of specific therapies for primary dysmenorrhea is the elevated level of self-treatment present in women who suffers from dysmenorrhea. This situation could end up in off target effects and the likely appearance of resistances.

Taking all together, herein, a new perspective for the interruption of dysmenorrhea derived pain is suggested. Prostaglandin F2α and its interaction with FP receptor have been observed to be highly related with myometrium contractions in those women who suffered from dysmenorrhea. Therefore, the interruption of this interaction by the utilization of PGF2α analogous could be an interesting strategy that in case of being effective could solve a problem that affects a noteworthy number of our population.

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