Novel strategies for treating biofilm and MRSA associated infections.

Memoria presentada por

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Que la presente memoria de Tesis Doctoral "**Novel strategies for treating biofilm and MRSA associated infections**" elaborada por Doña **JUANA MARÍA PRIETO** ha sido realizada bajo su dirección y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctor.

Y para que así conste, firman la presente en Pamplona, a 23 de octubre de 2020.

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Albert Einstein

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“Un poco más de persistencia, un poco más de esfuerzo, y lo que parecía irremediablemente un fracaso, puede convertirse en un éxito glorioso”

Elbert Hubbard

TABLE OF CONTENTS:

RESUMEN

A lo largo de los últimos años el desarrollo de nuevas moléculas con actividad antibiótica ha sufrido un drástico descenso. La práctica totalidad de los antibióticos que se utilizan actualmente fueron descubiertos entre las décadas de los 30 y 90, habiéndose comercializado únicamente cuatro clases de nuevos antibióticos en los últimos 30 años. Este factor, sumado al aumento exponencial de la prevalencia de infecciones asociadas a bacterias resistentes a antibióticos, nos ha sumido en lo que se conoce como "era post-antibiótica". Este escenario contempla incluso que infecciones que hasta el momento han sido controladas con tratamientos sencillos podrían poner en riesgo la vida del paciente por la ausencia de alternativas terapéuticas. En diciembre de 2014, en la revisión anual de resistencias antibióticas, el economista Jim O'Neil predijo que, si no se toman medidas, en el año 2050 las enfermedades por microorganismos multi-resistentes ocasionarán la muerte de 10 millones de personas en el mundo al año. Esta situación ha convertido la necesidad de desarrollar nuevas técnicas y aproximaciones terapéuticas para el control de infecciones causadas por bacterias multi-resistentes en una prioridad a nivel mundial. Especialmente preocupantes son las infecciones causadas por el grupo de patógenos denominado ESKAPE (*Entereococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanni, Pseudomonas aeruginosa* and *Enterobacter species*).

S. aureus tiene un gran peso específico en el problema sanitario y económico que suponen las bacterias ESKAPE ya que, pese a que se trata de una especie comúnmente asociada a la microbiota humana, este microorganismo es el agente etiológico de muy diversas enfermedades. Esto es en gran parte debido a su extrema capacidad adaptativa, en la que la formación de biopelículas de

diversa naturaleza, capaces de proteger a la bacteria frente al sistema inmune y el efecto de los antimicrobianos, tiene un papel crucial.

En esta tesis hemos trabajado en dos estrategias, un cribado fenotípico y otro frente a una diana específica, que podrían ayudar a ampliar las alternativas terapéuticas frente a *S. aureus*. En el primer capítulo, nos hemos centrado en la capacidad de esta bacteria para formar biofilms, y en el problema que esto implica. En nuestro intento de afrontar el problema con un abordaje diferente, nos hemos basado en el hecho de que la producción del biofilm, en especial el compuesto por el polisacárido PIA/PNAG, en muchas ocasiones es una ventaja para la bacteria, pero en otras puede ser su propio "talón de Aquiles". Con el objetivo de encontrar un compuesto capaz de incrementar los efectos negativos colaterales de la producción del biofilm e inhibir de forma selectiva a aquellas bacterias capaces de producir el polisacárido PIA/PNAG, hemos realizado un cribado a gran escala (HTS), en colaboración con la empresa Biomar, de compuestos y extractos procedentes de fermentaciones de microorganismos marinos. Los resultados han revelado la existencia de una sub-fracción (SF8) compuesta por tres moléculas que presenta una actividad inhibitoria específica sobre aquellas cepas que han iniciado el proceso de formación del biofilm polisacarídico. Además, los resultados obtenidos hasta la fecha sugieren que el efecto inhibidor de SF8 está mediado por una regulación de IcaC a nivel post transcripcional y la represión de proteína A, así como de otras proteínas de alto peso molecular.

En el segundo capítulo, nos hemos fijado un objetivo alternativo para "desarmar" a *S. aureus*, centrándonos en la participación del sistema de dos componentes (TCS) GraXRS en la capacidad de *S. aureus* para contrarrestar las barreras del sistema inmune innato del huésped. Con el objetivo de encontrar un medicamento en uso, capaz de bloquear GraXRS y hacer más susceptible a la

bacteria, hemos realizado un HTS con una colección de 1280 medicamentos aprobados por la agencia FDA cuyas patentes han expirado, utilizando una cepa reportera defectiva en todos los TCS no esenciales excepto GraXRS. El medicamento Vertepofin, cuya indicación aceptada es el tratamiento de la degeneración macular, es capaz de bloquear este TCS, mejorando la actividad anti-bacteriana mediada por las células polimorfonucleares. Su administración tópica en un modelo murino es capaz de reducir significativamente la carga bacteriana. Asimismo, se ha reforzado la conexión entre el sistema GraXRS y la señalización mediada por condiciones redox, ya que en nuestro cribado observamos que las moléculas antioxidantes y redox-activas son capaces de reducir la expresión del regulón GraXRS. El análisis del mecanismo molecular sugiere que el residuo redox-activo C227 de GraS participa en la inhibición ejercida por este fármaco. Teniendo en cuenta estos resultados, sugerimos incluir el fármaco Vertepofin en la lista de compuestos cuya indicación puede reconvertirse para sensibilizar a *S. aureus* y por tanto ser útil para combatir infecciones persistentes o resistentes a los antibióticos.

SUMMARY

In the course of recent years both development and commercialization of new antimicrobial drugs have undergone a very significant decline. Virutally all the antibiotics that are currently being used in clinics were discovered between 30´s and 90´s decades and only four new types of antimicrobial drugs have been brought into the market during the last 30 years. Such a premise, together with the fact that we are witnessing an exponential growth of infections associated to antibiotic-resistant bacteria, has plung us into in what is referred to as "post-antibiotic era". This scenario could turn common easy-to-cure infections into life-threatening diseases due to the lack of therapeutical alternatives; Actually, during the Annual Revision of Antibotic Resistance of 2014, it was predicted that, if community is not willing to undertake corrective measures, 10 million people could die from infectious diseases per year by 2050. Thus, development of novel techniques and alternative therapeutical approaches to combat resistant bacteria, specially those belonging to the ESKAPE family (*Enterococcus facecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacteri baumanni, Pseudomonas aureuginosa* and *Enerobacter*) are of major importance and extraordinary priority worldwide.

S. aureus poses a particular threat to economy and public health, since, though it is commonly associated to our normal microbiota, this species can be the ethiologic agent of a very wide range of diseases. This is mostly due to its impressive adaptative capacity, which mainly relies on an extraordinary network of signalling pathways for sensing and responding to environmental changes and the capacity to form biofilms that protect bacteria from the action of antimicrobials and the immune system.

In this thesis, we have applied a phenotypic (chapter I) and a target-focused (Chapter II) screening approaches, with the objective of finding molecules that

could somehow broaden the therapeutical alternatives against *S. aureus.* The first chapter is focused on the contradictory nature of PIA/PNAG, the major component of polysaccharidic staphylococcal biofilms. Though the production of this polymer protects bacteria and entails an unquestionable evolutionary advantage, it might also have some side effects in terms of bacterial fitness, which could be indeed enhanced or exploited to generate a lethal outcome. In collaboration with the spanish company Biomar, and with the hope of finding a molecule capable of targetting this bacterial “Achilles heel”, we have perfomed a High-Troughput-Screening (HTS) with a library of extracts and compounds of marine origin. Our results have revealed that a subfraction proceeding from the fermentation of a marine species, composed by three active compounds, exerts a specific inhibitory effect on those bacteria that had entered into the biofilm lifestyle and are producing PIA/PNAG as a part thereof. Up to date, analysis suggest that the mechanisms underlaying this inhibition are, at least in part, mediated by a post transcriptional inactivation of icaC protein and repression of Protein A and other high-molecular-weight proteins.

The second chapter is focused on the GraXRS Two Component System (TCS) as an alternative target for disarming *S. aureus*, since this pathway is crucial for bacterial resilience against the barriers of the host's innate immune system and thus has a pivotal role in *S. aureus* virulence. With the aim of finding new molecules capable of blocking GraXRS activity, we have screened 1280 off-patent FDA-approved drugs using a reporter strain lacking all non-essential TCSs but GraXRS. We have found that Vertepofin, a drug that is normally prescribed to treat macular degeneration, inhibits this TCS and it is indeed very efficient in enhancing PMN-mediated bacterial killing. Besides, the topical administration of this drug in a murine model significantly reduces the bacterial load. Likewise, the connection between the GraXRS signaling pathway and redox signalling has been strengthened by our findings, since we have observed that active

antioxidant and redox molecules are capable of reducing the expression of the GraXRS regulon. The analysis of molecular mechanisms underlaying Verterporfin effect suggest that the active C227 redox residue of GraS participates in the inhibition exerted by this drug. We therefore believe that it might be worth considering the drug Vertepofin as a candidate for sensitizing *S. aureus* and combating persistent or antibiotic-resistant infections.

INTRODUCTION

1. Staphylococcus aureus

Staphylococcus aureus is a gram-positive spherical bacterium, usually arranged in grape-like irregular clusters (see figure 1), which is commonly described as a non-motile, non-sporulated, facultative anaerobic microorganism. Relevant characteristics shown by this species also include a relatively high resistance to drying, extreme temperatures (up to 50ºC for 30 minutes), a wide pH range (4,8-9,4) and high-salt concentrations (up to 9% of sodium chloride), but a moderate susceptibility to certain chemicals like hexachlorophene, chlorhexidine or povidone-iodine instead. Regarding *S. aureus* major biochemical features, worth mentioning are its capacities to metabolize mannitol, glucose, xylose, lactose, sucrose, maltose and glycerol (Crossley *et al.*, 2009; Somerville and Proctor, 2009; Brooks *et al.*, 2013).

Most strains of *Staphylococcus aureus* are capable of producing the golden coloured carotenoid pigment staphyloxanthin, which acts as a virulence factor mainly due to its antioxidant properties that counteract the action of the reactive oxygen species produced by the host immune system. In addition, *S. aureus* is capable of producing surface polysaccharides that are important components of the staphylococcal cell envelope. These glycopolymers include capsular polysaccharide (CP), cell wall teichoic acid (WTA), and polysaccharide intercellular adhesin/poly-β(1-6)-N-acetylglucosamine (PIA/PNAG) and play distinct roles in *S. aureus* colonization and pathogenesis. Besides, colonies of *S. aureus* are β-hemolytic due to the production of several hemolysins, including α-hemolysin, β-hemolysin, γ-hemolysin and δ-hemolysin, which contribute to host cell damage. (Pelz *et al.*, 2005; Clauditz *et al.*, 2006; Somerville and Proctor, 2009; Brooks *et al.*, 2013).

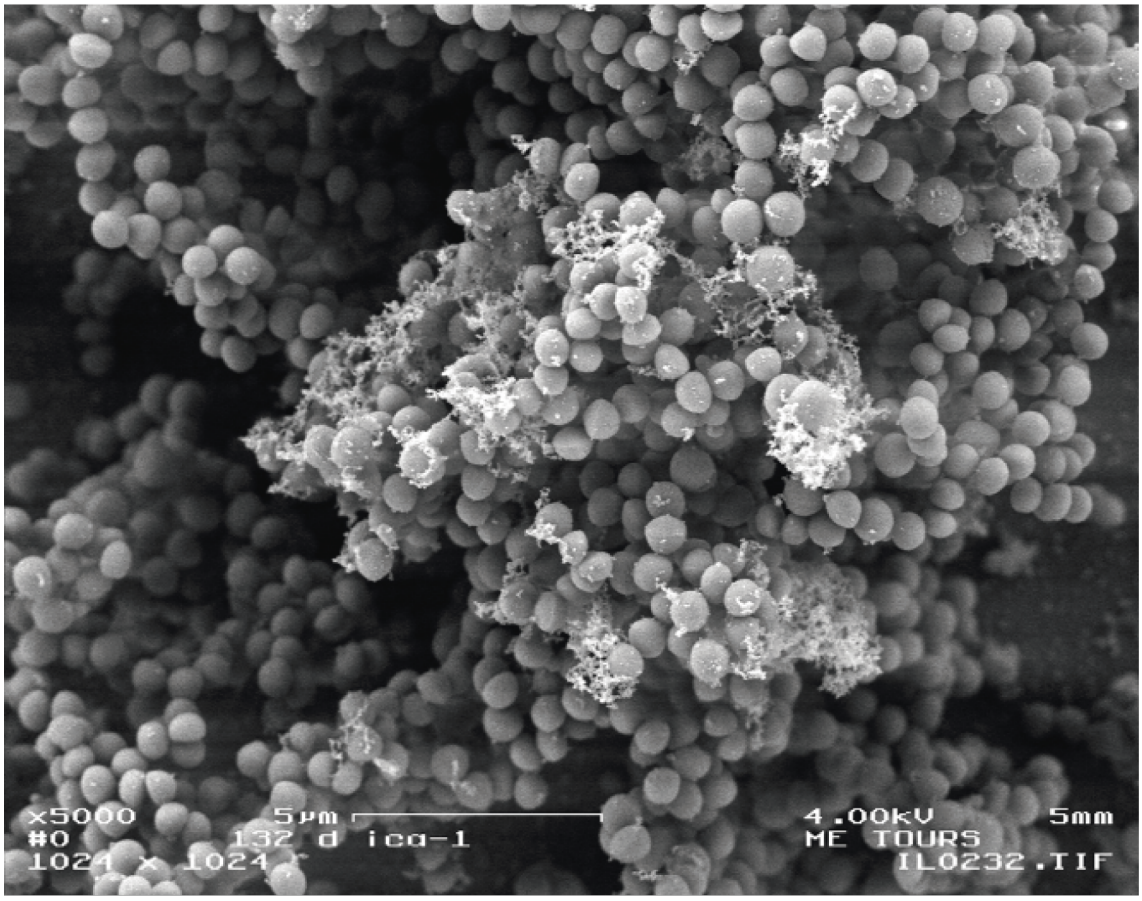

Figure 1. Scanning electron micrograph (SEM) of *Staphylococcus aureus*. Magnified 5000x.

Phylogeny

From a phylogenetic point of view, the species *S. aureus* is a member of the phylum Firmicutes, class Bacilli, order Bacillales, family Staphylococcaceae and genus *Staphylococcus*.

The genus *Staphylococcus* was initially classified on the basis of the colony colour, referring to the orange-yellow staphylococci as *S. aureus*, the white colonies *as S. albus* and the lemon-coloured species *as S. citreus*. However, pigment as the sole criterion for species classification was unsatisfactory, mainly because it was not a genetically stable character in many strains (Kloos, 1980). Afterwards, various molecular DNA-based methods requiring the use of several species-specific PCR primers, hybridization probes, multiple restriction enzymes, 16S rRNA gene sequencing, PCR restriction fragment length polymorphism (PCR-RFLP) and PCR-RFLP analysis of the 23S rRNA gene with two restriction enzymes were developed. However these conventional molecular typing approaches frequently struggle to discriminate between isolates in health-care environments (Ghebremedhin *et al.*, 2008). Nowadays, technological advances have turned whole genome sequencing (WGS) into the most promising method when it comes to distinguish clonal isolates. This method enables the entire genome of isolates to be compared, which enhances resolution significantly (Humphreys and Coleman, 2019). The affordability and increasing availability of WGS in recent years has enabled a more detailed study of previously undocumented transmission, as well as the overall and detailed analysis or the evolutionary route of resistance genes in *S. aureus* strains (Kuroda *et al.*, 2001; John *et al.*, 2019).

Apart from phylogenetic findings and classifications, in a simplified but more useful and well-accepted scheme, staphylococci are divided into two main groups on the basis of their ability to clot blood plasma (coagulase reaction). The first one, the coagulase-negative staphylococci (CoNS) group, represents a regular part of the microbiota of the skin and mucous membranes of humans and animals. The second one, the coagulase-positive staphylococci (CoPS) group,

consists of the most pathogenic staphylococcal species, where the major human pathogen is *S. aureus*. The genus consists of 47 species, 38 of which fulfil the categorization of coagulase-negative species, one of them includes both a coagulase-negative and positive subspecies, and the remaining species belong to the coagulase-positive group (Brooks *et al.*, 2013; Becker *et al.*, 2014).

2. Epidemiology

S. aureus is a frequent component of the human microbiota. Colonization commonly occurs after born and persists throughout life in a significant proportion of the population (approximately 20% of individuals are persistently colonized and 30% are transiently colonized), being the anterior nares, the throat, and the perineum the niches from which *S. aureus* can be cultured (Wertheim *et al.*, 2005).

S. aureus normally behaves like a commensal microbe that colonizes the host without causing disease but might turn into a dangerous pathogen due to its versatile and resilient nature. Indeed, *S. aureus* is capable of infecting almost every tissue and organ system in the human body, leading to diverse serious clinical conditions. Acute infections, such as bacteraemia and skin abscesses, are generally caused by planktonic cells and associated to the production of secreted toxins and exo-enzymes, while chronic infections, such as osteomyelitis, endocarditis, septicaemia and pneumonia, are normally associated to the biofilm or community lifestyle, which permits attachment and persistence on host tissues (like bone and heart valves) or on implanted materials (catheters, prosthetics joints or pacemakers, to name a few). Penetration into deeper tissue often occurs through invasive procedures like the introduction of catheters or artificial prostheses that are carried out in healthcare settings. Various host factors predispose to infection; These factors include loss of the normal skin barrier, presence of underlying disease (type 1 diabetes e.g.), acquired immunodeficiency syndrome or defects in neutrophils function. *S. aureus*-related infections are associated with increasing morbidity and mortality, longer hospital stays and often required surgical removal of infected devices, resulting in an expensive annuals cost (Sibbald *et al.*, 2006; Verbrugh, 2009; Lister and Horswill, 2014; Haag and Bagnoli, 2015; Al-Mebairik *et al.*, 2016; Moormeier and Bayles, 2017). The incidence of *S. aureus* bacteremia (SAB), in particular those cases associated to methicillin-resistant *S. aureus* (MRSA) strains increased dramatically in the period between 1960 to 2000 (Tong *et al.*, 2015). Even though

the prevalence has decreased in recent years, MRSA remains as one of the most important nosocomial pathogens, and noticeably, MRSA infections have emerged in the community. In Spain, *S. aureus* is, after *Escherichia coli*, the most frequent etiologic agent causing both nosocomial infections (10,06% of total hospital-acquired infections and 14,55% of hospital-acquired bacteremia) and community-acquired infections (8,94% of total community infections and 11,37% of community acquired bacteremia) (EPINE-EPPS, 2019).

The problem associated to staphylococcal infections is enhanced by the amazing ability of *S. aureus* to develop resistance to antibiotics. Infections caused by this pathogen were initially treated with penicillin since its introduction in the 1940s, but the appearance and rapid spread of methicillin-resistant *S. aureus* (MRSA) strains has eliminated the use of β-lactams as a treatment option. Actually, the phenomenon of antibiotic resistance was observed very soon. In 1961 the first MRSA strain was isolated, just 1 year after the introduction of methicillin. Vancomycin has long been a last resort antibiotic for multiple-drug-resistant *S. aureus* strains, but in 1996 a strain showing reduced sensitivity towards vancomycin was isolated, designated vancomycin intermediately resistant *S. aureus* (VISA). A few years later, in 2002, the first vancomycin resistant *S. aureus* (VRSA) emerged (Sibbald *et al.*, 2006; Assis *et al.*, 2017). As a result, *S. aureus* is nowadays considered as part of a dangerous group of bacteria that escape the lethal action of antibiotics. The group is composed by *Enterococcus faecium,* ***Staphylococcus aureus****, Klebsiella pneumoniae, Acinetobacter baumanni, Pseudomonas aeruginosa* and *Enterobacter species* and is referred to as “the ESKAPE bugs” (Rice, 2008).

Finally, worth mentioning is the fact that, apart from being a notorious human pathogen, *S. aureus* causes an array of infections with great economic livestock animals including cows, sheep, goats, poultry and rabbits. For instance, intramammary infection of dairy cows leading to mastitis, is a major economic burden on the global diary industry. The disease also affects small ruminants, which is a particular problem in regions that produce sheep and goat cheeses

and is also considered a major cause of lameness in the poultry industry, causing skeletal infections in commercial broiler chickens. Furthermore, this pathogen might cause skin abscesses, mastitis and septicaemia in rabbits (Fitzgerald, 2012).

3. Molecular pathogenesis

As mentioned earlier, *S. aureus* is recognized as a commensal colonizer of the skin and mucosa surface and also as an important pathogen. The success of *S. aureus* as both colonizer and pathogen and moreover to cause a wide range of infections in human and animals is largely due to its ability to adapt to different environment and to the extensive repertoire of mechanisms for virulence the bacterium is provide with. These virulence factors vary in their presence and specificity between clones and might be classified attending several criterions; In this thesis they are divided according to their mechanism of action: (1) adhesion and invasion; (2) Evasion and persistence; and (3) Toxins (Foster, 2004; Sibbald *et al.*, 2006; Gordon and Lowy, 2008; Al-Mebairik *et al.*, 2016). Each group is described below, summarized in table 1 and illustrated in figure 2.

Adhesion and invasion

The first step of *S. aureus* colonization involves bacterial adherence to host epithelial cells. This adhesion is mediated by surface proteins that are covalently attached to peptidoglycan, which are known as cell wall anchored (CWA) proteins. The precise repertoire of CWA proteins on the surface varies among strains and depends on growth conditions, but it is accepted that *S. aureus* can express up to 24 CWA proteins. The MSCRAMM (microbial surface component recognizing adhesive matrix molecules) protein family is also related to the attachment to components of the host extracellular matrix (ECM) such as fibrinogen, fibronectin and collagen. Through the acronym MSCRAMM was originally applied to surface proteins that mediate attachment to components of the host ECM, it is worth noting that many bacterial surface proteins are not MSCRAMM, while some MSCRAMM have additional functions other than promoting adhesion. MSCRAMM family includes clumping factor (*clfA* and *clfB*), collagen adhesin (*cna*), extracellular adherence protein (Eap), fibronectin-bindings proteins (FnBPA and FbBPB), biofilm associated protein (Bap), Iron-regulated surface determinant protein A (IsdA) or *S. aureus* surface protein G

(SasG). (Cucarella *et al.*, 2002; Vergara-Irigaray, Valle, Merino, Latasa, Garcia, Mozos, *et al.*, 2009; Foster *et al.*, 2014; Al-Mebairik *et al.*, 2016).

ECM adhesion step is a prerequisite for the internalization into non-professional phagocytic cells (NPPCs) like epithelial cells, endothelial cells, fibroblasts and osteoblasts. FnBPs can bind Fn molecules, and thus cluster α5β1 integrins on the cell surface to trigger the efficient intracellular signalling required for internalization. The signalling pathway of staphylococci internalization involves focal adhesion kinases (FAKs) and activated Src that subsequently recruit cortactin to promote actin polymerization and mobilize the endocytic machinery. Downstream of the FAK-Src pathway, the activation of PI3K and Akt is also important for the internalization of *S. aureus*. This internalization mechanism appears to be an active process on the cellular side, but *S. aureus* could also stimulate its own uptake by upregulating β1 integrin expression in the host cell via α-haemolysin secretion (Foster *et al.*, 2014; Goldmann *et al.*, 2016; Josse *et al.*, 2017).

Evasion and persistence

The innate immune system represents a first-line defence against invading pathogens and consists of three major effector mechanisms: (i) complement system, (ii) phagocytosis and (iii) antimicrobial peptides and enzymes production. The importance of these three effector mechanisms is different depending on the site of infection and on bacterial characteristics. Nevertheless, *S. aureus* has plenty of mechanisms to evade host innate immunity.

- Complement system represent the most “primitive” line of defence against infectious agents. The role of the complement system is to enhance binding and uptaking processes by phagocytic immune cells. *S. aureus* fights this mechanism by producing *Staphylococcus* protein A (SpA) (Forsgren and Sjöquist, 1966) and *Staphylococcus* binder of immunoglobulin (Sbi), two proteins that bind IgG in the wrong orientation, thereby blocking Fc receptor-mediated phagocytosis (Zhang *et al.*, 1998).

Besides that, *S. aureus* also produces *cna* (inhibits activation of the lectin pathway)(Kang *et al.*, 2013), Aureolysin (Aur, inhibits the de the deposition of C3 on the bacterial surface) (Laarman *et al.*, 2011), Eap (blocks the formation of lectin)(Woehl *et al.*, 2014), *Staphylococcus* complement inhibitor (SCIN, blocks C3b deposition and C5a production)(Suzan H M Rooijakkers *et al.*, 2005), Staphylokinase (SAK, remove opsonins IgG and C3b from the surface), SSL7 (interference with the production of C5a)(S.H.M. Rooijakkers *et al.*, 2005), Extracellular fibrinogen-binding protein (Efb, blocks C3 and C5 convertases) and its homologue extracellular complement-binding protein (Ecb, blocks C3 and C5 convertases and can build a "capsule-like" shield to prevent recognition of opsonins by FcR or CR)(Jongerius *et al.*, 2010; de Vor *et al.*, 2020).

- Phagocytosis: neutrophils are the most important effector cells in staphylococcal infections. They are recruited to the tissue by chemoattractants that are locally produced following infection by the bacterium (formylated peptides, leukotriene and platelet-activating factor) (Schiffmann *et al.*, 1975). *S. aureus* secrets proteins like chemotaxis inhibitory protein of Staphylococcus (CHIPS) (de Haas *et al.*, 2004).that evade priming and activation of neutrophils by blocking the interaction of chemoattractants with their neutrophil receptor, Moreover, FRP-like 1 inhibitory protein (FLIPr) and FLIPr-like block FPR1, FPR2, multiple FcRs and inhibit antibody-mediated phagocytosis (Stemerding *et al.*, 2013).
- Antimicrobial peptides and enzymes: after phagocytosis, bacteria are subjected to high levels of reactive oxygen species (ROS), reactive nitrogen species (RNS) and degranulation of antimicrobial products (lactoferrin, lysozyme, antimicrobial peptides (AMPs) and neutrophil serine proteases) in the phagosome. As response, *S. aureus* induces the expression of a large number of antioxidant enzymes like catalases, staphyloxantin or superoxide dismutase and uses different strategies to

avoid antimicrobial peptides (which target negatively charged bacteria), altering its surface charges or even degrading (Aur) and neutralizing (SAK) them (Liu, 2009).

Persistence is also a clinically relevant mechanism, allowing bacterial resilience against host defences or antibiotics. The capacity to persist on the host shown by *S. aureus* mainly lies in its ability to form small colony variants (SCVs) and display an aggregative behaviour known as biofilm. SCVs constitute a slow-growing auxotrophic subpopulation of bacteria with distinctive phenotypic and pathogenic traits that contribute to persistent and recurrent infections. *In vitro* assays have shown that SCVs are able to "hide" in host cells without causing significant host-cell damage, remaining protected from antibiotics and host defences. They can later revert to the more virulent wild-type phenotype, possibly resulting in recurrent infection (Gordon and Lowy, 2008; Liu, 2009; Melter and Radojevič, 2010).

Furthermore, *S. aureus* is capable of assembling sessile microbial communities known as biofilms. Within these multicellular structures, bacteria are attached to a surface or to other cells and embedded in a protective extracellular polymeric matrix. The composition of the scaffold varies amongst strains, but generally contain host factors, polysaccharides, proteins and extracellular DNA. Biofilms play an important role during infection, "sheltering" bacteria against several clearance mechanisms. Thus, biofilm matrixes can impede the access of certain types of immune elements like macrophages or antibodies and generate tolerance towards antibacterial agents. Beyond offering resistance to clearance mechanisms, biofilms are important in chronic diseases progression since individual cells can disperse from previously stablished scaffolds and either seed new sights of infection or mediate an acute process (Vuong *et al.*, 2004; Lister and Horswill, 2014). Following section describes further details regarding *S. aureus* biofilm.

Toxin-mediated diseases

Toxins are key virulence factors defined as molecules that increase the potential of a pathogen to cause disease through a direct interference with the host. The main *S. aureus* toxins can be divided into three major groups.

First group includes the pore forming toxins (PFTs), which by itself or in association with a receptor of host cell are able to produce a transmembrane channel. The PFTs group includes hemolysin-α, hemolysin-β, leukotoxins (LukDE, LukAB, Panton-Valentine leukocidin PLV) and phenol-soluble modulins (PSMs) (Grumann *et al.*, 2014).

The second group refers to exfoliative toxins (ETs), also known as epidermolytic toxins. ETs are extremely specific serine proteases secreted by *S. aureus,* which recognize and hydrolyse desmosome cadherins in the superficial layers or the skin. ETs include exfoliative toxin A/B/C/D (ETA, ETB, ETC, ETC) (Bukowski *et al.*, 2010; Mariutti *et al.*, 2017).

The third and last group comprises toxins known as superantigens (SAgs). These toxins activate a large fraction of T lymphocytes simultaneously by directly cross-linking certain T cell receptors in an MCH-independent manner. There are more than 23 staphylococcal SAgs toxins as toxic shock syndrome toxin (TSST-1), staphylococcal enterotoxins (SEA to SEE, SEG to SEJ, SEL to SEQ and SER to SET) and staphylococcal superantigen-like toxins (SEIK to SEIQ, SEU to SEIX) (Proft and Fraser, 2003; Grumann *et al.*, 2011; Grumann *et al.*, 2014; Otto, 2014).

Table 1. Summary of *Staphylococcus aureus* virulence factors.

Mechanism of action	Virulence Factor	References
Adhesion and invasion	ClfA, ClfB, FnbA, FnbB, Bap, SasG, Cna, Sdr, SraPBbp, Eap FnBPA, FnbPB IsdA, WTA	(Cucarella *et al.*, 2002; Vergara-Irigaray, Valle, Merino, Latasa, Garcia, Mozos, *et al.*, 2009; Foster *et al.*, 2014; Al-Mebairik *et al.*, 2016; Goldmann *et al.*, 2016; Josse *et al.*, 2017)
Evasion and persistence	SpA, Sbi, can, Aur, SCIN, SAK, SSL7, Efb, Ecb, CHIP, FLIPr, FLIPr-like Eap, staphyloxantin, katG, mprF, Dlt operon, Coa, capsule, SCVs, IcaACBD, IcaR, Rbf	(Forsgren and Sjöquist, 1966; Zhang *et al.*, 1998; Suzan H M Rooijakkers *et al.*, 2005; Liu, 2009; Jongerius *et al.*, 2010; Laarman *et al.*, 2011; Zecconi and Scali, 2013; Stemerding *et al.*, 2013; Kang *et al.*, 2013; Woehl *et al.*, 2014; McGuinness *et al.*, 2016)
Toxins	PSMs, ETA, ETB, ETC, ETD, SEAs, TSST, Hla, Hlb, PVL, LukDE, LukAB, SEs	(Proft and Fraser, 2003; Sibbald *et al.*, 2006; Gordon and Lowy, 2008; Bukowski *et al.*, 2010; Grumann *et al.*, 2011; Grumann *et al.*, 2014; Otto, 2014; Mariutti *et al.*, 2017)

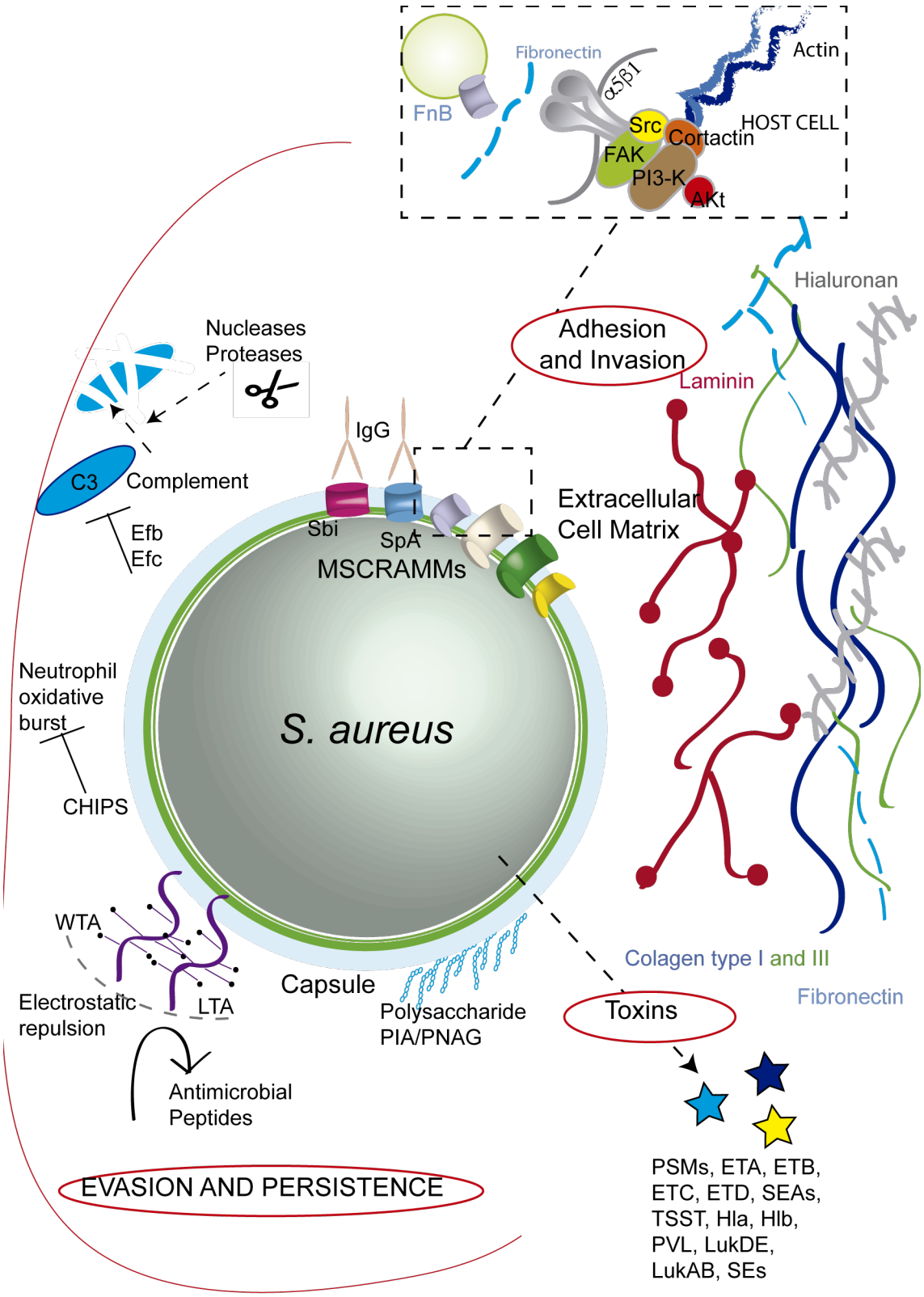

Figure 2. Schematic diagram of *S. aureus* virulence factors. Illustrated acording to the three main groups: adhesion and invasion, evasion and persistence and toxins.

4. Biofilm

Bacterial biofilm

In the 17th century, Antonie van Leeuwenhoek observed swarming "animalcules" deposited on living and dead matter for the first time. These "animalcules" in the tartar on his own teeth were indeed bacteria of the dental plaque, and represent the first documented evidence of the biofilm concept; (Percival *et al.*, 2011). The exhaustive observation of this particular microbial structure awaited the invention of the electron microscopy.

In general terms, biofilms are defined as a communities of microorganisms attached to biotic or abiotic surfaces, embedded by a matrix of extracellular polymers, acting as an independent functioning and homeostatically-regulated ecosystems (Percival *et al.*, 2000; Sadekuzzaman *et al.*, 2015; Jamal *et al.*, 2018). Now it is well recognized that almost all microorganisms in nature, under the appropriate conditions, have the ability to grow as part of a sessile biopolymer-enshrouded community referred to as biofilm. Biofilms communities differ from their planktonic (freely suspended) counterparts in terms of gene expression and protein production. The communication between neighbouring bacteria mainly occurs via quorum sensing, a social language and behaviour that enables interactions within bacterial communities. Sometimes interactions can be beneficial (metabolic cooperation, attachment allowing), but other times the relationship might be based on the competition for resources or natural nutrients (Elias and Banin, 2012).

There is a wide range of microbial biofilms depending on whether they are composed by single or multiple species, or according to the matrix composition, which is highly complex and might suffer great variations depending on environmental conditions (Donlan, 2002). Biofilm formation is a phenomenon that occurs in both ecological and clinical environments and leads to the development of beneficial communities or inconvenient disease-associated biofilms formed on medical devices (Costerton *et al.*, 1981). In humans, an estimated 65% of all hospital infections are associated to biofilms. Once established, these infections

are very difficult to eradicate due to their resilience to removal by host defense mechanisms and antimicrobials (Percival *et al.*, 2011). The biofilm-producing pathogen *Staphylococcus aureus* has become specially notorious for causing chronic infections associated to the biofilms formed on indwelling medical devices (Moormeier and Bayles, 2017).

Staphylococcus aureus biofilm

As it has just been stated, the capacity of *S. aureus* to form biofilms is an important virulence factor when it comes to device-related infections. Biofilm plays a relevant role by providing defense against several clearance mechanisms. The biofilm matrix can impede the access of certain types of immune defense, such as macrophages, which display incomplete penetration into the biofilm matrix (frustrated phagocytosis). Additionally, biofilm cells display increased tolerance to antibiotics, due to the presence of a diffusion barrier that slows down the infiltration of some antimicrobial agents but also because of the low metabolic rates of some cells, known as physiologically dormant or persister cells. Beyond offering resistance to clearance mechanisms, biofilm also plays an important role in the progression of chronic diseases since, following the establishment of a biofilm, individual cells can disperse from the original biofilm and either seed new colonization spots or mediate an acute infection process or even sepsis (dispersal model) (Lister and Horswill, 2014).

S. aureus can produce a multilayer biofilm embedded within a highly heterogenous glycocalyx or slime layer (see figure 3). Initially, it was thought that the slime was a mixture of teichoic acids (80%) and proteins (20%) (Hussain, 1993). In 1995, Mack *et al.* isolated the Polysaccharide Intercellular Antigen (PIA/PNAG) from staphylococcal extracellular matrix (Mack *et al.*, 1996), also known as polymeric *N*-acetyl glucosamine (PNAG) (Maira-Litran *et al.*, 2002). In general terms, *Staphylococcus* biofilms can be classified depending on the composition of the biofilm matrix as PIA/PNAG-dependent or PIA/PNAG-independent biofilm.

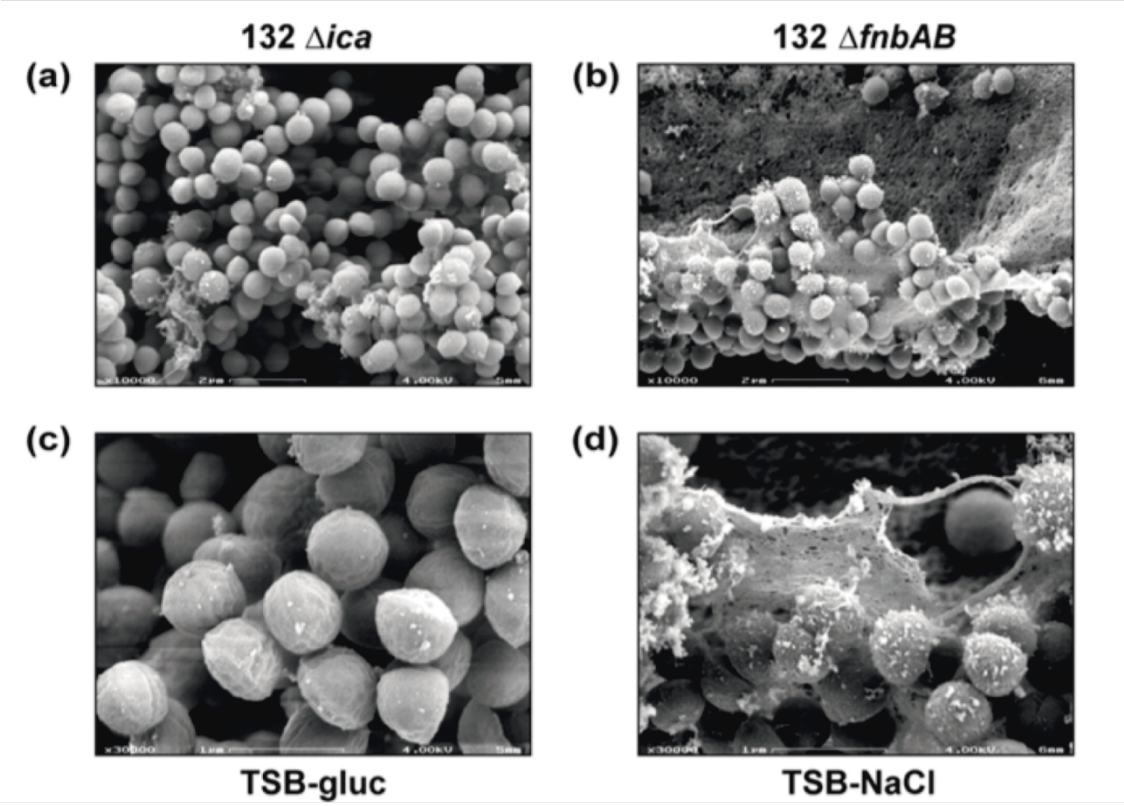

Figure 3. Scanning electron micrographs. (a, c) proteinaceous biofilm developed by the *S. aureus* strain 132 Δica mutant and (b, d) PIA/PNAG-dependent biofilm developed by the strain 132 ΔfnbAB mutant at ×10,000 (a, b) and ×30,000 (c, d) magnifications. (Vergara-Irigaray et al., 2009)

- **PIA/PNAG-independent biofilm matrix**

S. aureus can produce a biofilm matrix in which proteins usually take the responsibility for mediating cell-to-cell interactions and multicellular behaviour. The molecular mechanisms underlaying the production of protein-based biofilm matrix remain to be fully understood so far, probably because regulatory schemes behind biofilm formation might not be the same for all strains and the same strains may have multiple mechanisms depending on the environmental signals. In any case, increasing number of studies indicate that proteinaceous scaffolds are more common than previously anticipated (O'Gara, 2007; Taglialegna *et al.*, 2016).

Research groups leaded by J. Penades and I. Lasa described the biofilm-associated protein (Bap), an essential protein for both initial adherence and intercellular accumulation during PIA/PNAG-independent biofilm formation (Cucarella *et al.*, 2001). Although *bap* gene is present in only 5% of bovine isolates and appears to be absent in human clinical isolates of *S. aureus (Cucarella et al., 2004)*, this protein is member of a group of over 100 surface proteins with conserved structural and functional characteristics from several bacterial species (Lasa and Penadés, 2006; Latasa *et al.*, 2006). Bacterial cell surface-anchored proteins can assemble the matrix scaffold through homophilic interactions between identical molecules expressed on neighbour cells or through heterophilic interactions with other surface proteins or even with non-proteinaceous cell wall structures (Conrady *et al.*, 2008; Herman-Bausier *et al.*, 2015). Another strategy by which proteins can contribute to the formation of the matrix is through polymerization into functional amyloids fibers. Secreted proteins can assemble to form insoluble fibers, which constitute a strong platform able to mediate interactions between the neighbour cells and surfaces. Acting as a sensor, Bap is constitutively expressed and processed. When the pH of the medium becomes acidic and the concentration of calcium is low, the resulting fragments form insoluble amyloid-like aggregates, and when calcium concentration increases, metal-coordinated Bap adopts a more stable

conformation (Taglialegna *et al.*, 2016). Besides, *S. aureus* produces extracellular fibers in biofilm communities that consist of small peptides called phenol soluble modulins (PSMs). The accumulation of PSM peptides in fibers modulates their ability to disperse biofilms. Thus, PSMs fulfill dual and opposing roles that are modulated by amyloid-like aggregation (Schwartz *et al.*, 2012).

Furthermore, a substantial number of staphylococcal molecules have been subsequently associated to PIA/PNAG-independent biofilms. As stated before, microbial surface components recognizing adhesive matrix molecules (MSCRAMM) are peptidoglycan covalently linked adhesins containing the C-terminal sorting signal LPXTG motif. Among the MSCRAMMs, fibronectin (Fn)-binding proteins A and B (FnBPA and FnBPB) have been identified as key molecules in proteinaceous biofilms (Vergara-Irigaray, Valle, Merino, Latasa, Garcia, Mozos, *et al.*, 2009), while the *S. aureus* surface protein G (SasG) is involved in the biofilm accumulation (Corrigan *et al.*, 2007); and Protein A (*spa)*, another LPXTG protein, has also been associated with biofilm formation due to promotion of intercellular aggregation (Merino *et al.*, 2009). SraP (Serine-rich adhesin for binding to platelets) protein also contributes to biofilm formation by mediating attachment to a variety of host cells or bacteria themselves (Foster *et al.*, 2014).

Another important component of the staphylococcal biofilm is extracellular DNA (eDNA). The autolysis of a subpopulation of the biofilm cells and subsequent genomic DNA release must also occur early in cell attachment for proper biofilm formation. Due to the negative charge of the DNA polymer, eDNA potentially acts as an electrostatic polymer that anchor cells to a surface, to host factors, and to each other (Archer *et al.*, 2011; Lister and Horswill, 2014).

- **PIA/PNAG-dependent biofilm matrix**

Although multiple bacterial and external factors influence attachment and accumulation, production of an extracellular polysaccharide adhesin by *icaADBC* operon-encoded enzymes, termed polysaccharide intercellular adhesin (PIA/PNAG) or polymeric *N*-acetyl-glucosamine (PNAG), is currently the best

understood mechanism underlaying biofilm formation in staphylococci (Mack *et al.*, 1996; Maira-Litran *et al.*, 2002).

PIA/PNAG is composed of β-1,6-linked *N*-acetylglucosamine polymer. The first two genes of the *icaADBC* operon, *icaA* and *icaD* respectively, exert a primary role in the exopolysaccharide synthesis. *icaA* encodes for a transmembrane enzyme with *N*-acetylglucosaminyl transferase activity, necessary for the synthesis of the poly-*N*-acetylglucosamine polymer, *icaD* gene co-expression is also requiring for optimal activity (oligomers longer than 20 glucosamine units). Conversely, the product of the *icaC* gene appears to translocate the poly-*N*-acetylglucosamine polymer to the bacterial cell surface, while *icaB* product operates the deacetylation of the molecule. Deacetylation is relevant for the structural development of the exopolysaccharide-based biofilm, since such process permits the fixation of the polymer to the outer bacterial surface (Gerke *et al.*, 1998). figure 4 illustrates PIA/PNAG synthesis process.

The negative regulator termed intercellular adhesin locus regulator (*icaR*) gene governs the expression of the *ica* locus under the influence of SarA and the stress σ^B (Cerca *et al.*, 2008).

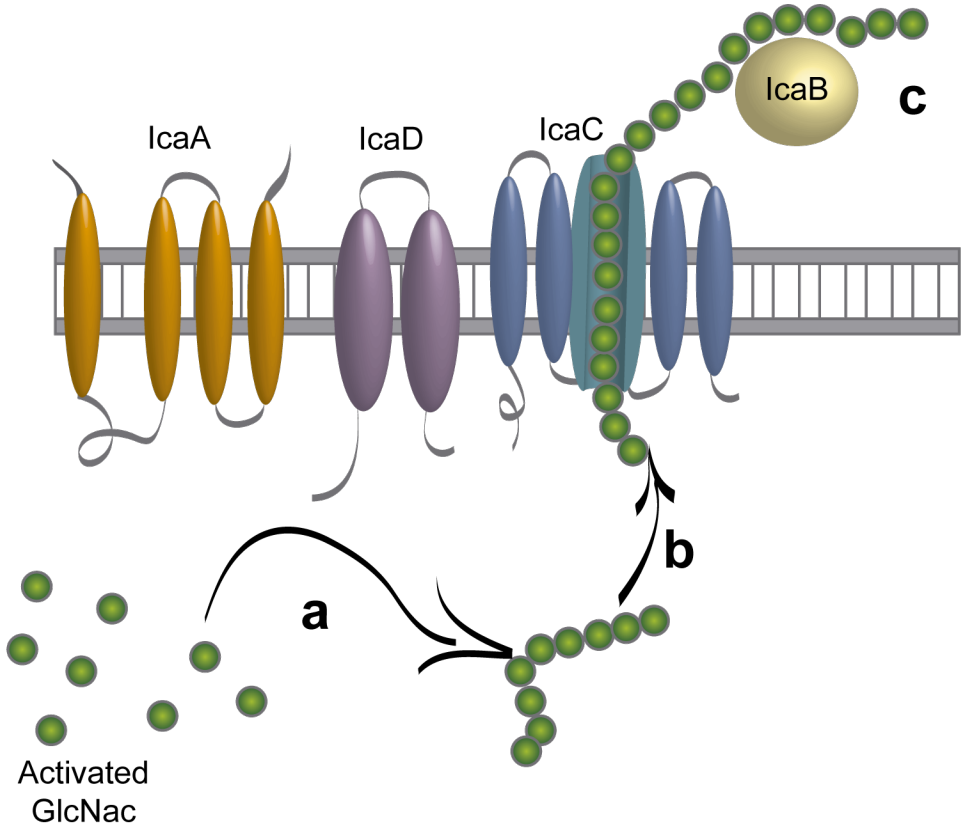

Figure 4. The exopolysaccharide PIA/PNAG synthesis by *icaADBC* locus encodes enzymes. a) N-acetylglucosaminyl transferase activity of IcaA and IcaD enzymes. b) poly-N-acetylgucosamine polymer translocation to the bacterial cell surface by IcaC. c) Deacetylation of the molecule by IcaB.

Stages of biofilm development

While the biofilm matrix composition varies amongst bacterial species and even in the same species or the same strains under different environmental conditions, a basic model of biofilm formation is widely recognized. The model consist of three sequential stages: (i) attachment, (ii) accumulation/maturation and (iii) detachment/dispersal (O'Toole *et al.*, 2000). Different stages are described below and illustrated in figure 5.

i. Attachment

To initiate biofilm formation, planktonic *S. aureus* cells first attach to a surface. The microorganism must be brought into close proximity to the surface, driven either randomly by a stream of fluid flowing over a surface as occurs for non-motile bacteria, or in a directed fashion via chemotaxis, twitching (pili) or swimming (flagella) motility. Once bacteria reached the surface, adhesion occurs through a variety of CWA proteins or surface molecules, which are specific for different host matrix substrates, through their appendages (like pili or flagella) or through other physical forces between cell and surface (like van der Waal's forces, electrostatic interactions or hydrophobic interactions) (O'Toole *et al.*, 2000).

ii. Maturation

The following stage is the proliferation and maturation of the biofilm. This process begins once irreversible attachment to the surface occurs and as long as a sufficient nutrient source existed. In this phase, bacteria replicate and synthesize extracellular polymeric substances comprising polysaccharides and proteins that form the extracellular matrix and maintain bacteria interacting with each other. At this moment, channels and mushroom-shaped structures are formed to facilitate nutrient delivery and oxygen circulation to deeper layers of the biofilm and waste removal. Cell-to-cell communication via quorum sensing is an important process at this stage (O'Toole *et al.*, 2000; Le *et al.*, 2014).

iii. Dispersal

Finally, to conclude the cycle, some bacteria detach from the biofilm and initiate the colonization of new niches. Three different dispersal strategies can be observed: swarming dispersal, clumping dispersal and surface dispersal. Mechanic forces, surfactant molecules (PSMs) and enzymes that degrade biofilm matrix molecules such as nucleases and proteases also stimulate this process. All of these dynamic detachment events could succeed in dispersing biofilm bacteria to new surfaces or to a susceptible host (Hall-Stoodley and Stoodley, 2005; Le *et al.*, 2014).

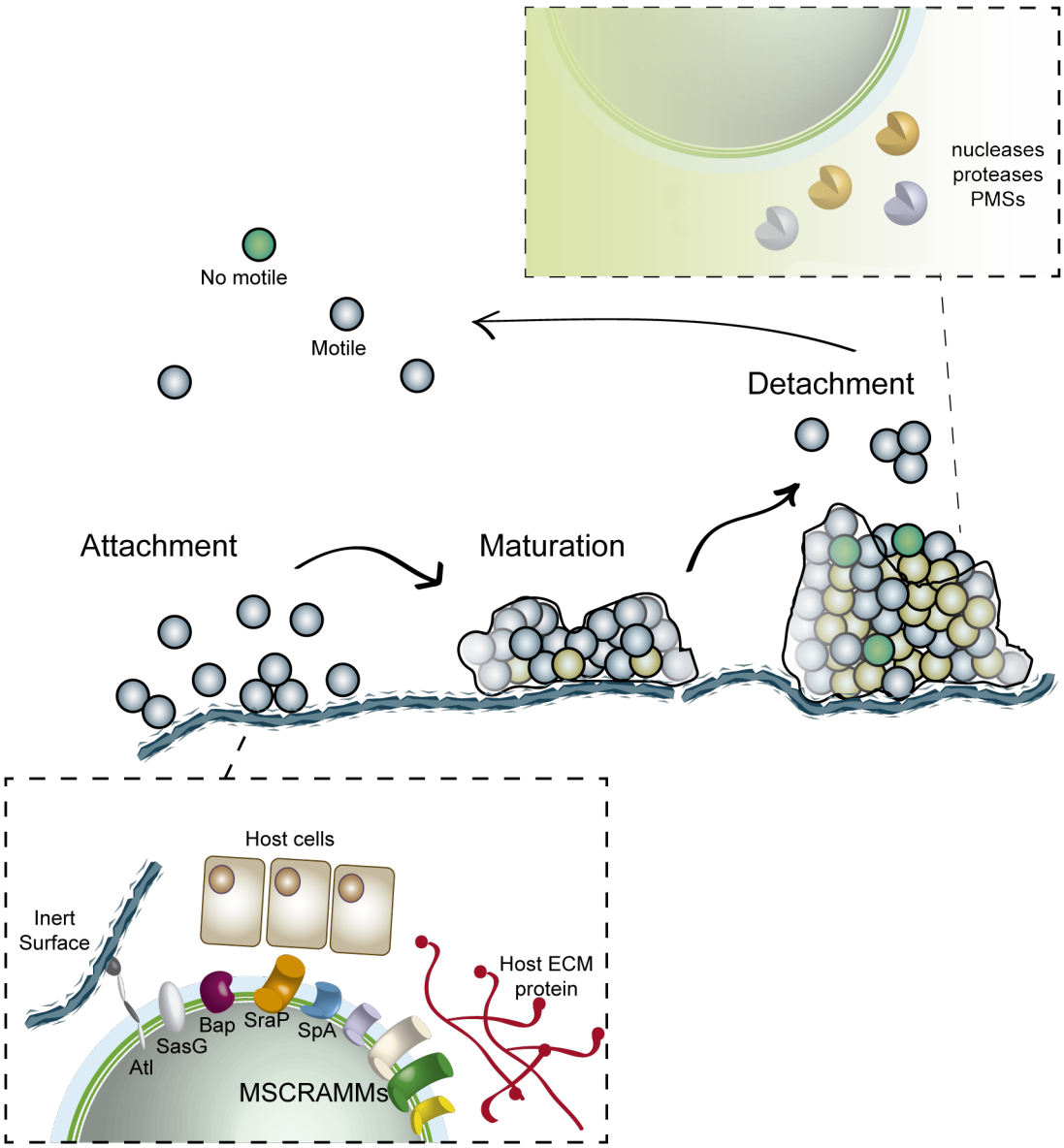

Figure 5. Biofilm life cycle. Biofilm formation follows sequentially steps of attachment, maturation and final detachment to re-enter into planktonic mode.

Biofilm regulation

A fine control of sessile and planktonic phenotypes is highly required to explain a well-coordinated and effective action in biofilm formation and disruption. Mechanisms for biofilm formation are enabled by stimuli from density of bacterial cells belonging to the same group and sharing the same pheromone system (termed as quorum sensing QS) and also from environmental stimuli, including presence of oxygen, glucose, ethanol, salts, certain antibiotics, osmolarity and temperature. The expression of biofilm phenotype is considered as a very complex process, in part because there is a multiplicity of factors that contribute to the biofilm extracellular matrix, but also because the biofilm production derives from a complicated equilibrium of production of extracellular polymeric substances and enzymatic reactions. So much so, and though it is assumed that environmental signals determine the biofilm composition, the molecular determinant underlying the choice of either a polysaccharide or protein-based biofilm matrix is not well understood; (Vergara-Irigaray, Valle, Merino, Latasa, Garcia, Mozos, *et al.*, 2009; Arciola *et al.*, 2015).

Focusing on the regulation of polysaccharidic biofilms (see figure 6), different mechanisms are known. As previously mentioned, PIA/PNAG is the principal component of this kind of biofilm. The production of this polysaccharide is regulated by environmental factors, such as the presence of glucose, NaCl, ethanol, osmolarity, temperature or antibiotics. Under anaerobic conditions, for instance, i*ca* operon is upregulated by the staphylococcal respiratory response regulator SrrAB via binding a DNA sequence upstream of *icaADBC* operon. Specially notable is the strong negative regulation conferred by *icaR, a* gene that encodes a transcriptional repressor with a central role in the environmental regulation of *ica* operon expression. Modification of the bacterial environment by the addition of NaCl or ethanol to the growth medium can activate the *ica* operon via separate regulatory pathways in an *icaR*-dependent manner. IcaR interacts with *icaADBC* promoter and inhibits the access of the ribosome to the Shine-

Dalgarno (SD) region. This process occurs after a complex post-transcriptional modulation mechanism which control *icaR* expression and subsequently PIA/PNAG production. (O'Gara, 2007; Mozos *et al.*, 2013). Apart from IcaR, putative binding sequences for TcaR (teicoplanin-associated locus regulator) have been identified in the promoter region of the ica operon, suggesting that this marR-type protein functions as a direct repressor of PIA/PNAG production.

An alternative regulatory mechanism involves phase variation of the poly-*N*-acetylglucosamine expression. Phase variation functions as a reversible on/off switch for a particular gene, that could be led by a slipped-strand mispairing mechanism. This slipped-strand mispairing occurs during DNA replication when there is mispairing between mother and daughter DNA strands in regions that contain simple nucleotide repeats, resulting in the addition or subtraction of one or more repeats that can bring about a change in transcriptional efficiency or shift the reading frame to alter or halt translation. Brooks *et al*., found that a RecA-independent expansion or contraction of a 4-nt tandem "ttta" repeat shifts the reading frame of *icaC*, leading to a premature stop codon, truncating the protein at 303 amino acids; 47 amino acids shorter than full-length protein. This mutation results in the complete inhibition of PIA/PNAG production (PIA/PNAG-negative phenotype) (Brooks and Jefferson, 2014). Additionally, it has also been observed in some *S. aureus* strains, that the insertion sequence IS256 contributes to the production of biofilm-negative variants through insertion/excision events into *icaC* and the *sarA* genes (Archer *et al.*, 2011; Kleinert *et al.*, 2017).

In the case of proteinaceous biofilms (see figure 6), development control is an intricate network of overlapping circuits involving two-component systems (TCS) and transcriptional and post-transcriptional, including RNA molecules. The accessory gene regulator (Agr) system plays an important role modulating of the expression of different virulence-associated genes. The main Agr effector molecule, RNAIII, downregulates genes that encode cell surface proteins (Spa, FnBPA, SasG, Coa) and upregulates exoproteins (PSMs, proteases and Hla) (Novick, 2003). Rot (repressor of toxins) is another key player within the biofilm

regulatory network promoting biofilm upregulation by proteases repression. Rot is regulated by Agr in presence of RNAIII (Mootz *et al.*, 2015). The staphylococcal accessory regulator (SarA) protein is a global transcriptional regulator with a profound impact on *ica*-independent biofilm production indirectly via *agr*-positive regulation (Rice *et al.*, 2006). Also Sigma factor B (Sig B) has a role in biofilm production by SarA and Agr-RNAIII regulation (Gotz, 2002; Valle *et al.*, 2003). Furthermore, there are other two-component systems that collaborates in the regulation of the biofilm formation, such as SaeRS (downregulates proteases in synergy with SarA), WalKR (upregulates both LytM and AtlA autolysis) and ArlRS (decreasing protease activity) (Toledo-Arana *et al.*, 2005; Dubrac *et al.*, 2007; Mrak *et al.*, 2012).

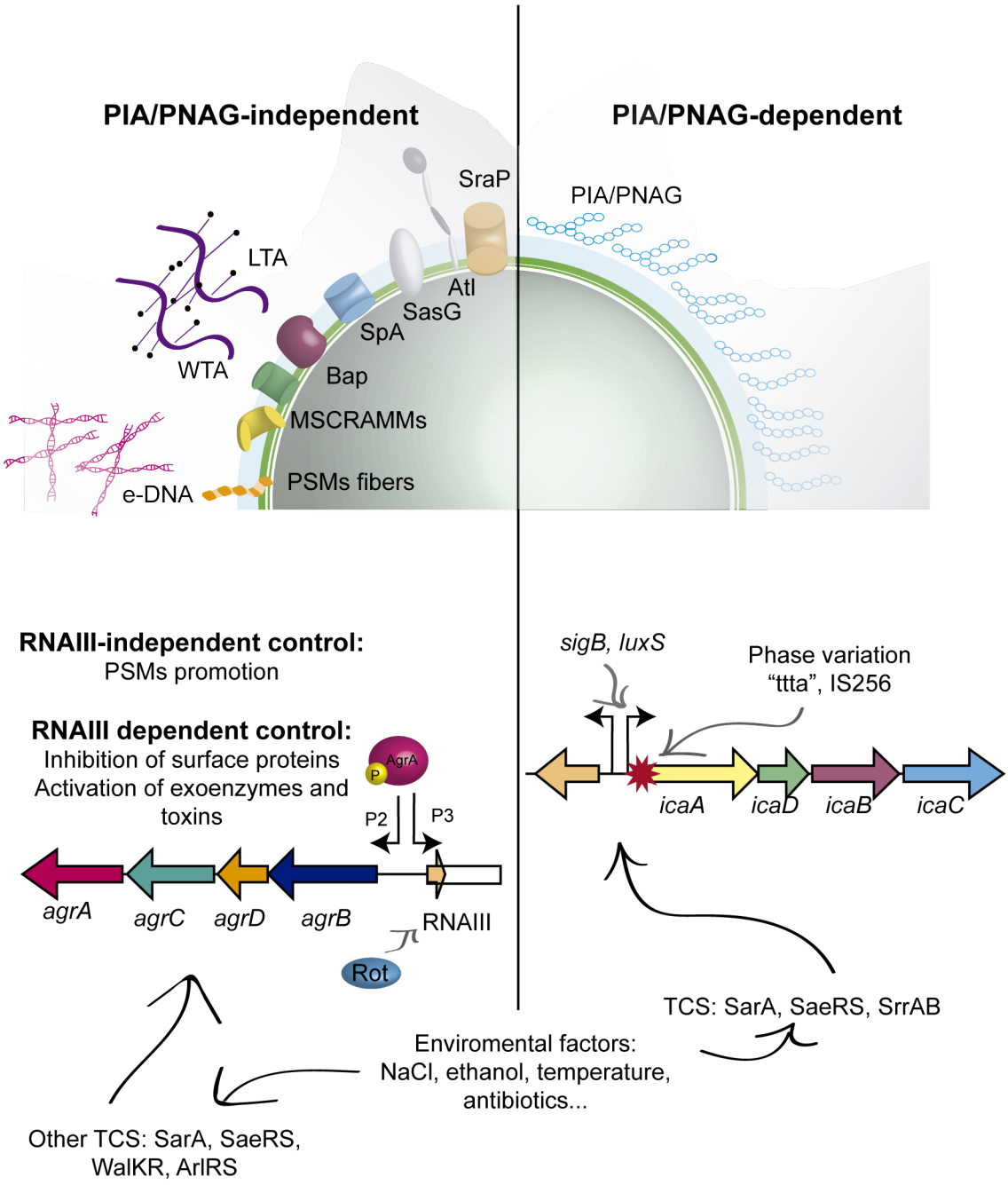

Figure 6. The regulation of biofilm formation. Schematic diagram of PIA/PNAG-independent (left side) and PIA/PNAG-dependent (right side) biofilm regulation.

Signal transduction system of S. aureus

In nature, bacteria are subjected to changes in local pH, osmotic pressure, temperature, redox potential, nutrient availability or exposure to toxic chemicals, to name but a few conditions. Focusing on *S. aureus*, this species shows an amazing ability to adapt to distinct environments, being able to survive in a wide range of niches and can thus cause a diverse spectrum of human diseases and animal infections. To successfully cope with selective pressure, bacteria have evolved simple but highly efficient signal transduction systems to regulate gene expression and respond accordingly. Thus, this capacity of coordinated expression of genes in response to environmental cues is a key factor that has determined the evolutionary success of this pathogen (Cheung *et al.*, 2004; Dubrac and Msadek, 2008; Capra and Laub, 2012; Mattos-Graner and Duncan, 2017; Villanueva *et al.*, 2018).

S. aureus utilizes three major classes of signal transduction systems:

a) Diguanylate cyclase and phosphodiesterase, which transmit internal or external signals by modifying the cyclic diguanylate (c-di-GMP) levels. Diguanylate cyclase are characterized by the presence of the GGDEF domain and they are responsible for the synthesis of c-di-GMP. Phosphodiesterases contain the EAL domain and they are responsible for the degradation of this molecule. The signalling molecule c-di-GMP, is a global regulator that play a role in process like biofilm formation, motility and by modulating the function of c-di-GMP binding molecules, proteins or RNA. *S. aureus* genome presents only one conserved domain GGDEF (GdpS) and a second protein with highly modified GGDEF domain (GdpP) but with phosphodiesterase activity. There is no evidence of any *S. aureus* genome encoding an EAL domain protein. (Karaolis *et al.*, 2005; Römling and Amikam, 2006; Jenal and Malone, 2006; Holland *et al.*, 2008; Corrigan *et al.*, 2011).

b) Proteins kinases/phosphatases, which upon receiving a stimulus bind and modify the phosphorylation status of target specific genes (Liebeke *et al.*, 2010).

c) Two-component signal transduction systems, which are composed by a sensor located on the membrane and a cytosolic receptor protein that triggers the bacterial response (Stock *et al.*, 2000). This mechanism is exposed in the following section.

Two-Component signal transduction systems

Two-component signal transduction systems or two-component systems (TCSs) are the predominant means by which bacteria sense and respond to environmental stimuli. In their most basic form, TCSs systems comprise a receptor membrane-bound protein, referred to as histidine kinase (HK), that sense a specific signal and translates that input into a desired output; and its cytosolic response regulator (RR) protein, required for inducing transcriptional adaptation. Upon receiving a stimulus, the HK catalyses an autophosphorylation reaction on a conserved histidine residue. This phosphoryl group is then transferred to a conserved aspartate on a cognate RR. Phosphorylation of the regulator usually drives a conformational change that activates its output response (see figure 7). In some cases, input signals may promote the phosphatase state rather than stimulating autophosphorylation (Yang and Inouye, 1993; Jin and Inouye, 1993; Casino *et al.*, 2010; Capra and Laub, 2012).

HK contains two highly conserved domains, the dimerization and histidine phosphotransferase (DHp) domain, which harbor the conserved histidine that is the site of both autophosphorylation and phosphotransfer reactions, and the catalytic and ATP biding (CA) domain. In addition, all HK are identified by unique signature sequence called H, N, G1, F and G2 boxes. The conserved amino acids of the N, G1, F and G2 boxes border the unique ATP-binding pocket and compose the transmitter domain. The most conserved residues are those used to anchor ATP within the binding site: an Asp in the G1 box and an Asn in the N box (Dutta *et al.*, 1999; Galperin, 2005; Wilke and Carlson, 2013).

RR are typically multidomain proteins, consisting of a well-conserved receiver or regulatory domain (at the N-terminal) and a variable effector domain (at the C-terminal). The conserved regulatory domain catalyzes the transfer of a

phosphoryl group from its cognate HK to one of its own aspartic acid residues. This phosphorylation promotes a stabilizing conformation capable of promoting activity of the effector domain. The variable effector domain elicits the specific output response of the system, most commonly transcriptional regulation (Gao *et al.*, 2007; Capra and Laub, 2012).

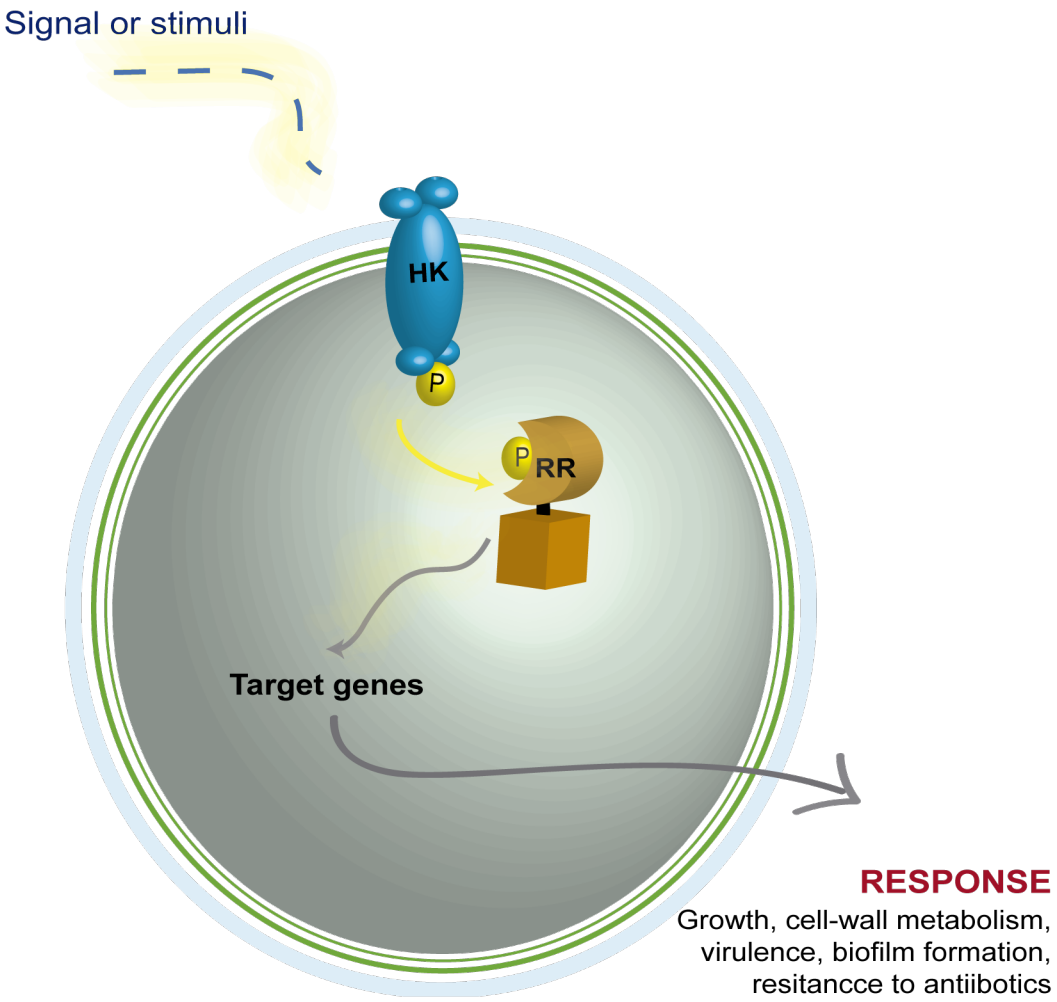

Figure 7. Overview of general two-component systems. In response to stimuli, HK auto-phosphorylates and transfers the phosphoryl group to the RR, which regulates target genes and effects changes in cellular physiology.

TCS in *S. aureus*

Most clinically relevant bacterial species usually contain multiple two-component HK-RR pairs. The sum of TCSs is proportional to the genome size, the diversity of environment in which the organism lives, and the complexity of the cellular differentiation. The number of TCSs seems to expand primarily through a mechanism of gene duplication and subsequent accumulation of mutations that insulate the new pathways from the existing TCS pathways. The final consequence of this evolutionary process is that bacteria gain the capacity to colonize new niches or improve the efficiency to grow under the condition of the niche they are colonizing (Galperin, 2005; Capra and Laub, 2012; Villanueva *et al.*, 2018).

S. aureus is a versatile pathogen that also presents several TCS. Most strains encode 16 TCS but might also harbor an additional TCS when the *mec element*, linked to the induction of methicillin resistance, is present in the chromosome (see table 2). These TCSs are involved in sensing a diverse array of environmental stimuli and contribute to the ability of *S. aureus* to adapt to the diverse environments it encounters during its life cycle.

Even though our understanding of staphylococcal gene regulation by TCS has made significant progress in the last decade, there are many issues that remain to be understood. Current knowledge about the genes affected by most of the TCS is the result of studying mutants in the respective sensor kinase, response regulator and auxiliary genes. Nevertheless, the precise nature of signal(s) that are sensed and their relevance to bacterial physiology for most *S. aureus* TCSs have not been fully uncovered.

Although we have still not reached the point where the function of two TCSs is totally deciphered, *S. aureus* TCS are commonly grouped according to their major function (Haag and Bagnoli, 2015):

- Regulation of virulence gene expression: two TCS, a*grCA* and *saeRS,* are known as global regulators of virulence-related gene expression.

- Response to AMPs and cell wall damage: Antimicrobial peptides (AMPs) interact with microbial membranes, leading to cell lysis or formation of transient pores, though which AMPs are transported into the cell. *S. aureus* contains three TCS, *vraSR, graXRS* and *braRS,* that mediate the response to the exposure to AMPs.
- Cell wall metabolism, autolysis and cell death: bacterial growth and replication requires an exquisite control to coordinate DNA replication machinery, cell wall biosynthesis and remodeling. *S. aureus* employs three TCSs, *arlRS, lytSR* and the crucial *walkRK,* to regulate cell wall metabolism.
- Respiration, fermentation and nitrate metabolism: *S. aureus* is a facultative anaerobe that can grow without oxygen using either anaerobic respiration with nitrate as the terminal electron acceptor or by fermenting carbohydrates. Three TCS of *S. aureus* synchronize the response to environmental oxygen levels in order to fine-tune respiratory activity and divert energy fluxes into different metabolic pathways; These are *srrAB, nreCBA* and *airRS*.
- Nutrient sensing and metabolism: the availability of nutrients and micronutrients is a key determinant of the microenvironment in which a bacterium resides and is essential for bacterial metabolism and survival. *S. aureus* harbors three TCS responsible for nutrient sensing and metabolism regulation. These are *hssSR, kdpDE* and *phoRP*.

Table 2. TCSs of *S. aureus*.

	Name	Alternative name	Genes (MW2)	Major function
TCS1	*walKR*	*yycFG, vicRK, micAB*	walR/walk MW0020 M0021	Bacterial cell composition
TCS2	*tcs2**		*	Kdp-like, potassium transport
TCS3	*tcs3*		MW0200 MW0199 MW0198	Unknown function
TCS4	*lytSR*		lytR/lytS	Murein hydrolase activity
TCS5	*graXRS*	*aspRS*	graX graR graS	CAMP sensing and virulence
TCS6	*saePQRS*		saeP saeQ saeR saeS	Secreted factors mostly involved in immune evasion
TCS7	*tcs7*		MW1206 MW1207 MW1208 MW1209	Specific to staphylococci, unknown function
TCS8	*arlRS*		arlR arlS	Adhesion, autolysis, multidrug resistance and virulence genes
TCS9	*srrAB*	*srhSR, resED*	scpA scpB rluB srrA srrB	Aerobic and anaerobic respiration
TCS10	*phoPR*		phoP phoR	Phosphate assimilation
TCS11	*yhsSR*	*yheSR*	yhcS yhcR	Oxygen sensing
TCS12	*vraSR*		MW1827 yvqF vraS vraR	Cell wall biosynthesis
TCS13	*agrBDCA*		agrB agrD agrC agrA	Exo- and cell protein synthesis, quorum sensing
TCS14	*kdpDE*		kdpD kdpE	Potassium transport
TCS15	*hssRS*		hssR hssS	heme sensing
TCS16	*nreABC*		narG narH narJ narI nreA nreB nreC	Nitrogen assimilation/oxygen regulatory protein NreC
TCS17	*braRS*	*nsaRS, bceRS*	MW2546 braR braS	Bacitracin efflux/influx/sensing

* System present in some *S. aureus* strains, which carry SCCmec element.

Sensory deprived strain

Given the uncertainty surrounding several TCS function, in a previous study a genetic reductionist approach was applied on two genetically unrelated *S. aureus* strains in order to generate mutant derivatives that lack the whole TCS signaling network. The process involved the sequential deletion of the 15 non-essential TCSs of *S. aureus* except *walKR* TCS, whose complete deletion is lethal. The results obtained in such work demonstrated that *S. aureus* remains viable after the deletion of the 15 non-essential TCSs and might be even deprived from all of them, including WalK, under non-replicating conditions. Besides, the experiments carried out with this unique strain, referred to as ΔXV, and its TCS-restored derivatives, confirmed the concept of TCSs as self-sufficient modules that confer a specific advantage under particular environmental conditions (Villanueva *et al.*, 2018).

More deeply, phenotypic analysis of the ΔXV mutant strain revealed indistinguishable growth levels at 37 and 44ºC in comparison to those showed by the wild type strain, and similar metabolic capacities. However, ΔXV strain lost the capacity to reduce nitrite, showed a slight growth arrest at 28ºC, a decreased capacity to survive in the environment and higher sensitivity to detergents. Moreover, in the absence of TCSs, *S. aureus* is unable to invade eukaryotic cells and colonize organs, rendering the bacteria avirulent in a mouse infection model (Villanueva *et al.*, 2018).

The TCS-deficient strain is an extremely useful tool, which will allow the study of TCS function without counteracting or “hindering” effects exerted by other TCSs. With the help of ΔXV, every TCS might be individually analyzed and thus determine the specific signal recognized by the TCS and the precise relevance of the TCS to bacterial physiology. Besides, this strain will be really useful to identify the regulon of each TCS or in the hard work of finding antimicrobials that specifically block TCS functions. In this thesis we will focus on TCS5 (*graXRS*).

GraXRS

The GraXRS (for Glycopeptide-resistance-associated) TCS might also be referred to as antibiotic peptide sensor (ApsRS). GraXRS was identified by Cui *et.* al. in 2005 while studying the transcriptomic profiles of *S. aureus* strains showing different degrees of vancomycin resistance. Thus, expression of *graS* gene was significantly higher in strains that showed increased resistance to the antibiotic. Furthermore, it was observed that overexpression of *graS* in vancomycin sensitive strains increased the resistance to this antibiotic. As a result, GraXRS was linked with resistance to vancomycin (Cui *et al.*, 2005).

GraXRS is also connected with control of resistance to cationic antimicrobial peptides (CAMPs) through the synthesis of enzymes that increase bacterial cell surface positive charge, by D-alanylation of teichoic acids and lysylination of phosphatidylglycerol, leading to electrostatic repulsion of CAMPs. GraXRS requires the ABC transporter *vraFG* for conferring resistance to CAMPs (Falord *et al.*, 2012).

Recently, GraXRS has also been associated to virulence and cell-wall metabolism. Transcriptomic approaches have revealed that, in contrast to many TCSs, GraXRS does not regulate its own expression, but affects the expression levels of 248 genes, some of which are major regulators of virulence gene expression, colonization factors and exotoxin-encoding genes. Modification of teichoic acid with D-alanine by the products of the *dlt* operon protects S*. aureus* against major antimicrobial host defense molecules such as defensins. Furthermore, acidic exposure, as inside macrophage phagolysosomes, evokes GraS signaling, which in turn elicits an adaptive response that endows the bacteria with increased resistance to antimicrobial effectors so that *S. aureus* can regulate with GraXRS its surface properties in order to overcome innate host defense (Meehl *et al.*, 2007; Kraus *et al.*, 2008; Falord *et al.*, 2011; Flannagan *et al.*, 2018).

The *graXRS* genes are located immediately upstream of the ABC transporter genes *vraF* and *vraG*, being one of four TCS system loci that are in proximity to

ABC transporter genes (see figure 8A). Interestingly, this close relationship between TCS and ABC transporter was only observed in firmicutes. The GraXRS system shows high similarity to the BceRS TCS of *Bacillus subtilis*, similar to its *S. aureus* homolog, the *bceRS* genes, that also are located immediately upstream of the ABC transporter system (Falord *et al.*, 2011; Falord *et al.*, 2012; Haag and Bagnoli, 2015). So, it can be said that GraXRS is a five-component signal transduction system, whose components are described below.

The membrane-bound HK, GraS, is a 346 amino acid protein that consists of a membrane bound domain (spanning 1-63 residues) and a cytoplasmic domain referred to as kinase domain (spanning residues 110-346), that harbors the catalytic domain (residues 181 to 346). The cytoplasmic domain of GraS does not have autokinase activity (Muzamal *et al.*, 2014).

The second component of GraXRS is the cytosolic protein that acts as its cognate RR. GraR is a 224 amino acid protein that present a conserved aspartate residue at position 51. This residue is essential for its activity (Falord *et al.*, 2012). A highly conserved ten-base-pair palindromic sequence (5' ACAAATTTGT 3') located upstream from GraR-regulated genes was shown to be essential for transcriptional regulation and induction by GraR, suggesting that this could be a likely GraR binding site (Falord *et al.*, 2011; Falord *et al.*, 2012).

The third component, GraX, is the auxiliary protein. GraX is a cytosolic protein with 308 amino acids that contains a weakly hydrophobic putative transmembrane segment (residues 216 to 236) and a suggested extracellular C terminus. GraX play a role as a bridge protein between its HK (GraS) and RR (GraR), interacting with GraS (see figure 8B) (Falord *et al.*, 2012; Muzamal *et al.*, 2014).

Finally, the ABC transporter proteins VraF and VraG are 254 and 630 amino acids proteins respectively, located immediately upstream of the *graXRS* genes which are required for conferring CAMPs resistance (see figure 8B) (Kuroda *et al.*, 2000; Muzamal *et al.*, 2014).

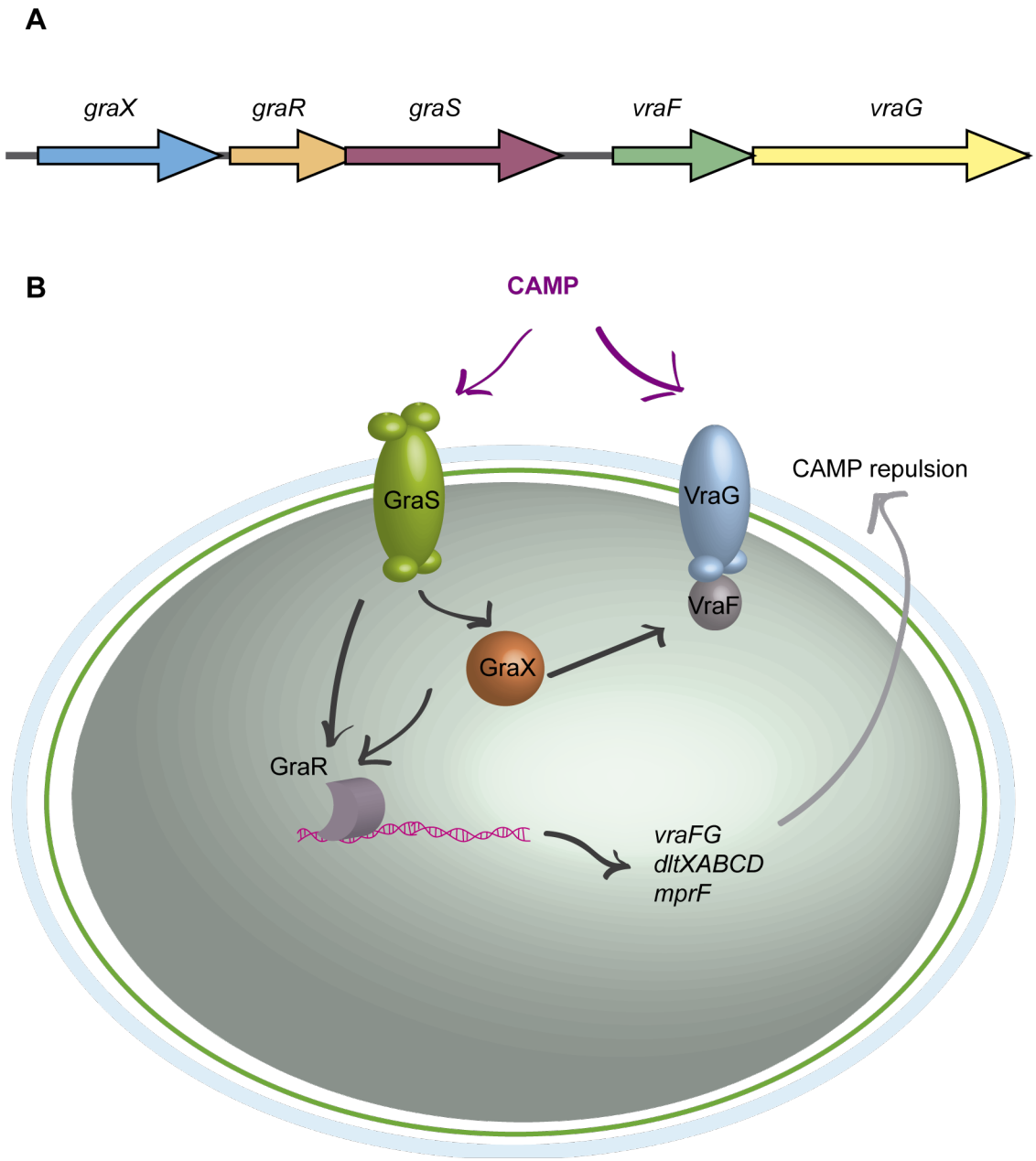

Figure 8. GraXRS TCS. A) The *graXRS* and *vraFG* locus of *S. aureus* MW2. B) GraXRS signal transduction network. GraS and VraG sense the stimuli (CAMPS) and the signal is transduced to GraS through a mechanism that likely involves interaction between VraG and GraS. Activation of the GraSR systems leads to increaed transcription of the *vraFG* operon, *dltXABCD* operon and *mprF* gene, generating a response (CAMP resistance by repulsion).

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OBJETIVES

The specific objectives of this thesis are:

1. Finding novel molecules capable of turning PIA/PNAG production into a lethal process for *S. aureus*.

2. Following a drug repurposing methodology, screen a collection of FDA-approved drug for inhibition of the GraRS two-component systems of *S. aureus*, using a singular strain that lacks the whole TCS machinery.

CHAPTER I.

A different slant in anti-biofilm drug discovery

Abstract

The polysaccharide PIA/PNAG is one of the major components of staphylococcal biofilms. Contrary to what it might seem, production of PIA/PNAG does not always confer a selective advantage, proof of which are the multiple on-off mechanisms that regulate its expression. In this study, with the aim of finding novel molecules capable of turning PIA/PNAG production into a lethal process for *S. aureus*, we have performed a High Throughput Screening assay in which several PIA/PNAG overproducer strains and their *icaADBC* mutant counterparts were grown in the presence of a collection of marine extracts and compounds provided by the Spanish company BIOMAR. We have selected a sub-fraction (SF8) composed by malayamicin, lumichrome and soyasaponin that specifically inhibits the growth of those strains capable of producing PIA/PNAG-dependent biofilm. Furthermore, this study aims at an initial approach to characterize the molecular mechanism underlaying the selected extract. Results obtained up to date suggest that the inhibitory effect exerted by the sub-fraction is mediated by a post transcriptional *icaC* down regulation and protein A repression, among other high weight molecular proteins.

Introduction

Conventional antibiotics fail to successfully treat biofilm-associated-chronic infections, being *S. aureus* one of the etiologic agents that is mostly perceived as a serious threat to human health. Since we are unfortunately immersed in the post-antibiotic era, current therapeutical measures tend to pursue the inhibition of biofilm formation and virulence factors instead of bacterial death (Jaśkiewicz, Neubauer, Kazor, Bartoszewska, & Kamysz, 2019).

Over the last years, great progress in the eradication and dispersal of staphylococcal biofilms has been made, mostly helped by the gradually better understanding of the molecular mechanisms that govern such multicellular behavior. The principal anti-biofilm strategies under study today contemplate different fronts like the inhibition of attachment using small molecules like aryl rhodanines, chelators or silver ions and nanoparticles, disruption of the biofilm architecture through molecules like Cis-2-Decenoic acid or a mixture of D-aminoacids dispersion, degradation of matrix component by enzymes like dispersin, DNase or other lytic enzymes encoded by bacteriophages, inhibition of quorum sensing, modification of Two-Component-Systems signalling pathways, or even the generation of a protective immune response via anti-biofilm vaccines (P. Y. Chung & Toh, 2014; Verderosa, Dhouib, Fairfull-Smith, & Totsika, 2019).

One of the major components of staphylococcal biofilms is the polysaccharide PIA/PNAG. The production of this high molecular weight polymer depends on the proteins encoded by the *icaADBC* intercellular adhesion locus, an operon that is subjected to strict regulation, both at transcriptional and post-transcriptional levels. Despite the undoubted role of this polysaccharide in staphylococcal persistence, adaptation and pathogenesis, production of PIA/PNAG does not always confer a selective advantage, proof of which are the on-off mechanisms like phase-variation that regulate its expression (Arciola, Campoccia, Ravaioli, & Montanaro, 2015). Actually, when *S. aureus* is subjected to several subcultures, PIA/PNAG-negative phase variants quickly increased in

number relative to PIA/PNAG over-expressers. Bearing this premise in mind, the rationale behind our approach here was that PIA/PNAG production might be considered as a bacterial "Achilles heel" and thus, over-synthesis and/or accumulation of PIA/PNAG-intermediate proteins-metabolites could become toxic or alternatively render a status of high susceptibility to antimicrobials. So, in our attempt to somehow look at the anti-biofilm paradigm from another perspective, we have carried out an alternative phenotypic, instead of target-focused, drug discovery approach to find compounds that could specifically be lethal for those bacteria that were initiating the biofilm lifestyle. Indeed, phenotypic screenings, understood as testing molecules to see if they exert the desired effect on a cell, leaving the precise target or mechanism aside, are having something like a renascence these days, since, though they do not always lead to best-in-class drugs, they definitively entail some advantages for generating first-in class drugs (Swinney, 2013). Using a screening platform composed by PIA/PNAG overproducer strains and their *icaADBC* mutant counterparts, we have tested the capacity of a library of extracts and compounds of marine origin to specifically kill bacteria upon entering into biofilm-growth phase. The basis of the assay was as simple as selecting those molecules that were capable of exerting specific growth inhibition of PIA/PNAG positive strain but had hardly any effect on the PIA/PNAG negative ones.

The idea of using marine extracts and compounds in this study came as a result of a collaboration with the Spanish company Biomar. Though terrestrial plants and microorganisms are of global and paramount importance in drug discovery, marine biodiversity is assumed to be even higher, being nowadays conceived as a major source of high added value molecules for treating human diseases (Khazir, Mir, Mir, & Cowan, 2013). Evidence of this conception is represented by the wide array of anti-staphylococcal compounds and extracts possessing antibacterial and/or anti-biofilm activities that have already been obtained from marine sponges and microbes (Balasubramanian, Harper,

Shopsin, & Torres, 2017; Gomes et al., 2014; Kildow, Conradie, & Robson, 2012; Palomo et al., 2013; Rahman & Richardson, 2010; Stowe et al., 2011).

Though we have not yet been able to identify a singular compound capable of exerting a PIA/PNAG-dependent antibiotic effect, the present study describes the finding and characterization of a chemical subfraction obtained from a marine microbe, composed by Lumichrome, Soyasaponin and Malayamicin, that specifically inhibits those bacteria producing PIA/PNAG. Preliminary studies aimed at the understanding of the molecular mechanisms underlaying the effect of sub-fraction TA-15-A-A112CHV-F.9/10.SF8 indicate that post transcriptional regulation of *ica* operon and *ica*-conditional repression of proteinA and other (LPXTG) high molecular weight proteins could be crucial to exploit PIA/PNAG-associated fitness cost.

Results

High Throughput Screening platform

In order to develop a robust phenotype-screening platform, we first tested the capacity of PIA/PNAG synthesis of nine previously characterized strains and selected strong biofilm formers that could be easily distinguishable from their *ica*ADBC mutant counterparts. Upon characterizing their multicellular behaviour through a standard microtitter-plate protocol, three pairs of strains were selected: (I) the clinical isolate 15981 showing a strong PIA/PNAG-dependent biofilm production and its derivative PIA/PNAG defective mutant, (II) the clinical strain ISP479r, also capable of producing a polysaccharidic biofilm, and its derivative defective mutant and (III) *S. aureus* strain 132, together with the derivative mutant. This last strain was chosen due to its ability for producing a polysaccharidic or proteinaceous biofilm depending on the presence of high concentrations of salt or glucose respectively (Vergara-Irigaray, Valle, Merino, Latasa, Garcia, Mozos, et al., 2009).

In order to optimize the screening protocol, the effect of different temperatures (28ºC and 37ºC) of incubation, initial inoculum (1:200, 1:100 and 1:40) and incubation times (24h and 36h) was first examined. As shown in figure 1, differences between biofilm positive and negative strains were especially notable when the assay was performed at 28ºC for 36h. By contrast, incubation at 37ºC for 24h led to a more discriminative outcome when the proteinaceous biofilm formed by strain 132 was analyzed (figure 1).

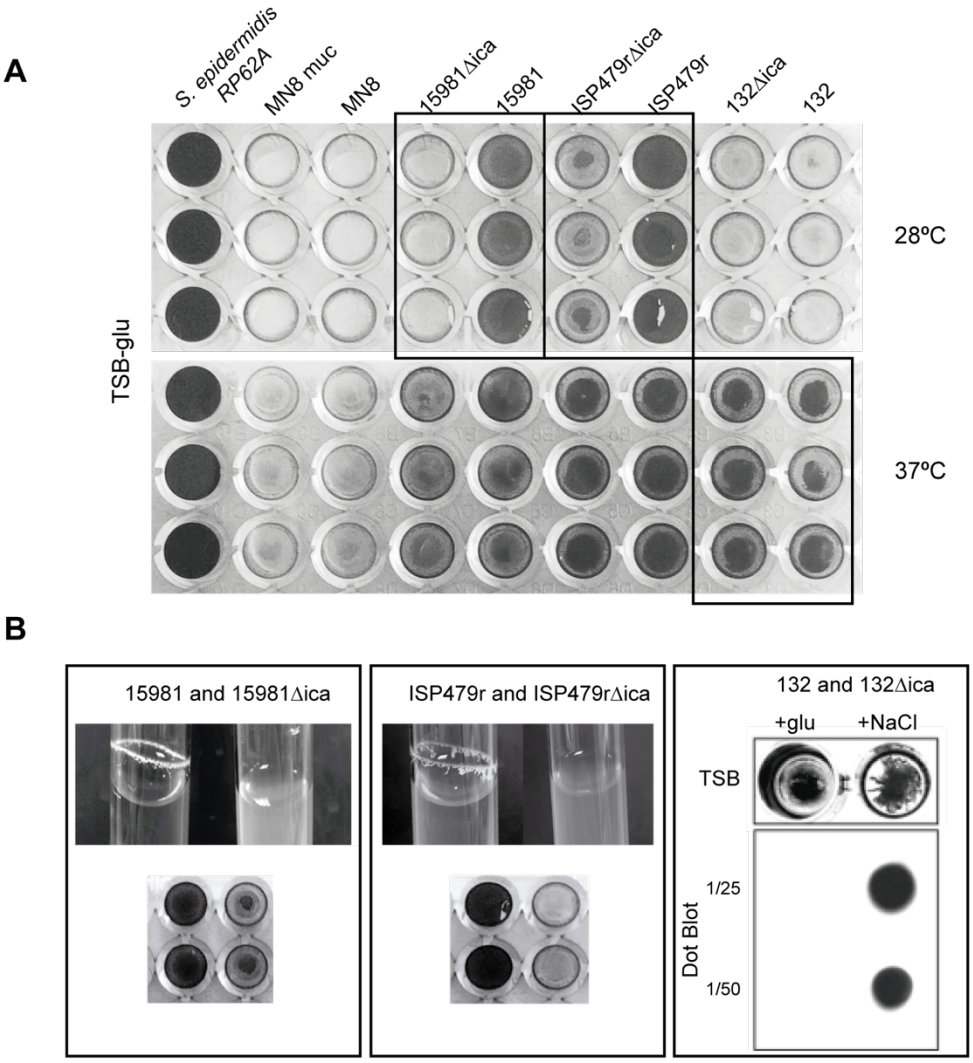

Figure 1. Anti-PIA/PNAG production screening platform. A) Quantification of the capacity of *S. aureus* and *S. epidermidis* strains to produce biofilm in 96-well polystyrene plates in TSB-glu at two different temperatures using crystal violet staining method. B) Biofilm phenotype in crystal tubes and 96-well plates shown by the three pairs of selected strains.

Screening and extract selection

In collaboration with the company Biomar, the ability of more than 60,000 crude extracts and 10,000 natural compounds to affect *S. aureus* 15981 was assessed. Attending to the specific inhibition of biofilm formation or growth of the wild type strain, a first sub-collection of 29 extracts was generated.

The inhibitory effect of the 29 extracts was newly tested using the three pairs of clinical strains (15981, ISP479r, 132, together with the *ica* mutant strains) at two different concentrations (40 and 400 ng/ml). This secondary screening enabled us to verify that the effect of the majority of selected extracts was rather variable and dependent on the genetic background. Furthermore, the sub collection of extracts tended to lead to a more tenuous outcome in this second screening round. In accordance to its anti-biofilm potential, five extracts were finally selected, four of them displaying an "orthodox" inhibitory effect on multicellular behaviour and one of them actually showing the desired specific antibiotic effect against the strains that produce PIA/PNAG (figure 2).

AA-AW-P-K005SPI: This extract exhibited a high anti-biofilm activity at the lower concentration, showing inhibition rates over the 50%. Curiously, this effect was not exhibited when bacteria were exposed to the high dose (400ng/ml), at which the biofilm formation was not reduced, or it was only decreased by a 20%. Bacterial growth, whether *ica* operon was present or absent, remained unaffected by the presence of this extract.

HT-16-50-AA02, AA-99-B-L020GMA and AA-99-K023: These extracts presented anti-biofilm activity at the high concentration (400ng/ml) without altering the biofilm formation at the low dose (40ng/ml). Bacterial growth, independently of PIA/PNAG synthesis was not inhibited by the presence of the extracts.

TA-15-A-A112CHV: This extract was especially interesting and fitted with our goal since it showed antibiotic activity, being such growth inhibition accentuated on those strains capable of forming a PIA/PNAG-depending biofilm.

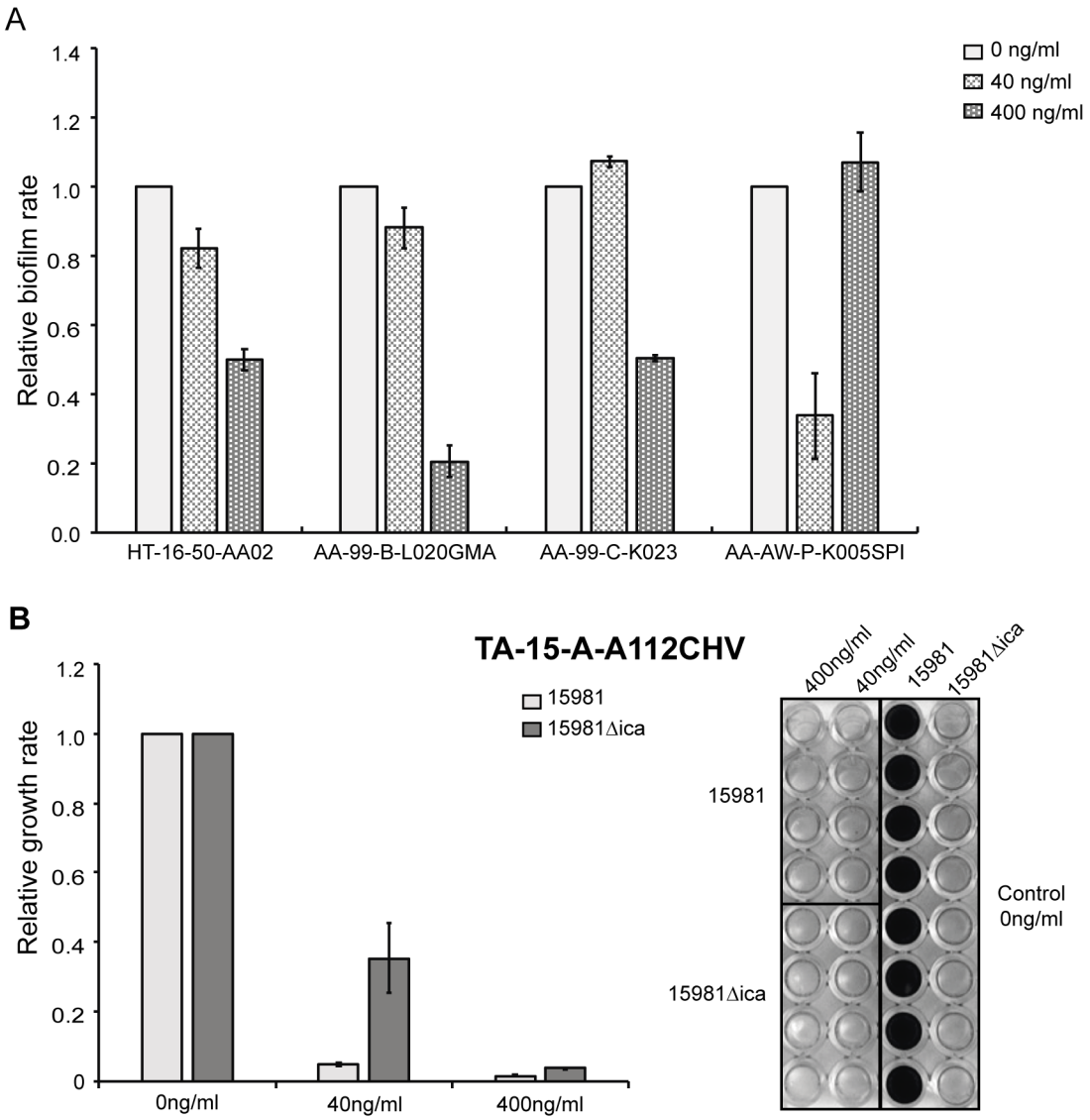

Figure 2. Secondary screening. A) Relative biofilm formation of *S. aureus* 15981 strain in the presence of selected extracts. B) Relative growth rate of *S. aureus* 15981 and 15981Δica strains in the presence of TA-15-A-A112CHV extract. Effect of this extract on biofilm formation is also illustrated.

Dose-response analysis

Dose-response assessment is a critical step when it comes to evaluate how viable the following processes of fractionation and molecule identification steps might be. To further characterize the behavior of the previously selected extracts in this regard, 15981 and its *ica* minus derivative strain were exposed to a wide range of extract concentrations. These experiments were carried out using microtiter plates and OD_{600} values were subsequently measured.

As a result, it was found that extracts referred to as AA-99-B-L020GMA, AA-99-C-K023 and TA-15-A-A112CHV provided a linear response (figures 3C, 3D and 3E). By contrast, in the case of the extract referred to as HT-16-50-AA02, it was noticeable that concentrations ranging from 4 to 32 ng ml^{-1} led to biofilm inhibition, even with 2-fold higher bacterial growth rates, but, unexpectedly, high dosages led to the opposite multicellular behaviour. Something similar was observed when different concentrations of AA-AW-P-K005SPI were tested; This extract displayed strong anti-biofilm activity from 4 to 32ng ml-1, but the effect was lost at higher concentrations (figure 3B). In view of these results, both HT-16-50-AA02 and AA-AW-P-K005SPI were discarded when further investigation was planned.

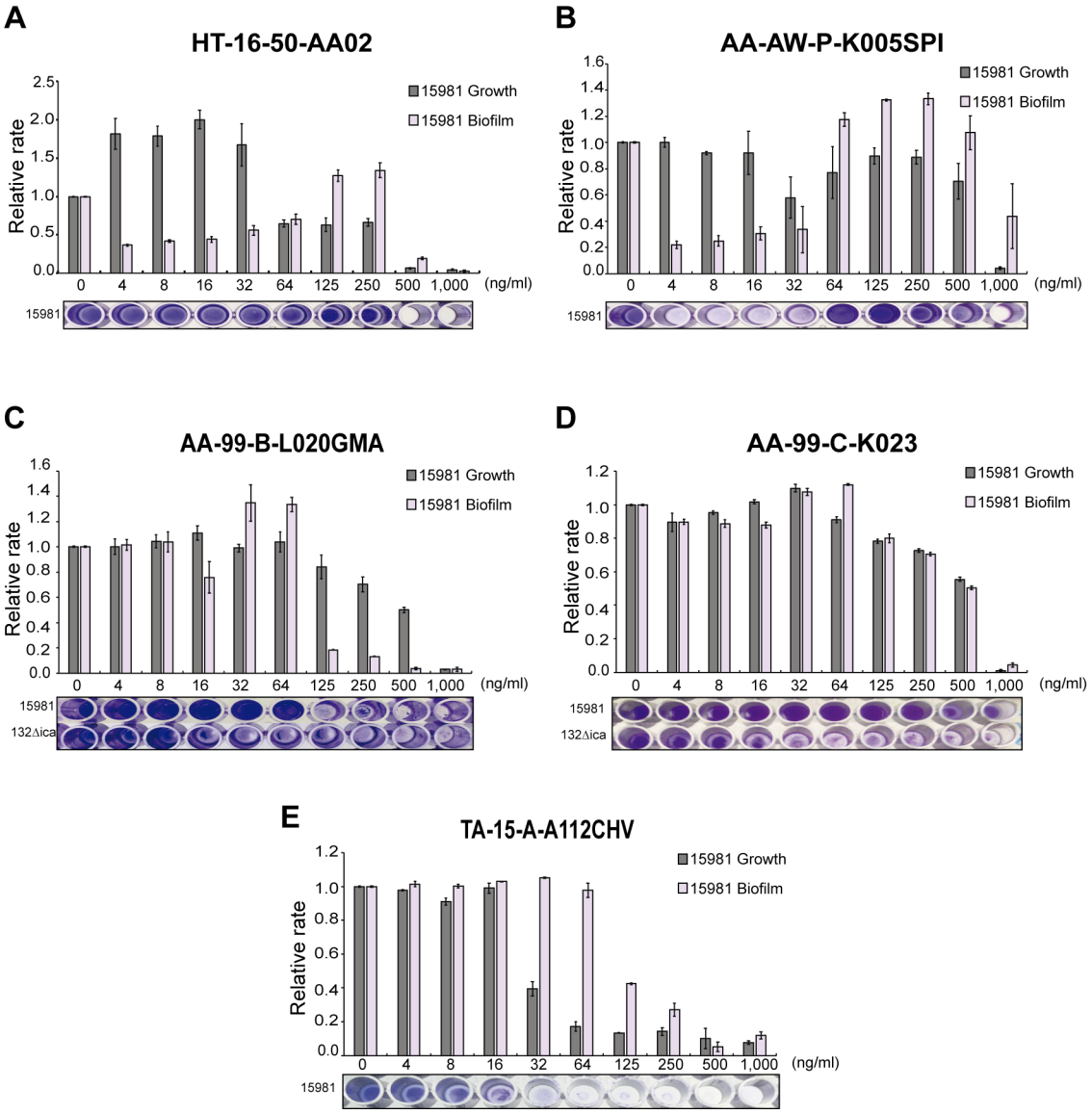

Figure 3. Dose response relationship. Graphics showing relative growth and biofilm rates of *S. aureus* 15981 strain in the presence of a concentration range up to 1000ng/ml of (A) HT-16-50-AA02, (B) AA-AW-P-K005SPI, (C) AA-99-B-L020GMA, (D) AA-99-C-K023 or (E) TA-15-A-A112CHV. Corresponding crystal violet staining of resulting biofilm on polystyrene wells is also shown below each graphic. In the case of AA-99-B-L020GMA and AA-99-C-K023 extracts, the effect on a strictly proteinaceous biofilm, formed by *S. aureus* 132Δica strain, is additionally indicated.

Batch reproducibility analysis

Once extracts referred to as HT-16-50-AA02 and AA-AW-P-K005SPI had been discarded due to lack of a dose-response relationship, the reproducibility between fermentation batches of AA-99-B-L020GMA, AA-99-C-K023 and TA-15-A-A112CHV extracts were analysed. To do so, new batches were obtained from independent fermentation processes at Biomar facilities and *S. aureus* 15981 strain was simultaneously cultured in the presence of the old and new batches of each extract.

Unfortunately, the concentration needed to get the same anti-biofilm activity level with the new AA-99-B-L020GMA extract was significantly higher when compared to the original extract (figure 4A) and, curiously, new AA-99-C-K023 batch showed no activity at all (figure 4B). Hence, both extracts were discarded due to the lack of reproducibility between batches.

TA-15-A-A112CHV was the only extract whose independent batches displayed the same effect and thus its different effect over PIA/PNAG positive and negative genetic backgrounds was further analysed. With such purpose, both *S. aureus* 15981 and its *ica* lacking derivative were simultaneously tested in the presence of a wide range of concentrations of the extracts using a microtiter-plate growth assay. As shown in figure 4C**,** doses ranging from 32 to 64 ng mL^{-1} caused a very significant inhibition of the wild type strain, while the *ica* minus derivative hardly suffered a slight growth arrest. These results suggest that extract TA-15-A-A112CHV exerts a specific antibiotic activity over PIA/PNAG producing cells.

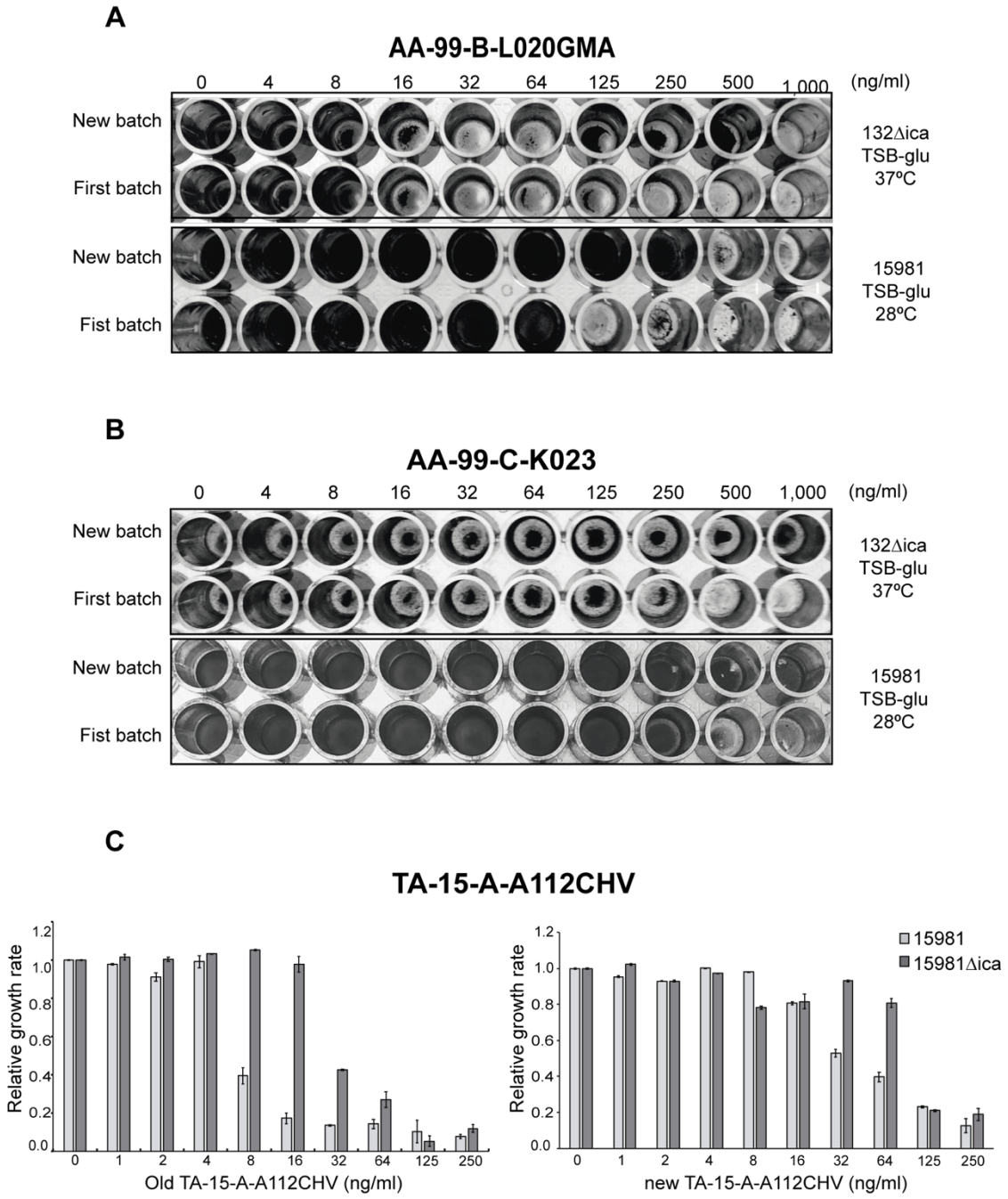

Figure 4. Analysis of fermentation batches reproducibility. Crystal violet staining of the biofilm formed by *S. aureus* 15981 and 132Δica strains in the presence of serial concentrations of two independent batches of (A) AA-99-B-L20GMA and (B) AA-99-C-K023 extracts is shown. (C) Growth rates exhibited by *S. aureus* 15981 (light grey bars) and 15981Δica (dark grey bars) strains in the presence of different concentrations of two independent batches of TA-15-A-A112CHV extract are plotted in graphics.

Characterization of TA-15-A-A112CHV

PIA/PNAG specificity:

In order to determine the extent of PIA/PNAG specificity of this extract, we took advantage of the ability of strain *S. aureus* 132 to alternatively form a protein or polysaccharide-based matrix, depending on environmental conditions. To do so, an ordinary microtiter-plate growth test was performed using glucose or salt-supplemented TSB medium, to which serial dilutions of TA-15-A-A112CHV were added. In the first case the biofilm matrix would be primarily constituted by proteins whether the second condition leads to PIA/PNAG production. As shown in figure 5, strain *S. aureus* 132 showed a higher degree of susceptibility to TA-15-A-A112CHV in comparison to that previously showed by *S. aureus* 15981 and, according to our hypothesis, doses ranging between 2 and 8 ng ml^{-1} had a significant inhibitory effect when conditions favoured the production of PIA/PNAG but hardly affected those bacteria forming a protein-dependent biofilm.

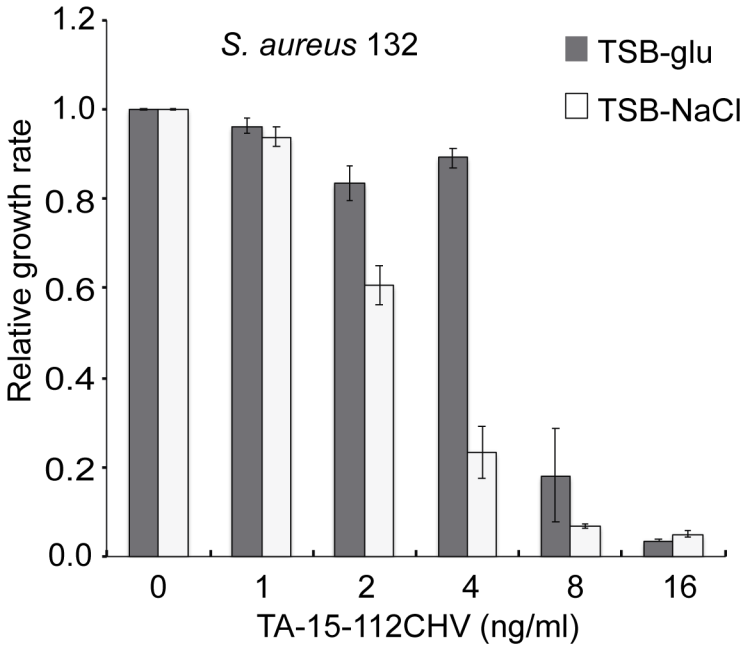

Figure 5. TA-15-A-A112CHV specificity over PIA/PNAG production. Growth rates exhibited by strain *S. aureus* 132 cultured in the presence of glucose, in order to obtain a proteinaceous matrix (grey bars), or salt, leading to a polysaccharidic biofilm (white bars), both in combination with TA-15-A-A112CHV extract at different concentrations is plotted in a graphic.

Fractions

As a step forward in TA-15-A-A112CHV characterization, the team of Biomar chemists provided us with 10 extract fractions, whose PIA/PNAG-selective antibiotic activity was tested once again in different concentrations using a microtiter-plate growth assay. Results shown in figure 6 clearly evidenced that fraction 11/12 (referred to as TA-15-A-A112CHV-F.11/12) contained the compound(s) responsible for the differential inhibitory effect.

Upon selecting TA-15-A-A112CHV-F.11/12 as the active fraction, an additional extraction step was carried out at Biomar facilities and 10 sub-fractions (referred to as TA-15-A-A112CHV-F.11/12.SF1 to TA-15-A-A112CHV-F.11/12.SF10) were analysed applying the same methodology as described before. As a result, we obtained two sub-fractions (referred to as TA-15-A-A112CHV-F.9/10.SF7 to TA-15-A-A112CHV-F.9/10.SF8) capable of exerting the PIA/PNAG-specific antibiotic effect. As shown in figure 6, SF8 displayed lower PIA/PNAG-differential MIC values, suggesting that this was actually the fraction that contained the active compound(s) or a higher concentration of it(them). In view of such an outcome, this fraction was newly analyzed through FAB mass spectrometry, obtaining three potential active compounds: (I) Soyasaponin, (II) Lumichrome and (III) Malayamicin. However, to our surprise, none of three compounds, under the conditions tested and whether alone or in different combinatory formulations, were capable to reproduce the effect exerted by SF8.

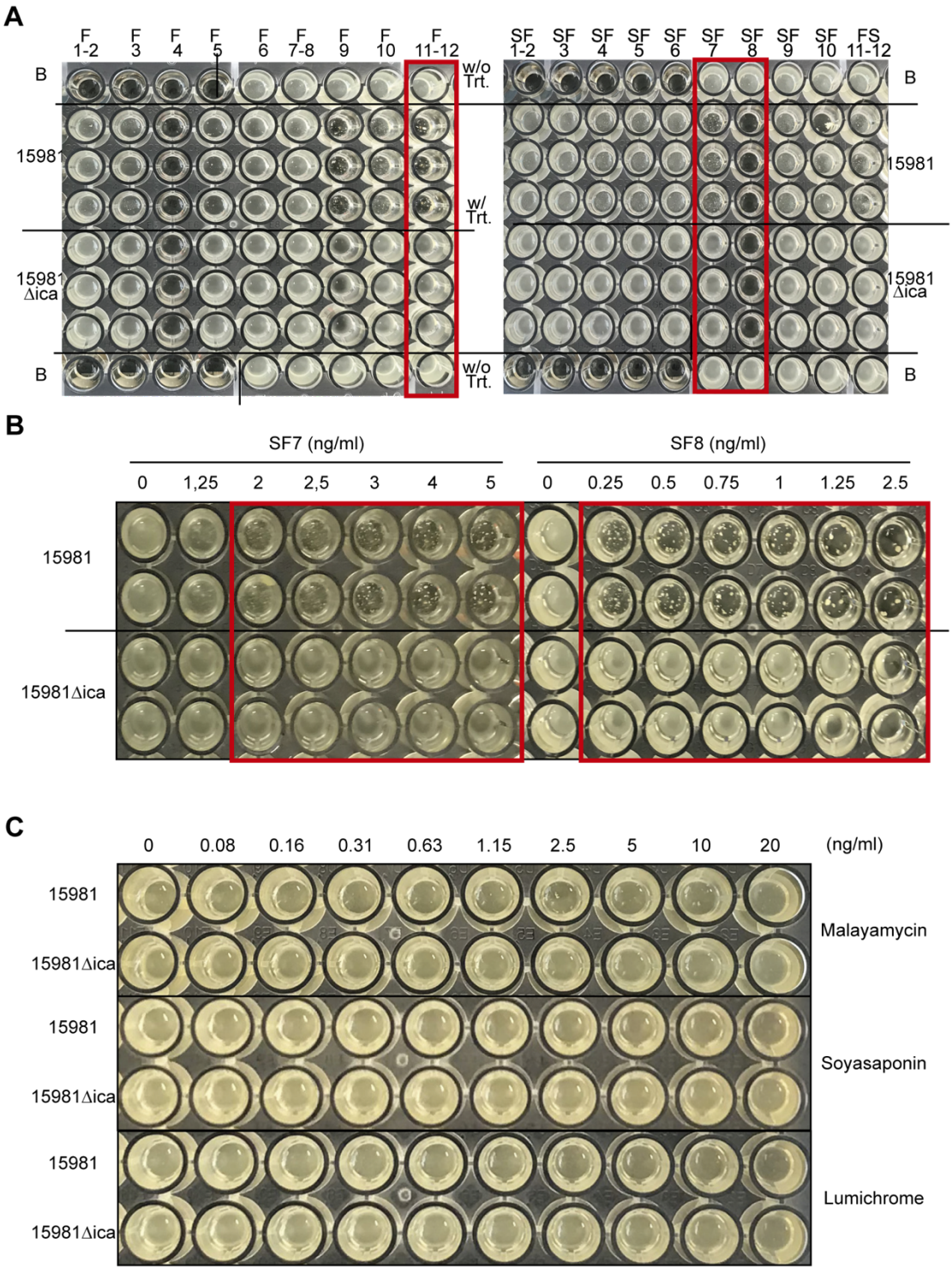

Figure 6. Fraction and sub-fraction screening. (A) Pictures of 96 well plates showing anti-PIA/PNAG activity of different TA-15-A-A112CHV fractions (left) and subfractions (right). Further analysis with (B) sub-fractions 7 and 8 and (C) compounds Malayamicinm, Soyasaponin and Lumichrome at different concentrations is also shown using the same layout.

Preliminary approach for understanding the molecular mechanisms underlaying PIA/PNAG-specific lethal effects

In order to understand how PIA/PNAG synthesis machinery was responding to the presence of TA-15-A-A112CHV-F.9/10.SF8, both transcriptional and translational activities of *ica* operon were analyzed. Transcriptional characterization was carried out using a *S. aureus* 15981 strain derivative, previously transformed with a plasmid that harboured an *ica*A *p*romoter-*lac*Z transcriptional fusion. *S. aureus* 15981 Δ*ica* strain that contained the same reporter plasmid was used as a negative control. Curiously, measurements of beta galactosidase activity in the absence and presence of a sub-inhibitory concentration of SF8 did not differ significantly, while addition of Soyasaponin and Lumichrome resulted in a slight increased transcriptional activity of the operon responsible for PIA/PNAG synthesis (figure 7A).

On the other hand, translation of IcaC protein was assessed using the *S. aureus* 15981 wild type and *ica* minus derivative strains whose IcaC protein had been tagged with a 3XFlag epitope at its carboxi-terminal region. When these strains were grown in the presence of SF8 or its constituent compounds, the post-transcriptional effect of SF8 became evident, since IcaC protein was hardly detected via western blotting. Soyasaponin had a much less clear effect on icaC translation, while lumichrome seemed to lack any effect at this level. Nevertheless, this experiment allowed us to detect that SF8 makes the band corresponding to protein A disappear, but only in the case of the *ica* positive genetic background. Apart from protein A, and also restricted to the case of the strain capable of synthesizing PIA/PNAG, the protein profile analysed by coomassie staining obtained after the exposure to SF8 also lacked some high-molecular-weight proteins (figure 7).

Thus, these results suggest that SF8, but not its constituent compounds individually, exerts a post transcriptional inhibitory effect on *ica* operon expression, while is also capable of inhibiting the expression of Protein A and some other high molecular weight proteins.

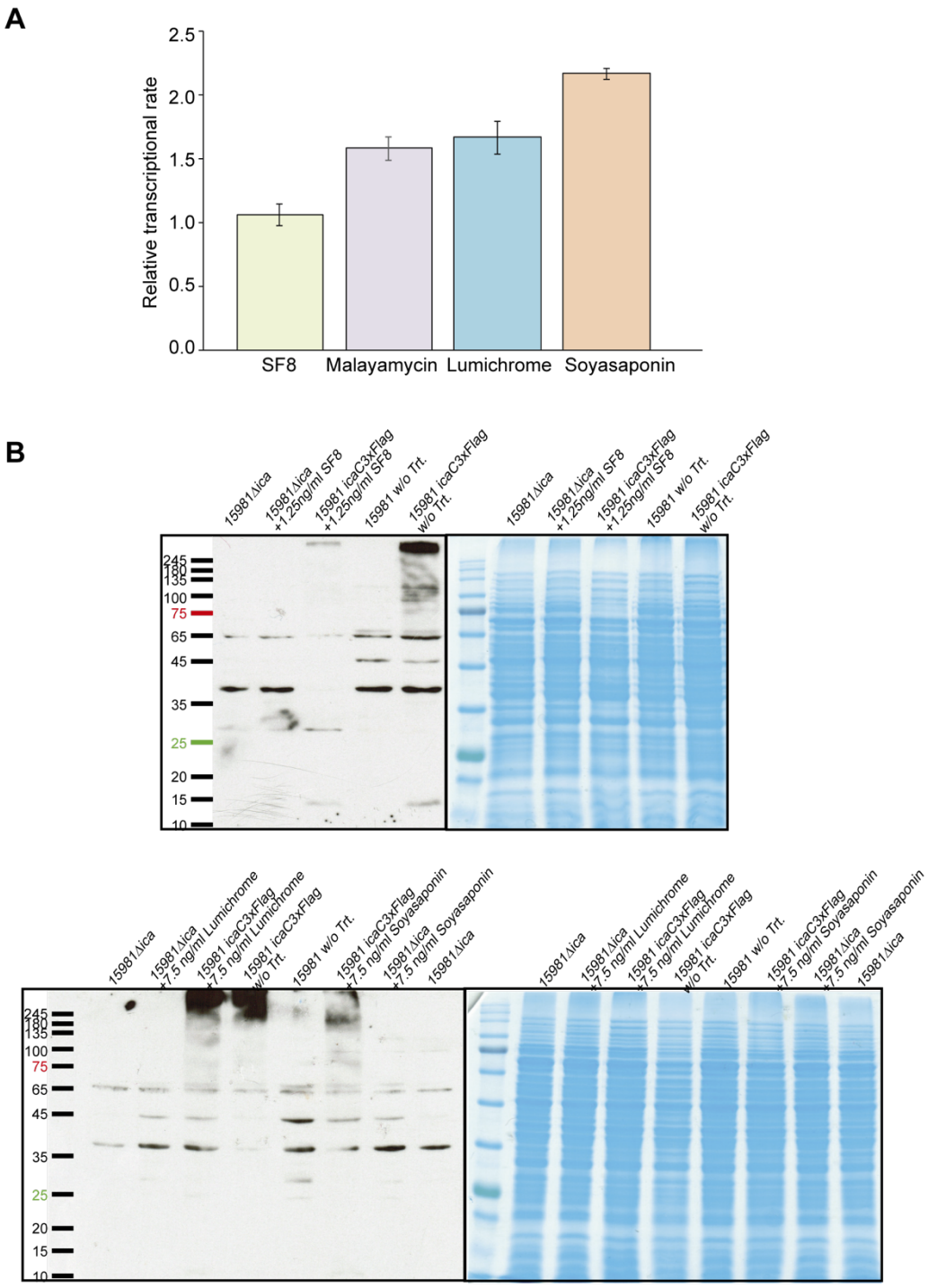

Figure 7. Transcriptional and translational activity of *icaADBC* operon. (A) Relative transcriptional of *icaA* gene and (B) IcaC protein expression in *S. aureus* 15981 strain grown in the presence of SF8 at 1,25 ng/ml or Malayamicin, Lumichrome and Soyasaponin at 7,25 ng/ml is presented. Transcriptional activities are plotted in a graphic in which beta galactosidase values given by a *ica-promoter*: *lacZ* fusion have been relativized to those showed by the wild type strain grown in the absence of any sub-fraction or compound. IcaC expression is illustrated via western blot images. Corresponding protein profiles stained with coomassie blue are also shown.

Discusion

When it comes to anti-biofilm drug discovery, two main premises must be taken into account. First, the multiplicity of methods that can be used for studying biofilm growth might make difficult to choose the most reliable one; on the other hand, ordinary inhibition-based screenings enable rapid identification of the new drug candidates. However, this method might not be the most appropriate for particular *in vitro* growth pattern, such as the anchored or steady-state status found in biofilm matrixes (Jaśkiewicz et al., 2019).

With the aim of somehow overcoming these limitations and trying a different approach, we decided to focus on the contradictory nature of PIA/PNAG polysaccharide synthesis. Beyond doubt is the fact that this major component of the staphylococcal biofilm mediates virulence both through its contribution to matrix assembly and immune evasion. This is probably the main reason why evolutionary forces have definitively favoured the conservation and inter-species spread of the locus encoding it. However, evidence suggests that while PIA/PNAG might protect bacteria from the immune system under certain circumstances, it might also be the target for an effective immune response (Brooks & Jefferson, 2014; Cerca et al., 2007; Maira-Litrán et al., 2012). In the same context, it has been shown that overproduction of the polysaccharide can entail a significative fitness cost and it is actually not infrequent to isolate PIA/PNAG negative revertant strains of *S. aureus* and *S. epidermidis* from patients (Brooks & Jefferson, 2014; Juárez–Verdayes, Rodríguez–Martínez, Cancino–Diaz, & Cancino–Diaz, 2013; Martin-Lopez et al., 2002). Behind all these phenomena there is a finely orchestrated regulatory system that includes repressors like IcaR, CodY or Sigma B and activators such as SarA or GraRS (Jefferson, Cramton, Götz, & Pier, 2003; Majerczyk et al., 2008; Merino et al., 2009; Valle, Echeverz, & Lasa, 2019; Valle et al., 2003) and even reversible mechanisms for truncation of *ica* operon mediated by the insertion of sequence element IS256 into *icaA* or *icaC* genes (Loessner, Dietrich, Dittrich, Hacker, &

Ziebuhr, 2002), or the Rec-A independent addition or subtraction of non-multiples of three repeat shifts in the *icaC* ORF (Brooks & Jefferson, 2014). This extreme regulatory scenario has always prompted us to think that PIA/PNAG synthesis carries a “weakness” connotation, which could really be exploited to combat biofilm-associated infections.

S. aureus 15981 and ISPr strains have been extensively used as models for staphylococcal virulence, their multicellular behaviour is well characterized, and their sequence is available. *S. aureus* 132 is also a “veteran” strain, included in this platform due to its particular bivalent multicellular behaviour, which allows to differentiate those effects on protein-based biofilm from those dependent on polysaccharide production (Vergara-Irigaray, Valle, Merino, Latasa, Garcia, Ruiz de los Mozos, et al., 2009).

Why use marine natural extracts and compounds for the screening? While it is true that antimicrobial compounds from marine sources have not yet been developed into clinical trial phases, there are plenty of studies underscoring the great potential of marine microorganisms, especially those adaptable to extreme conditions like deep seabed, for the production of novel metabolites and anti-infectives like polyketides or cyclic peptides (Pereira et al., 2020). Thanks to a collaboration with the Spanish company Biomar, their AquaE and AquaC collections, containing more thant 1,000 compounds and 40,000 extracts respectively, were tested against *S. aureus* 15981 strain and its *ica* minus derivative.

As a result, from the first screening, 29 extracts were classified because showed potential for exerting a differential inhibition on PIA/PNAG positive strains. It was kind of disappointing, though not unexpected, the result obtained when these extracts were newly tested using the whole set of strains, since most of them showed a tenuous effect, which was also dependent on the genetic background. Upon applying this second screening step, five extracts were selected, two of which were additionally discarded due to the lack of dose-response relationship. When batch reproducibility for the three remaining extracts

was analyzed, only the new fermentation batch leading to extract TA-15-A-A112CHV showed a consistent outcome. The BIOMAR team of chemical experts performed several TA-15-A-A112CHV fractionation and sub-fractionation steps, which allow us tu find out the SF8 from F9/10, which was capable of showing the inhibitory effect on PIA/PNAG positive strains growth without affecting the *ica* negative counterpart.

Mass spectrometry results indicated that SF8 was indeed composed by three compounds: Malayamicin, Lumichrome and Soyasaponin. To our knowledge, there is no available information about the effect of Malayamicin on microorganisms, except for a patent concerning the biocidal compounds Malayamicin and its isomer desmethylmalayamycin A, which show inhibitory effects on different fungi, virus and cancerous cells (https://patentscope.wipo.int/search/es/detail.jsfdocId=MX124710&tab=NATIONALBIBLIO).

Lumichrome instead, is a riboflavin derivative which has been studied due to its capacity to behave as a signal for plant growth (Dakora, Matiru, & Kanu, 2015) or activate LasR bacterial quorum sensing receptor (Rajamani et al., 2008). This molecule is also considered as a potential antibacterial agent due its photosensitizing effects (Bergh, Bruzell, Hegge, & Tønnesen, 2015; Martins et al., 2008) but especially interesting is the recent work describing an approach very similar to ours in which lumichrome showed strong inhibitory activity against *Staphylococcus aureus* Sortase A, a transpeptidase responsible for anchoring surface proteins to the peptidoglycan cell wall, without affecting cell viability (B. Chung, Kwon, Shin, & Oh, 2019). With regard to soyasaponin, this substance has been shown to exert synergistic effects on the antimicrobial activity of β-lactam antibiotics against β-lactamase-producing *Staphylococcus aureus* strains (Horie, Chiba, & Wada, 2018).

Unfortunately, the desired biofilm-specific effect displayed by SF8 did not seem to rely on a unique compound, since, under the conditions tested and neither alone nor in different combinatory formulations, any of them was capable

to reproduce the effect exerted by SF8. This results certainly opens up a more complicated scene for developing a clinically viable formulation. However, and with the aim of getting some knowledge about how it would be possible to reinforce the bacterial weakness associated to PIA/PNAG production, we thought it was worth analyzing the potential molecular mechanisms underlaying the observed effect. When transcriptional and translational effects on *ica* operon were tested, it became evident that SF8 was acting at a postranscriptional level, since bands corresponding to Ica protein complex practically disappeared from blot images. These same western blot assays offered an even more intriguing result, revealing that SF8 inhibits the expression of protein A and the activity of Ica operon is actually required for obtaining such effect. Protein A is a very common and relevant surface protein that binds Immunoglobulin G and simplifies bacterial transmission, allowing it to take hold of the host in a shorter space of time (Falugi, Kim, Missiakas, & Schneewind, 2013; Winstel, Missiakas, & Schneewind, 2018). Indeed, when the effect of lumichrome was assessed individually through western blot, a slight inhibitory effect on protein A was obtained, reinforcing the previously mentioned observation made by Chung and colleagues (B. Chung et al., 2019). Apart from protein A, some other high molecular weight proteins were inhibited by SF8 exclusively in the *ica* positive genetic background, as stated by the protein profiles analysis via coomassie staining. These evidences have raised some crucial questions. Are those additionally inhibited proteins members of the LPXTG family? How is the inhibition of LPXTG proteins connected with Ica operon? In this regard, and since all post transcriptional silencing mechanisms depend on IcaC protein, would it be possible that accumulation of the other proteins encoded within the *ica* locus had an extra function and could mediate the effect exerted by SF8? This hypothesis has already been proposed by L. Brooks and K. Jeffersson (Brooks & Jefferson, 2014). Up to date, we have no certain answers for such questions but studies to determine genome sequences of SF8-resistant clones are underway. Hopefully, though our work might not end up, or at least in a short-term basis, in a

pharmaceutical formulation, elucidation of mechanisms underlaying PIA/PNAG collateral effects could pave the way to novel antimicrobial and antibiofilm therapies.

Experimental procedures

Bacterial strains, plasmids and culture media

Bacterial strains and plasmids are listed in table 1 and table 2 respectively. *Escherichia coli* XL1blue strain was grown in LB broth and agar (Scharlau). *Staphylococcus aureus* strains were grown in trypticase soy broth (TSB) (VWR Chemicals), trypticase soy agar (TSA), trypticase soy broth with 0.25% of glucose (TSB-glu), Mueller Hinton (MH) (Pronadisa) and B2 medium (1% casein hydrolysate, 2.5% yeast extract, 2.5% NaCl, 0.1% K_2HPO_4, and 0.5% glucose [w/v]). When required for growth or selection, medium was supplemented with the appropriate antibiotic at the following concentrations: ampicillin (Am) 100 µg ml^{-1}, chloramphenicol (Clo) 10 µg ml^{-1} and 20 µg ml^{-1}.

DNA manipulations

Plasmids were isolated using NucleoSpin® Plasmid kit (Macherey-Nagel) according to the manufacturer protocols.

Plasmids were transformed into *Escherichia coli* XL1Blue strain by electroporation and then introduced first into the restriction-deficient *Staphylococcus aureus* strain RN4220 using a previously described protocol (Lee, 1995), and transferred to other strains also by electroporation. *S. aureus* electro-competent cells were produced as described before (Schenk and Laddaga, 1992).

Compounds and extracts libraries

The compounds and extracts tested belongs to two libraries of the Spanish company Biomar. The first one is composed of 1,000 compounds obtained through the fermentation of marine microorganisms and by isolation of the pure compound. This library contains several natural products that are also presented in other libraries. However, the marine origin and the high taxonomic dereplication degree implemented for the microorganism selection, leads the presence of at least 25% of compounds that could be considered as new products. The second library consists in more than 60,000 crude extracts. These extracts are generated

through the fermentation of marine microorganism in specific cultures designed by the company to promote the production of secondary metabolites, that are extracted in a cocktail of organic solvents developed to extract compound with a wide range of polarity.

PIA/PNAG-dependent biofilm quantification assay

The PIA/PNAG-depending biofilm-forming capacity was tested in microtiter wells as previously described (Heilmann *et al.*, 1996).

Briefly, *S. aureus* strains (PIA/PNAG producer and PIA/PNAG defective strains simultaneously) were cultivated overnight in TSB-glu at 28ºC. The culture was diluted 1:40 in TSB-glu and supplemented with the appropriate crude extract or purified compound at the following concentration: extracts at 40 ng ml^{-1} and 400 ng ml^{-1} or compounds at 0.05 ng ml^{-1}, 0.5 ng ml^{-1} and 5 ng ml^{-1}. 96-well polystyrene microtiter plates (BioLite Thermo Scientific) were inoculated with 200 µl of the previously prepared cell suspensions and the plates were incubated for 36 h at 28ºC. After the cultivation, to quantify the growth, the optical density at 650nm (OD_{650nm}) was determined using a microplate reader (MultiSkan GO Thermo Scientific). Then, the wells were gently washed twice with water and air-dried. The remaining surface absorbed cells or the individual wells were stained with crystal violet for 5 min at room temperature. Next, the microtiter plates were rinsed again twice with water, dried in an inverted position and photographed. To quantify the biofilm formed, the crystal violet-stained cells were resuspended in 200 µl of ethanol-acetone (80:20 v/v) solution and the optical density at 595nm (OD_{595nm}) was determined. Each assay was performed in triplicate and repeated at least three times.

PIA/PNAG-independent biofilm quantification assay

For quantification PIA/PNAG-independent biofilm-forming capacity, a test similar to that described for PIA/PNAG-dependent biofilm was used. Sterile 96-well polystyrene microtiter plates from the same manufacturer (BioLite Thermo Scientific) were inoculated with 200 µl of an overnight culture (37 ºC in TSB-glu)

diluted 1: 40 in TSB-glu supplemented with the appropriate extract or compound at the identical concentrations. The plates were cultivated 24 h at 37 ºC, and after the incubation the process was performed analogous to the process carried out for the PIA/PNAG-dependent assay. Each assay was performed in triplicate and repeated at least three times.

Doses-response assay

To confirm and to determine the potential of selected extracts and compounds (from the primary screening), chosen drugs were serially diluted from 1,000 ng ml^{-1} to 0 ng ml^{-1} into triplicate rows, and assayed with *S. aureus* 15981 and 15981Δica strains by the already described PIA/PNAG-dependent biofilm quantification assay.

β-galactosidase assay

To quantify *icaADBC* operon translation level overnight cultures were diluted 1:40 in TSB-glu supplemented with the appropriate concentration of each extract. 12-well plates were inoculated with 2 ml of cells suspension and incubated for 36 h at 28 ºC. *S aureus* strains were harvested by centrifuging 2ml of culture samples (2 min 20,000 g). Cells were resuspended in 1 ml of Z buffer (Jeffrey H. Miller, 1972) with 100 μl of chloroform and 50 μl of 0.1 % of SDS, and lysed by incubation at 28 ºC for 5 min. Assay was performed as previously described and β-galactosidase specific activity was expressed as Miller units OD_{650nm}^{-1} or as Miller units g of $cells^{-1}$ (Jeffrey H. Miller, 1972). Briefly, to initiate the reaction 200 µl of 4 mg ml^{-1} *ortho*-Nitrophenyl-β-galactoside (ONPG) was added to each sample, and when a faint yellow was observed the reaction was stopped adding 500 µl of 1M Na_2CO_3. Miller units were calculated as described previously attending to OD_{600nm}, OD_{420nm}, OD_{550nm} and reaction time measurements (Li *et al.*, 2012). All experiments were carried out in triplicate.

SDS-PAGE and Western Blotting

For detection of *icaC* translate level after the incubation with the extract or compound, an overnight culture of the 15981 icaC3xFlag strains was diluted 1:40

in TSB-glu supplemented with the appropriate concentration of extract or compound and incubated for 36 h at 28 ºC in 12-well plates (Multiwell 12-Well FALCON). Two ml of bacterial cultures were centrifuged at 10,000 g for 1 min, pellets were washed twice with PBS and resuspended in 100 μl of lysis buffer (50mM Tris HCl pH 7.4, 250mM NaCl, 0.5% Triton X-100, 10% glycerol, 1mM DTT, PMSF) with 2 μl of Lysostaphin 1 mg ml^{-1} (Sigma) and 3 μl of Nuclease (Pierce) and incubated during 2 h at 37 ºC. After the lysis step, the amount of protein was determined by BCA method (BCA Thermo Scientific) according to the manufacture's protocol. Protein concentration of different samples was equalized by adding phosphate-buffered saline (PBS). The protein samples were mixed with 1 volume of Laemmli buffer and denatured by boiled at 95ºC for 5min. The samples were electrophoresed in 12 % sodium dodecyl sulphate (SDS)-polyacrylamide duplicated gels at 120 V for 2 h.

One of each gel was stained with Coomassie (Gel Code™ Blue safe protein stain Thermo Scientific) as loading control. The other was transferred onto Hybridization Nitrocellulose membrane (Millipore) by semi-dry electroblotting. Membranes were blocked overnight with 5 % skimmed milk in PBS containing 0.1 % of Tween 20 (Fisherbrand) and incubated with anti-FLAG antibodies labelled with phosphatase alkaline (Sigma) diluted 1:500 for 2 h at room temperature. 3XFLAG labelled IcaC protein was detected with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) following the manufacture's recommendations.

PIA/PNAG quantification

Cell surface PIA/PNAG exopolysaccharide levels were quantified as previously described (Cramton *et al.*, 1999; Mozos *et al.*, 2013). Briefly, overnight cultures of the strains tested were diluted 1:40 in TSB-glu supplemented with the appropriate concentration of extract and 2 ml of these cell suspensions were used to inoculate sterile 12-well polystyrene microtiter plates (FALCON). After 36 h of static incubation at 28 ºC, the same number of cells of each strain was

resuspended in 50μl of 0.5 M EDTA (pH 8.0). Then, cells were incubated for 5 min at 100 ºC and centrifuged 17,000 g for 5 min. Each supernatant was incubated with 10 μl of proteinase K at 20 mg ml^{-1} (Thermo Scientific) for 30 min at 37 ºC. Next, 10 μl of Tris-buffered saline (20mM Tris-HCl, 150mM NaCl [pH7.4]) containing 0.01 % of bromophenol blue were added to each sample, and 5 μl of each samples were spotted on a nitrocellulose membrane using a Bio-Dot microfiltration apparatus (Bio-Rad). The membrane was blocked overnight with 5 % skimmed milk in PBS with 0.1 % of Tween 20, and incubated for 2 h with specific anti-PNAG antibodies diluted 1:10,000 (Maira-Litran *et al.*, 2005). Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G antibodies (Jackson ImmunoResearch Laboratories, Inc., West-grove, PA) diluted 1:10,000 and developed using the SuperSignal West Pico Chemiluminescence Substrate (Thermo Scientific).

Table 1. Strains used in this study.

Strain	Characteristics	Reference
Escherichia coli		
Xl1Blue	Cloning assay cell	(Stratagene)
Staphylococcus aureus		
RN4220	A mutant of S. aureus strain 8325-4 that accepts foreign DNA	(Novick, 1990)
15981	Clinical strain; biofilm positive; *rsb*U+	(Valle *et al.*, 2003)
15981 Δica	15981 with deletion of the *icaADBC* operon	(Toledo-arana *et al.*, 2005)
ISP479r	ISP479 with *rsbU* restored	(Toledo-arana *et al.*, 2005)
ISP479r Δica	ISP479r with deletion of the *icaADBC* operon	
132	MRSA clinical strain; biofilm positive	(Vergara-Irigaray, Valle, Merino, Latasa, Garcia, Ruiz de los Mozos, *et al.*, 2009)
132 Δica	132 with deletion of the *icaADBC* operon	(Vergara-Irigaray, Valle, Merino, Latasa, Garcia, Ruiz de los Mozos, *et al.*, 2009)
MN8	Clinical strain.	(Schlievert *et al.*, 1982)
MN8 muc	Spontaneous mutant of MN8	(McKenney, 1999)
15981 icaC3xFlag	15981 strain carrying 3xFlag tag epitope at *icaC*	(Vergara, 2009)
15981 PicaA53	15981 strain carrying the pSA14::PicaA53 plasmid	(Mozos *et al.*, 2013)
15981Δica PicaA53	15981 Δica strain carrying the pSA14::PicaA53 plasmid	This study
Staphylococcus epidermidis		
RP62A		

Table 2. Plasmids used in this study.

Plasmid		
pSA14	Plasmid used for transcriptional fusions with *E. coli lacZ*	(Falord *et al.*, 2011)
pSA14::PicaA53	pSA14 containing *icaA* promoter	(Mozos *et al.*, 2013)

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CHAPTER II.

Inhibiting the Two-Component System GraXRS with Verteporfin to combat *Staphylococcus aureus* infections

Abstract

Infections caused by *Staphylococcus aureus* pose a serious and sometimes fatal health issue. With the aim of exploring a novel therapeutic approach, we chose GraXRS, a Two-Component System (TCS) that determines bacterial resilience against host innate immune barriers, as an alternative target to disarm *S. aureus*. Following a drug repurposing methodology, and taking advantage of a singular staphylococcal strain that lacks the whole TCS machinery but the target one, we screened 1.280 off-patent FDA-approved drug for GraXRS inhibition. Reinforcing the connection between this signaling pathway and redox sensing, we found that antioxidant and redox-active molecules were capable of reducing the expression of the GraXRS regulon. Among all the compounds, verteporfin (VER) was really efficient in enhancing PMN-mediated bacterial killing, while topical administration of such drug in a murine model of surgical wound infection significantly reduced the bacterial load. Experiments relying on the chemical mimicry existing between VER and heme group suggest that redox active residue C227 of GraS participates in the inhibition exerted by this FDA-approved drug. Based on these results, we propose VER as a promising candidate for sensitizing *S. aureus* that could be helpful to combat persistent or antibiotic-resistant infections.

Introduction

Though the undeniable efficiency of anti-infective measures like vaccines and antimicrobials have made us believe that infectious diseases are nowadays under control, nothing could be further from the truth. An increasing number of studies alert that unless actions are taken, infections caused by antibiotic-resistant bacteria will kill an extra 10 million people a year worldwide by 2050 (Kelly and Davies, 2017).

Staphylococcus aureus is one of the bacterial species whose manage is especially challenging due to the emergence of methicillin-resistant (MRSA) strains. With a population-weighted mean of invasive MRSA strains of about 17% in terms of European prevalence (Rasigade *et al.*, 2014), the major health care concern related to MRSA incidence lies in the limitations of currently approved treatments, which, in turn, leads to high rates of morbidity and mortality even in industrialized nations.

Despite of being an inoffensive colonizer of the nasal epithelium of one-third of the general population (Wertheim *et al.*, 2005), *Staphylococcus aureus* might become a dangerous life-threatening pathogen when it defeats host immune system, crosses the epithelial barrier and get access to deeper tissues like blood, dermis, gastrointestinal tract, heart valves or bones (Wertheim *et al.*, 2005; Balasubramanian *et al.*, 2017). This biological versatility is based on a highly orchestrated regulation of circuits that sense a plethora of environmental signals and modulate gene expression for fine tuning crucial traits like cell-wall structure, biofilm formation or resistance to antibiotics. The core feature of such circuits is the two-component-signaling transduction system (TCS) (Stock *et al.*, 2000), which is actually one of the most conserved and effective mechanisms in nature for coupling external stimuli and gene expression. In its most basic form, a canonical TCS normally consists of a membrane-bound histidine kinase and a cytosolic response regulator that, once phosphorylated, elicit appropriate

changes in the cell by regulating gene expression, protein interactions, or enzymatic activity (Capra and Laub, 2012).

Over the last decade, the scientific community has gained in-depth knowledge of the genes affected by specific staphylococcal TCSs, giving rise to a vast body of bibliography and information about mutants in the respective sensor kinases, response regulators and auxiliary genes. Thus, the pivotal role of AgrCA and SaeRS TCSs on virulence gene expression or the involvement of BraRS and GraXRS in antibiotic resistance has been studied in great detail (Kawada-Matsuo *et al.*, 2011; Falord *et al.*, 2011; Boyle-Vavra *et al.*, 2013; Haag and Bagnoli, 2015). Furthermore, since TCSs are a matter of life and death to bacteria and they are not present in host´s cells, these regulatory pathways have always been listed as promising antibacterial targets. Precisely based on the assumption that solution to present-day therapeutic limitations might somehow lie on impairing the way *Staphylococcus* senses and integrates environmental stimuli, significant effort in the form of ambitious High Throughput Screenings (HTSs) and Structure-Based Virtual Screenings (SBVSs) has been done to find new molecules with inhibitory effects on staphylococcal sensor kinases (Bem *et al.*, 2015). However, with few exceptions such as the molecule named walkmycin B (Okada *et al.*, 2010), biochemical screens normally identify a high number of compounds acting through nonspecific inhibitory mechanisms and thus render non-viable drugs in terms of clinical application (Hilliard *et al.*, 1999; Gotoh *et al.*, 2010; Bem *et al.*, 2015).

With the aim of designing a whole-cell drug discovery tool that could complement *in silico* docking and crystallographic analysis of kinase-ligands structure in TCS-targeting approaches, we decided to explore the potential of a recently developed staphylococcal strain that lacks the whole non-essential TCS machinery (ΔXV strain) (Villanueva *et al.*, 2018). Among all the TCSs whose individual contribution to a specific *S. aureus* phenotype has been defined, and based on its overall responsibility for resistance to host defenses like

polymorphonuclear cells (PMNs) or cationic antimicrobial peptides, we selected GraXRS as a candidate of therapeutic target (Kraus *et al.*, 2008; Falord *et al.*, 2011; Yang *et al.*, 2012; Cheung *et al.*, 2014; Chaili *et al.*, 2016). Additional evidence supporting our choice was given by a recent work showing that *S. aureus* uses this regulatory system to sense and adapt to the acidified phagolysosome in macrophages (Flannagan *et al.*, 2018), but also by several previous studies unveiling the potential of GraXRS to impact the bacterial capacity to colonize and survive on aortic valves in a rabbit endocarditis infection model (Cheung *et al.*, 2014) or to play a crucial function in a murine model of systemic infection (Kraus *et al.*, 2008). Most of these preceding articles conclude that mechanisms underlying GraXRS activity are related with changes in bacterial surface charge via its target downstream gene *mpr*F and the operon *dlt*ABCD (Falord *et al.*, 2011; Yang *et al.*, 2012; Muzamal *et al.*, 2014; Cheung *et al.*, 2014).

In this study, we have used the *S. aureus* strain deprived of fifteen TCS (Villanueva *et al.*, 2018) and isogenic derivatives containing exclusively the GraXRS TCS as a whole-cell platform to identify drugs that specifically target this signaling pathway. Upon evaluating the GraXRS-blocking activity of 1280 FDA-approved off-patent drugs, we found that molecules with antioxidant activity as acetylsalicylic acid, ascorbic acid, the porphyrin derivative verteporfin, or the flavonoid hesperidin, are capable of inhibiting the activity of GraXRS-dependent promoters. Among all the compounds, only verteporfin made a significant contribution to the susceptibility of *S. aureus* to human PMNs-mediated killing and rendered lower levels of bacterial colonization when its effect was assessed using an *in vivo* murine model. Though further analysis is needed to fully understand the precise molecular targets of verteporfin, data presented in this work suggest that the redox-active cysteine of GraS is required for this molecule to exert its inhibiting effect. Altogether, our results enlighten the potential of verteporfin as a supplement and(or) alternative antimicrobial therapy and provide

evidence that this compound could be included in a recently described category of drugs known as “Potential Drugs for Repurposing against Infectious Agents”.

Results

Design and validation of a GraXRS-focused screening platform

With the aim of designing a highly specific GraXRS-targeting screening assay, we first restored the GraXRS TCS into the chromosome of *S. aureus* ΔXV strain, which only contains the essential WalKS TCS system in its genome. The resulting *S. aureus* ΔXV Gra-RES strain, together with the corresponding *S. aureus* MW2 wild type strain and the single GraXRS mutant derivative, were transformed with two different reporter plasmids in which *lac*Z expression depends on the GraXRS-regulated promoters of *mpr*F and *dlt*X(Falord *et al.*, 2011). As shown in figure 1, transcriptional activity of reporter genes was barely detectable in the GraXRS deficient strains (single and multiple ΔXV mutants), whereas *mpr*F and *dlt*X-based reporter constructs were highly induced in the GraXRS containing strains (wild type and ΔXV GraRES). As an additional test for evaluating the behavior of the reporter strain-set, positive response of the GraXRS-dependent promoters to the presence of sublethal concentrations of colistin was also analyzed. Noticeably, and confirming the concept of TCS as self-sufficient modules previously envisioned (Villanueva *et al.*, 2018), *lac*Z expression driven by *dlt*X promoter was essentially equal in the wild type and *graXRS* restored genetic backgrounds. This result validates both the use of the GraXRS restored strain and *dltXP*::*lacZ* transcriptional fusion for high-throughput-screening designs aimed at the discovery of GraXRS-blocking drugs and molecules.

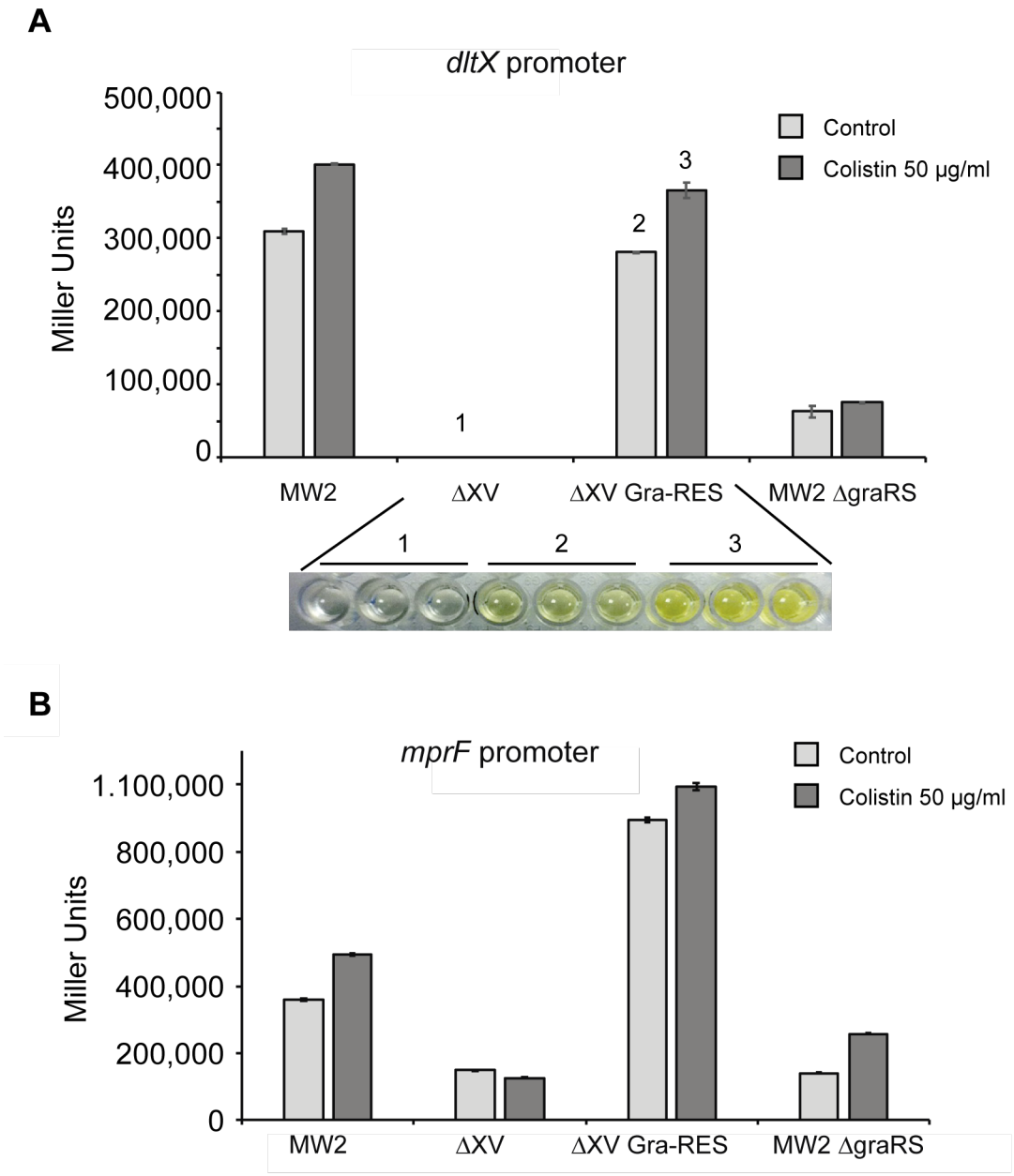

Figure 1. Validation of GraXRS-dependent transcriptional fusions. Graphic illustration of GraXRS-dependent activity of (A) *dltX* and (B) *mprF* promoters in *S. aureus* MW2 wild type, ΔXV, ΔXV Gra-RES and ΔgraRS strains. Transcriptional activity in TSB medium (light grey bars) and TSB supplemented with colistin 50 µg/ml (dark grey bars) is presented. Means and standard deviations values are shown from at least three independent experiments. A visual example showing different degrees of GraXRS activity ((1)ΔXV, (2)ΔXV Gra-RES and (3)ΔXV Gra-RES in the presence of colistin) in the 96-well format is also shown.

Primary and secondary screenings

From the initial screening of the 1.280 drugs included in the Prestwick library (http://www.prestwickchemical.com/libraries-screening-lib-pcl.html), we selected 77 compounds that led to reduced activity of *dltX* promoter by more than 55% as determined by beta-galactosidase activity. Because the intention of this work was to repurpose those FDA-approved drugs that specifically targeted GraXRS-mediated signaling pathway, and this TCS has been shown to be crucial for bacterial growth under acidic conditions (Villanueva *et al.*, 2018), the capacity of the selected compounds to affect OD_{600} values at pH 5,5 was tested, expecting a significant growth arrest in the presence of GraXRS-blocking compounds. Following a similar approach, the dose-response behavior of selected compounds was evaluated. To do so, both bacterial growth and *dltxP* transcriptional activity were quantitatively assessed in the presence of variable concentrations, ranging from 0 to 20 μM, of selected drugs (figure 2C).

In order to reinforce the involvement of GraXRS TCS in the phenotypic outcomes rendered by the drugs to be chosen, their ability to down regulate the GraXRS-dependent alternative *mpr*F promoter was considered as an additional selective criterion.

To verify the specificity of GraXRS for the selected compounds, we restored a different TCS, *saeRS*, into the ΔXV genome. In this case, the reporter *lac*Z gene was transcriptionally fused to *sec*4 promoter, which has previously described as part of SaeRS regulon (Liu *et al.*, 2016). No shift in the transcription levels of *sec4P::lacZ* fusion appeared when ΔXV Sae-RES were incubated in the presence of each selected compound.

Finally, and though equivalency between wild type and GraXRS restored strains in terms of GraXRS-mediated sensing had been previously validated, the possibility that other TCSs or derived circuits absent on ΔXV Gra-RES could somehow affect *dltX* transcriptional activity was considered. In order to address

this issue, the MW2 wild type strain was transformed with the *dlt*X-derived reporter plasmid and beta-galactosidase activity was measured in the absence and presence of mentioned compounds.

As shown in figure 2, the whole consecutive screening process significantly restricted the number of active compounds from almost 80 to 5. Acetylsalicylic acid (ASA), hesperidin (HES), ascorbic acid (VITC), verteporfin (VER) and troglitazone (TGZ) were capable of inhibiting bacterial growth under acidic conditions, affected *mprFP* activity negatively and exerted a suppressing effect on *dltXP* in the wild type genetic background. Among this final group of drugs, TGZ displayed an additional negative impact on SaeRS, and thus was firstly considered as a potential multi-target drug. However, since this molecule was withdrawn in 2000 due to high risk of hepatotoxicity, only ASA, HES, VITC and VER were contemplated as real candidates for therapeutic reposition. Curiously, though these four compounds show no common (known) pharmacological features, they are all chemically classified as redox-active drugs.

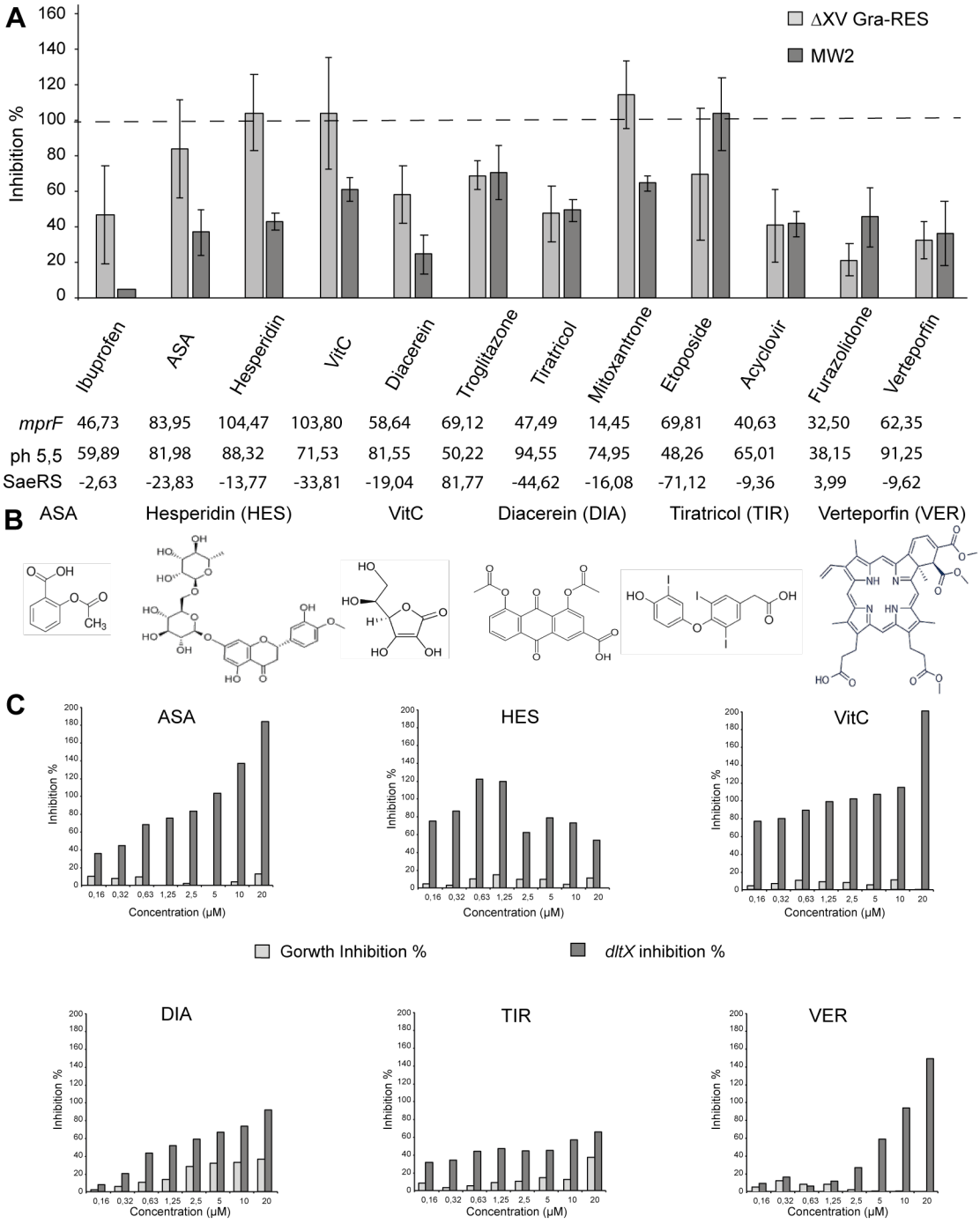

Figure 2. Screening results. (A) Graphic illustration of GraXRS-dependent activity of *dltX* promoter in the presence of selected drugs (10 μM) in *S. aureus* ΔXV Gra-RES (light gray bars) and MW2 wild type strains (dark grey bars). Data are presented as relative M.U. values obtained in the absence of any compound for *S. aureus* ΔXV Gra-RES and MW2 wild type strains respectively. Values exceeding 100% correspond to those cases where the observed transcriptional activity is lower to that showed by ΔXV and/or bacterial growth is inhibited. Standard deviations values correspond to at least three independent experiments. Results regarding additional selection criteria including the repression of the GraXRS-dependent alternative *mprF* promoter, significative inhibition of wild type strain under acidic growth conditions (pH 5,5) and transcriptional co-inhibition of the SaeRS-dependent *sec4* promoter are presented. (B) Structure of selected drugs included in drug bank profiles (https://www.drugbank.ca) is shown. (C) Graphic illustration of Dose Dependency profiles in terms of *dltX* inhibition exhibited by selected compounds. Data are presented as relative M.U. values obtained in the absence of any compound for *S. aureus* ΔXV Gra-RES (dark grey bars). Relative percentage of survival in the presence of increasing doses of compounds is also included (light grey bars).

In vitro effect of selected compounds: Phagocytosis and killing of *S. aureus* by human PMNs

Deepening into the anti-virulence potential of selected drugs, we next proceeded with an *in vitro* assay in which the bacterial susceptibility to phagocytosis and killing by human polymorphonuclear cells was assessed. Considering that GraXRS has been shown to have a pivotal role for *S. aureus* to resist PMN attack (Flannagan *et al.*, 2018), we isolated this cellular fraction from peripheral human blood and the effect of ASA, HES, VITC and VER on MW2 susceptibility to these immune cells was assessed. In all cases the process was boosted by opsonic antibodies naturally present in human sera due to unavoidable exposure to *S. aureus.* After incubating PMNs-*S. aureus* MW2 suspensions for 30 minutes in the absence or presence of the four selected compounds (5 μM each), removal of extracellular bacteria via gentamicin exposure, and subsequent lysis of eukaryotic cells at basic pH, bacterial viability was estimated via plate counting. Data shown in figure 3 prompted us to conclude that the presence of ASA, HES and VITC caused a slight increase in the sensitivity to PMN attack, while the effect of VER on reducing the number of surviving intracellular bacteria was substantially obvious and statistically significant (figure 3). As a result, from this *in vitro* approach, we chose VER as the sole candidate to proceed with the next assays.

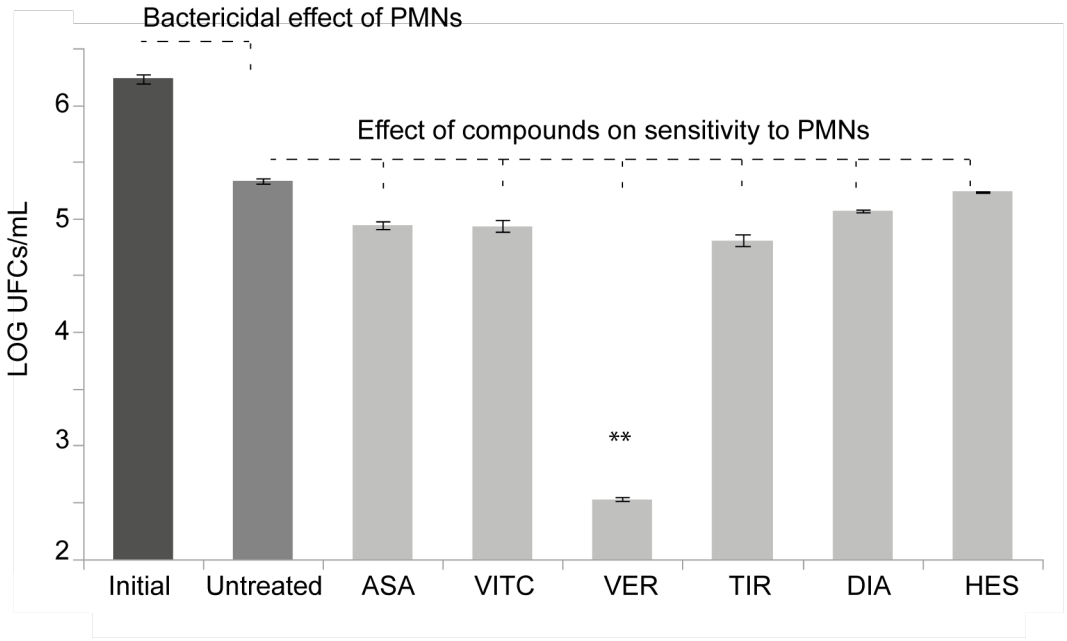

Figure 3. Effect of selected drugs in the susceptibility to phagocytosis and killing by human PMNs. Graphical scheme of bacterial counts expressed as log values after incubating PMNs and *S. aureus* MW2 wild type suspensions for 30 min in the presence or absence of four selected compounds (5 μM each), subsequent gentamicin treatments and lysis of eukaryotic cells (see Materials and Methods section). Means and standard deviations are presented, illustrating the bactericidal effect of PMNs and the additional contribution of ASA, VITC, VER, TIR, DIA and HES. In the case of VER, two asterisks denote an associated p value of 0,006 when ANOVA and Tukey´s pairwise post hoc tests were applied according to prior normality and homoscedasticity tests.

Effect of Verteporfin in a murine model of wound infection

Following on from the *in vitro* testing, we next evaluated VER using the murine model of wound (or surgical site) infection. In this *in vivo* approach, a silk suture contaminated with *S. aureus* MW2 or MW2Δ*graXRS* strain ($4{,}5x10^5$ CFUs cm^{-1}) was used for sewing up a previous incision on the back of the mice. VER was topically applied 2 and 24 hours after suture implantation using hydrogel-based formulations that contained either no active ingredient or the porphyrin under study at a low (0,125 mg/kg) or a high (2,5 mg/kg) dose. Assessment of the infection was performed by counting viable bacteria in tissue homogenates that were obtained upon animal euthanasia, 24 hours after the last treatment.

As shown in figure 4, the significantly lower quantity of viable bacteria present in tissue samples that had been infected with the GraXRS deficient strain unveiled the critical role of this TCS in surgical wound infections. When the effect of VER was assessed, data showed that topical administration of this drug significantly reduced the bacterial load in a dose-dependent manner. Noticeably, application of VER at a low dose led to a similar degree of bacterial colonization to that followed by the implantation of sutures that had been contaminated with the GraXRS lacking strain. While such a result reinforces the potential of VER as a GraXRS inhibitory drug, its specificity is suggested by the fact that the observed outcome after treatment of wounds infected with the wild type strain is certainly evident, but it becomes almost imperceptible in the case of Δ*gra*XRS-associated infection. Equally certain, however, is that high doses of VER could exert a GraXRS-independent and/or antibiotic effect.

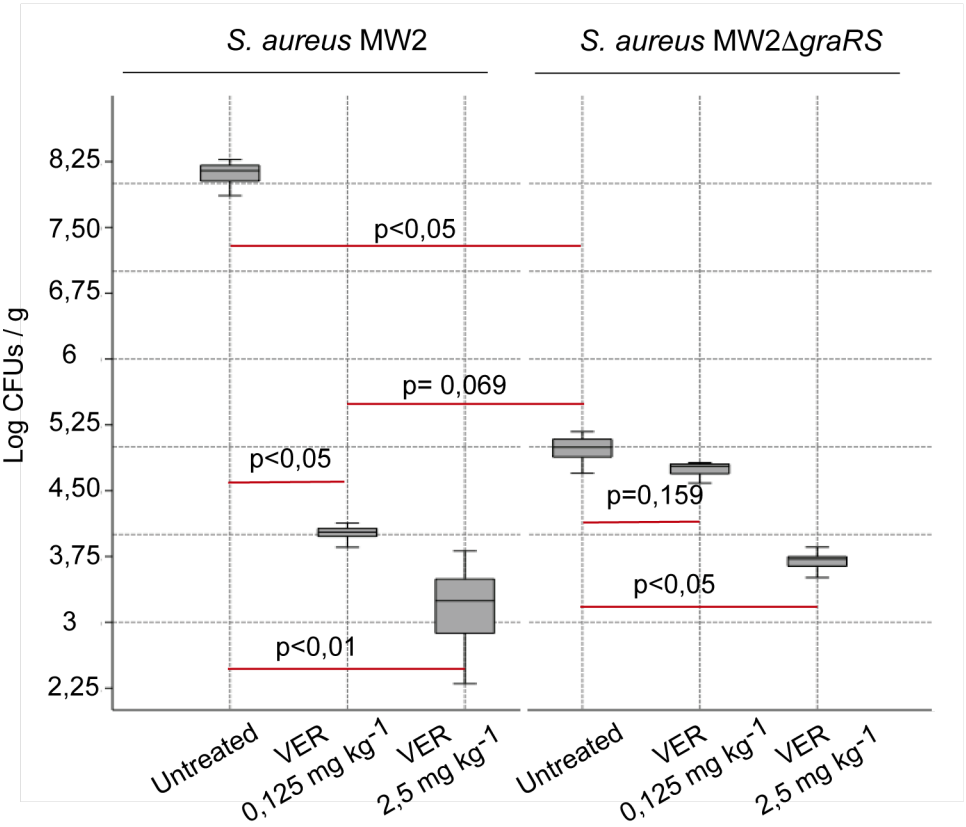

Figure 4. Effect of VER in a murine model of surgical wound infection. BOX-PLOT illustration (PAST statistical package) of bacterial counts expressed as log values per gram of wound tissue homogenates. Different treatments include the topical administration of hydrogel-based formulations with two doses (0,125 and 2,5 mg kg-1) of VER, 2 and 24 hours after making an incision that was immediately infected with a surgical suture contaminated with $4x10^5$ CFUs of *S. aureus* MW2 wild type or Δ*graRS* strain. Hydrogel without any active pharmaceutical ingredient was applied to control mice. All the animals were euthanized 24 hours after the last treatment. Apart from visual interpretation in accordance to IQR overlapping, data were statistically analyzed via Kruskal-Wallis and post hoc Mann-Whitney test. Corrected p-values corresponding to relevant post hoc statistical comparisons are also shown.

Exploring the mechanistic basis of verteporfin

Because VER is a tetradentate chelating porphyrin(Eales *et al.*, 2018) that might be involved in redox sensing, just like heme complex, and such molecules are normally sensed through thiol-based switches, we also analyzed the contribution of the single redox-active cysteine present in GraS, C227, to VER effect (Shimizu *et al.*, 2019). To do so, C227 was mutated to S or A in ΔXV Gra-RES background and transcriptional activity of *dltXP* in the resulting ΔXV Gra-RES S(C227-S) and ΔXV Gra-RES S(C227-A) strains was measured. An additional strain in which GraS H129 amino acid, the residue that undergoes phosphorylation upon activation of the kinase, had been mutated to Q was also constructed and included as a reference of complete GraXRS inactivation. As shown in figure 5A**,** data verified that replacement of cysteine by another residue had a negative impact on GraXRS activity, being such an outcome dependent on the polarity of the substituted amino acid. Thus, C227-A (non-polar) GraS isoform led to a lower degree of transcriptional activity of *dltXP* in comparison to that showed by the isoform in which C227 had been mutated to the polar amino acid serine. In accordance with this observation, bacterial growth arrest under acidic conditions, a phenotype that strictly reflects GraXRS status, also showed dependence on the mutation polarity (fig. 5B). When the effect of VER was assessed, transcriptional data revealed that GraXRS repression led by mutations was far from being as drastic as the one achieved by VER (fig. 5A), discarding the possibility of considering the C227 redox switch as the exclusive mechanism underlying VER effect. However, impairment of the signaling via C227 resulted in the insensitivity to VER, fact that suggests that intermolecular cysteine-disulfide-bond formation is required, though not entirely, for VER to have a blocking GraXRS-dependent effect.

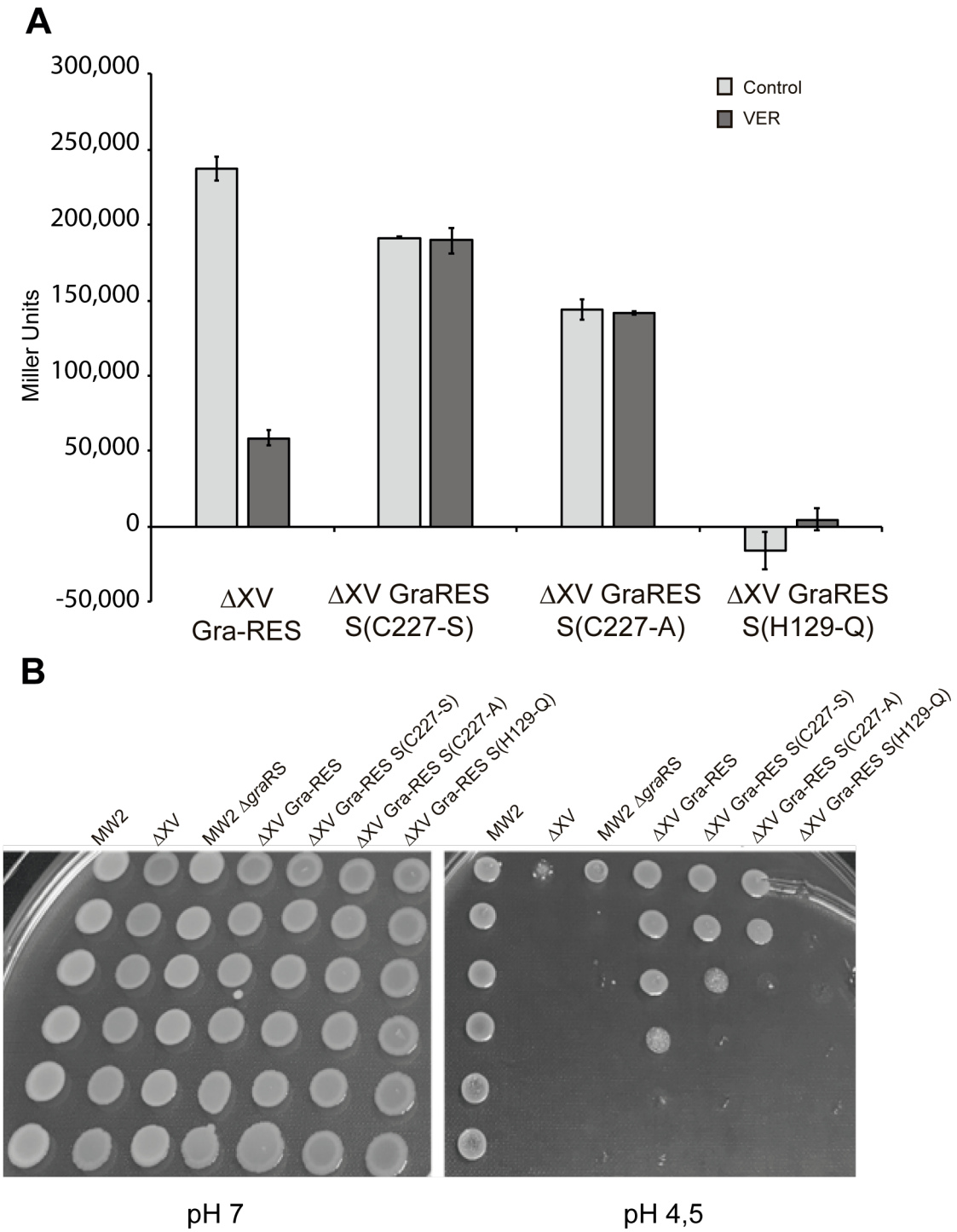

Figure 5. C227-mediated redox switch involvement in the sensitizing effect exerted by VER. (A) Transcriptional activity of GraXRS-dependent *dltX* promoter in *S. aureus* ΔXV Gra-RES, ΔXV Gra-RES S(C227-S), ΔXV Gra-RES S(C227-A) and ΔXV Gra-RES S(H129-Q) strains is illustrated both in the presence (dark grey bars) or absence (light gray bars) of VER (10 μM). Means and standard deviations values are shown form at least three independent experiments. (B) Pictures illustrating the capacity of serially-diluted suspensions of bacterial strains *S. aureus* MW2 wild type, ΔXV, ΔgraRS, ΔXV Gra-RES, ΔXV Gra-RES S(C227-S), ΔXV Gra-RES S(C227-A) and ΔXV Gra-RES S(H129-Q) to grow in neutral (left) and acidified (right) TSA medium are also included in this figure.

Discussion

Current strategies aimed at antimicrobial discovery prioritize innovative concepts like alternative molecular mechanisms of action, new natural product sources, pro-drugs, or even approved compounds that were originally intended for other therapeutic indications, as it is the case of the present work (Statement of Antimicrobial drug discovery, EASAC-2014 (van der Meer *et al.*, 2014)). Particularly speaking of *S. aureus,* and discarding the essential TCS WalKR/S as the antibiotic target *per excelence*, continuous efforts are being made toward the discovery of inhibitors of TCS involved in virulence and biofilm formation (for review (Thangamani *et al.*, 2016; Kong *et al.*, 2016; Yeo *et al.*, 2018)). Impairment of these non-essential biological pathways has the advantage of requiring a lower plasmatic dose compared with a MIC, reducing the tendency to resistance and minimizing side effects on neutral and beneficial microbiota that colonize treated human or animal hosts (Thangamani *et al.*, 2015). Up to date, several novel and previously approved drugs with the capacity to exert an inhibitory effect on Agr, SaeRS, and ArlRS TCSs have been described (for review (Thangamani *et al.*, 2016; Kong *et al.*, 2016; Yeo *et al.*, 2018)) but, to our knowledge, GraXRS had never been considered as a target for disarming *S. aureus*. The involvement of this TCS in the resistance to antimicrobial peptides and macrophages had already been envisioned in several occasions (Li *et al.*, 2007; Falord *et al.*, 2011; Falord *et al.*, 2012), but it has just been recently proven that GraXRS is entirely responsible for the response to pH inside acidified macrophage phagolysosomes (Flannagan *et al.*, 2018; Villanueva *et al.*, 2018). These premises led us to consider GraXRS as a clear target to counteract *S. aureus* response to innate host immunity and impede replication of the pathogen in the acute stage of systemic infection.

Taking advantage of the *S. aureus* strain that lacks its complete sensorial TCS network (Villanueva *et al.*, 2018), we developed a series of reporter strains that could be helpful for selecting compounds capable of blocking transcriptional

activity of the GraXRS-dependent *dltX* promoter. Working with ΔXV Gra-RES strain gave us the opportunity to perform a bioassay where bacterial sensing entirely depends on GraXRS (and WalkRS), thus reducing the probability of selecting off-target drug candidates. Proof of this last claim is the fact that among selected drugs, only one of them (Troglitazone) displayed a GraXRS-SaeRS multi target effect.

At the time of deciding the type of compound to be tested for the identification of GraXRS inhibitors, we, as many other researchers, opted for the drug-repurposing approach. This strategy is based on the identification of "off" antimicrobial targets for drugs that were approved for other clinical diseases (Thangamani *et al.*, 2015), hence bypassing the financial and regulatory barriers that have to be overcome to bring a drug to market. At present day, this concept of repurposing has gained renewed interest and a novel category of drugs known as "Potential Drugs for Repurposing against Infectious Agents" is exponentially thriving (Miró-Canturri *et al.*, 2019) By way of example, the old antimalarial drug chloroquine is being tested as SARS-CoV-2 inhibitor (Gautret *et al.*, 2020). Though the precise mechanistic basis of their effect remains to be completely elucidated, current candidate PDRIAs targeting *S. aureus* SaeRS and/or AgrTCSs are floxuridine, streptozotocin and diflunisal (Khodaverdian *et al.*, 2013; Hendrix *et al.*, 2016; Yeo *et al.*, 2018).

The screening methodology applied here consisted in the analysis of changes in *dltXP* transcriptional activity, followed by several consecutive steps where additional criteria like the effect on an alternative GraXRS-dependent promoter, TCS-selectivity, or the determination of bacterial growth in the presence of selected drugs under acidic conditions were applied. The overall process ended up in the selection of five candidate drugs: acetylsalicylic acid (ASA), hesperidin (HES), ascorbic acid (VITC), verteporfin (VER) and troglitazone (TGZ). Curiously, all compounds are classified as redox-active drugs. While ASA, HES, VITC and TGZ are commonly sorted as antioxidant

molecules, VER can induce oxidative stress through the production of free radicals or be alternatively combined with soluble metals and display a redox potential similar to that showed by the heme complex (Eales *et al.*, 2018). These observations are in agreement with the previously unveiled connection between GraXRS and oxidative stress, evidenced by the deciphering of the GraXRS regulon and the proved essentiality of this TCS in staphylococcal resistance to redox compounds like paraquat or H_20_2 (Falord *et al.*, 2011). Furthermore, a recent RNA-seq transcriptomic approach has just corroborated the involvement of GraXRS, collectively with VraSR, SaeRS, MgrA, SigB or Fur, in the cell response to thiol-oxidative stress (Loi *et al.*, 2018; Loi *et al.*, 2019).

After proving that VER was the only compound really capable of sensitizing bacteria against the effect of human PMNs, this compound was further examined using a murine model of surgical wound infection. This model has been previously used to assess the effect of systemic and topical antimicrobial agents, finding a close correlation with efficacy in clinical trials with human subjects. Noticeably, our results provided further evidence concerning the critical role of GraXRS in skin and wound infections and insinuated a pharmaceutical potential of VER as a novel local treatment for *S. aureus* infections. Since bacterial count after the infection with the wild type strain and subsequent treatment with 0,125 mg kg^{-1} of VER was quite similar to that proceeding from the infection with a GraXRS negative strain, the effect exerted by the porphyrin derivative seems to be highly dependent on the activity of this TCS.

When the possible path(s) of how VER inhibits the activity of GraXRS was envisaged, we thought of VER as a heme-like porphyrin capable of binding iron in different oxidation states (Eales *et al.*, 2018). Recent transcriptomic studies conducted with the constitutively-active forms of staphylococcal kinases have unveiled the involvement of GraXRS in the regulation of heme synthase A (MW_RS05355; (Rapun-Araiz et al., 2020)), fact that led us to attach importance to the chemical mimicry between both molecules. Since porphyrins are normally

involved in thiol-based molecular switches (Shimizu *et al.*, 2019), the unique potentially redox-active residue in GraS, C227, was considered a potential molecular target of VER. To address this question, the impact of C227 mutations to S and A on GraXRS activity and VER sensitivity was assessed. To our knowledge, the results achieved in this work by punctual mutations of C227 have evidenced for the first time that this cytosol-located redox-active residue actually participates in GraS kinase activity. This line of reasoning, which has support from studies that have characterized other bacterial kinases like AcrB (Malpica *et al.*, 2004), showed that GraS degree of silencing depended on the polar nature of the amino acid that substituted C227 and suggested the involvement of this redox-switch as a potential molecular target concerning VER effect. However, and though C227 substitutions led to insensitivity to VER, the inhibitory outcome yielded by mutations was not as drastic as the one achieved by exposure to VER, suggesting that additional molecular paths must be involved in this process. An additional candidate that might be considered is Stk1, the unique serine-threonine kinase which cross-phosphorylates GraR (Fridman *et al.*, 2013) and shows homology with OXR1, one of the recently discovered mammalian target of VER that, curiously, is also related to oxidative stress (AlAmri *et al.*, 2018). However, in accordance with Fridman et al (Fridman *et al.*, 2013), we have verified that both Stk1 and GraS-mediated phosphorylation on GraR as T128, T130, T149 and D51 respectively are equally required for full *dltX* expression (data not shown), fact that seriously complicates the use of our reporter systems when it comes to holding Stk1 accountable for intervening on VER effect.

Could VER be considered a viable antimicrobial candidate? Though we are fully aware that further *in vitro* and *in silico* studies helping to understand the whole molecular scenario underlaying VER effect and alternative *in vivo* approaches or definition of strategic dosages are some pending issues to claim a novel anti-virulence pharmaceutical indication for VER, we are convinced that this drug presents some strengths, apart from those inherent to anti-virulence

drugs, that might be worth considering. In terms of pharmacology, for instance, VER excretion is dependent on hepatic function, while many antibiotics primarily undergo elimination via kidney filtration. Thus, VER could be administered to patients suffering from kidney disease, or to those who were being concomitantly treated with antibiotics that are prone to cause nephrotoxicity (e.g. vancomycin) (Elyasi *et al.*, 2012). On the other hand, though side effects associated to parenteral administration of VER include hypersensitivity reactions or blood pressure alteration, these symptoms have lower mean severity ratings in comparison to those showed by many antibiotics (ema.europa.eu/Find medicine/Human medicines/European public assessment reports). Finally, taking into account that we have also observed that VER-containing topical formulations are effective and that further studies might confirm that chemical mimicry between porphyrin derivatives and heme group could actually be harnessed for disarming *S. aureus,* our results may be considered as a step forward in re-proposing VER as a plausible alternative in combating antimicrobial resistance.

Material and Methods

Bacterial strains, plasmids, oligonucleotides and culture media

Bacterial strains, plasmids and oligonucleotides (purchased to IDT) are listed in table 1, table 2 and table 3 respectively. *Escherichia coli* XL1blue strain was grown in LB broth and LB agar. *Staphylococcus aureus* strains were grown on trypticase soy broth (TSB), trypticase soy agar (TSA), trypticase soy broth with 0.2% of glucose (TSB-glu), Mueller Hinton (MH) and B2 medium (1% casein hydrolysate, 2.5% yeast extract, 2.5% NaCl, 0.1% K_2HPO_4, and 0.5% glucose [w/v]). When required for growth or selection, medium was supplemented with 5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside (XGal) and/or the appropriate antibiotic at the following concentrations: erythromycin (Eri) 10 μg ml^{-1}, ampicillin (Am) 100 μg ml^{-1}, chloramphenicol (Clo) 10 μg ml^{-1} and 20 μg ml^{-1}..

DNA manipulations

Plasmids were purified using NucleoSpin Plasmid kit (Macherey-Nagel) according to the protocol provided by the manufacturer. PCR fragments and enzymatic reactions were purified using GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Scientific). FastDigest restriction enzymes, Rapid DNA ligation kit, Dreamtaq DNA polymerase and Phusion DNA polymerase were supplied by Thermo Scientific and used according to provided instructions. Sequence verification of PCR-amplified products and plasmid constructions was performed by Stab Vida. Transformation of *Staphylococcus aureus* was performed following previously standardized protocols (Schenk and Laddaga, 1992; Lee, 1995).

Allelic exchange of chromosomal genes

To generate markerless deletions, two fragments of at least 500 bp that flanked upstream (primers A and B, table 3) and downstream fragments (primers C and D, table 3) of the region to be deleted were amplified by PCR. Amplified

products were digested using the corresponding restriction enzymes (table 3), purified and cloned by ligation into pMAD shuttle vector. To restore individual TCSs into □XV chromosome, a fragment containing the two flanking regions used to generate the deletion (Villanueva *et al.*, 2018) and the original TCS sequence were amplified by PCR using chromosomal DNA from MW2 strain as template and oligonucleotide pair *gra* or *sae* 1- 3. For restoration of the TCS with single amino acid substitutions at C227, *graRS* was amplified using MW2 chromosomal DNA as template and a two-step PCR protocol. First, the oligonucleotides A and G or H were used for generating two overlapping PCR products, while a second amplification step with A and D oligonucleotides using both purified PCR products as templates generated *graRS* S(C227-A) and *gra*RS S(C227-S) isoforms. Such DNA fragments were purified, digested with the corresponding enzymes (see Table 3) and inserted by ligation into the pMAD shuttle vector (Arnaud *et al.*, 2004). Homologous recombination experiments were performed as previously described (Valle *et al.*, 2003). Final plasmidless erythromycin sensitive white colonies were tested by PCR using primers E and F (Table 3).

Reporter plasmid construction

pSA14 was used for the construction of different reporter plasmids. Promoter regions of *mprF*, *dltX* and *sec4* were amplified by PCR using oligonucleotides described in table 3. PCR fragments were purified and cloned into pSA14 through restriction enzymes to generate transcriptional fusion with *lacZ*. GraRS-dependent reporter plasmids were transformed via electroporation into *S. aureus* MW2, MW2Δ*gra*XRS, ΔXV, ΔXV Gra-RES and ΔXV Gra-RESΔ*gra*X, while plasmid harboring Sae-dependent *sec4* promoter was inserted into MW2, ΔXV and ΔXV Sae-RES strains. To analyze *mprF, dltX* and *sec4* expression, ON cultures were chemically lysed beta-galactosidase activity was measured.

High throughput beta-galactosidase-based screening

Screening of the 1280 off-patent FDA-approved drugs in Prestwick Chemical drug library (Prestwick Chemical) was based in a method for beta-galactosidase assays in 96 well plates. Working solutions of the compounds were prepared in 50 µl of sterile distilled-deionized water at a concentration of 20 µM, and combined with 50 µl of a 1:30 dilution on 2x TSB medium of an overnight (ON) culture of *S. aureus* strains, thus generating 100 µl of 1:60 cell dilution on 1x TSB at a final concentration of 10 µM of each drug. Plates were incubated during 24 h at 37ºC and, upon incubation, OD_{600nm} was measured (Multiskan Go; Thermo Scientific). Bacterial cells were subsequently lysed by the addition of 100 µl $well^{-1}$ of Z buffer supplemented with lysostaphin (0.5 mg ml^{-1}) during 2 hours at 37ºC; Next, 30 µl $well^{-1}$ of Ortho-Nitrophenyl-beta-galactoside (ONPG, 4 mg ml^{-1}) was added and, when required, the reaction was stopped with 100 µl $well^{-1}$ of 1M Na_2CO3. OD_{420} and OD_{550} values were finally recorded for Miller Units calculation. Untreated reporter ΔXV-GraRES and ΔXV strains were included in every plate as internal controls. Experiments were carried out in triplicate

Phagocytosis and killing of *S. aureus* by human PMNs

Phagocytosis and killing of *S. aureus* by human neutrophils in presence of selected compounds was determined as described before (Peschel *et al.*, 2001). Polymorphonuclear cells (PMNs) were isolated from healthy human heparinized-defibrinated blood (Seralab Logistics) using Ficoll-Plaque PREMIUM (GE-Healthcare) according to manufacturer´s protocol and resuspended at a final concentration of $1x10^7$ PMNs ml^{-1} in HBSS supplemented with human serum. *S. aureus* strains were cultured to the early stationary phase and 10 ml of culture were centrifuged, washed twice with sterile PBS and resuspended in Hank´s Balanced Salt Solution (HBSS) supplemented with human serum at a final concentration of $4x10^5$ bacteria ml^{-1}. Finally, 0.2 ml of PMNs solution was mixed with 0.2 ml of *S. aureus* solution and 600 µl of HBSS supplemented with human

serum. Compounds were added at a final concentration of 5 µM. After incubation at 37ºC for 30 minutes, each sample was treated with gentamicin 100 mg ml^{-1} and then 100 µl of each mixture were added to 1 ml of pH11 solution. Finally, serial dilutions were plated on TSA to determine the number of colony-forming units (CFU) in presence of the different compounds. All data were referred to initial CFU number.

Mouse infections models

The experimental animal study was reviewed and approved by the ‘‘Comité de Ética, Experimentación Animal y Bioseguridad’’ of the Universidad de Navarra-Centro de Investigación Médica Aplicada (CIMA). Work was carried out at the CIMA animal facility under the principles and guidelines described in the ‘‘European Directive 86/609/EEC’’ for the protection of animals used for experimental purposes. Six-week-old female swiss mice (20-25 g) were obtained from ENVIGO and confined in groups of 6 animals.

The model was performed as previously described (McRipley and Whitney, 1976). Briefly, 10 cm fragments of commercial brailed silk (TC-15, Lorca Marín) were contaminated with 4x 10^6 CFU cm^{-1} of *S. aureus* MW2 or *S. aureus* MW2Δ*gra*XRS strains by immersion for 30 minutes. Fragments were then blotted- dried. One day prior to the experiment, the interscapular skin was shaved using a sharp razor. On the day of the infection, superficial wounds were produced on the exposed back surface though a longitudinal midline incision of 2 cm approximately. The skin of either side of the incision was retracted, and the wound was infected by stitching it with contaminated suture and a suturing needle. Wounds were topically treated 1 h and 8 h after infection with approximately 100 µl of hydrogel formulations containing 0,125 and 2,5 mg/100 µl. The hydrogel base without any active substance was applied in the control group. Treatments were repeated 24 hours after infection and mice were sacrificed 24 h after the last application. Finally, the wounded tissue was

resected, homogenized in PBS, and dilution series of homogenates was plated on TSB agar for enumeration of CFU (output). After an overnight incubation at 37ºC, CFU gr of tissue^{-1} were calculated and expressed as $\log_{10}$.

Statistical analysis

Data generated by PMN-mediated killing assay were compared using ANOVA, applying Tukey's pairwise as post hoc test. Data obtained from the bacterial counts in the murine model were treated and compared using Kruskal-Wallis test, Mann-Whitney pairwise and Dunn's post hoc tests. All tests were two-sided, and the significance level was 5%. The statistical analysis was performed with Past and R softwares.

Table 1 Strains used in this study

Strain	Characteristics	Reference
Escherichia coli		
Xl1Blue	Cloning strain (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)])	Stratagene
Staphylococcus aureus		
RN4220	Restriction deficient transformation recipient	(Novick, 1990)
MW2	Community-acquired strain of MRSA isolated in 1998 in North Dakota, USA.	(Baba *et al.*, 2002)
MW2Δ*gra*RS	Markerless mutation of *graRS* genes	(Villanueva *et al.*, 2018)
ΔXV	MW2 Δ*hptRS* Δ*lytSR* Δ*graRS* Δ*saeRS* Δ*MW1208-MW1209* Δ*arlRS* Δ*srrAB* Δ*phoPR* Δ*yhcSR* Δ*vraSR* Δ*agrBDCA* Δ*kdpDE* Δ*hssRS* Δ*nreBC* Δ*braRS*	(Villanueva *et al.*, 2018)
ΔXV Gra-RES	Restored ΔXV::*gra*RS strain	This study
ΔXV Gra-RES S(C227-A)	Restored ΔXV::*gra*RS strain with C227-A single amino acid substitution in GraS	This study
ΔXV Gra-RES S(C227-S)	Restored ΔXV::*gra*RS strain with C227-S single amino acid substitution in GraS	This study
ΔXV Gra-RES S(H129-Q)	Restored ΔXV::*gra*RS with H129-Q single amino acid substitution in GraS	This study
ΔXV Gra-RES Δ*gra*X	Markerless mutation of *graX in* ΔXV Gra-RES strain	This study
ΔXV Sae-RES	Restored ΔXV::saeRS strain	This study
MW2 *mpr*Fp	MW2 carrying pSA14::*mprF*p; Eri^R	This study
MW2 *dlt*Xp	MW2 carrying pSA14::*dlt*Xp; Eri^R	This study
MW2 *sec4*p	MW2 carrying pSA14::*sec4*p; Eri^R	This study
ΔXV *mpr*Fp	ΔXV carrying pSA14::*mpr*Fp; Eri^R	This study
ΔXV *dlt*Xp	ΔXV carrying pSA14::*dltX*p; Eri^R	This study
ΔXV *sec4*p	ΔXV carrying pSA14::*sec4*p; Eri^R	This study
ΔXV Gra-RES *mpr*Fp	Restored ΔXV::*gra*RS strain carrying pSA14::*mpr*Fp; Eri^R	This study
ΔXV Gra-RES *dlt*Xp	Restored ΔXV::*gra*RS strain carrying pSA14::*dlt*Xp; Eri^R	This study
MW2 Δ*gra*RS *mpr*Fp	MW2 Δ*gra*RS carrying pSA14::*mpr*Fp Eri^R	This study
MW2 Δ*gra*RS *dlt*Xp	MW2 Δ*gra*RS carrying pSA14::*dlt*Xp Eri^R	This study
ΔXV Sae-RES *sec4*p	Restored ΔXV::*Sae*RS carrying pSA14::*sec4*p Eri^R	This study

Table 2. Plasmid used in this study

Plasmid	Characteristics	References
pMAD	*E. coli – S. aureus* shuttle vector with a thermosensitive origin of replication used for allelic replacement	(Arnaud *et al.*, 2004)
pMAD::*gra*RES	pMAD plasmid containing the allele for chromosomal restoration of *gra*RS TCS	This study
pMAD::*sae*RES	pMAD plasmid containing the allele for chromosomal restoration of s*ae*RS TCS	This study
pMAD:: Δ*gra*X	pMAD plasmid containing the allele for markerless deletion of *gra*X gene	This study
pMAD::*gra*RES S(C227-A)	pMAD plasmid containing the allele for chromosomal restoration of *graRS* S(C227-A) isoform	This study
pMAD::*gra*RES S(C227-S)	pMAD plasmid containing the allele for chromosomal restoration of *graRS* S(C227-S) isoform	This study
pMAD::*gra*RES S(H129-Q)	pMAD plasmid containing the allele for chromosomal restoration of *graRS* S(H120-Q) isoform	This study
pSA14	pM4 derivative carrying the promoterless *E.coli lac*Z gene for constructing transcriptional fusions	(Falord *et al.*, 2011)
pSA14::*mpr*Fp	pSA14 containing the *mpr*F promoter region	(Falord *et al.*, 2011)
pSA14::*dlt*Xp	pSA14 containing the *dlt*X promoter region	This study
pSA14::*sec*4p	pSA14 containing the *sec4* promoter region	This study

Table 3. Oligonucleotides used in this study

Oligonucleotide	Sequence
Restoration of TCS	
gra-E	GGGCCATAAAAAGCCTCCAG
gra-F	GTAGCTTCCGACTTGTGAGCC
gra-A (EcoRI)	CCGGGAGCTCGAATTCCAAATAGATATTGCTGTATTCTTTATCGACCCAAC
gra-D (BamHI)	GGGCGATATCGGATCCAACGCCACCTAAAACACTTTGTACAC
G C227A Rv	CTAATAATCATACGGCACCATTTTATATC
H C227A Fwd	GATATAAAATGGTGCCGTATGATTATTAG
G C227S Rv	TCTAATAATCATACGAGACCATTTTATATC
H C227S Fwd	AGATATAAAATGGTCTCGTATGATTATTAG
G H129-Q Rv	GTTTTTATGTCTTGCACAAATTCTG
H H129-Q Fwd	CAGAATTTGTGCAAGACATAAAAAC
sae-E	AGTACAATTTGATGATGGTGTTGGTG
sae-F	GATTTCACAGCACCCCTAGC
sae-A (BamHI)	GGGCGATATCGGATCCCAAAAGGGTTATTTGAATGGATAGGC

sae-D (NotI)	CCATGGCATGCATCGCTGTTCACATAACACTACAAATCGC
graX A (BglII)	CACAGATCTGGTTGGTTATTGAGTGGTACATTTG
graX B (XhoI)	CACCTCGAGCTAAAATACTCCTTTAAACTGTAACC
graX C (XhoI)	CACCTCGAGGGTGATATGGATGCAAATAC
graX D	GGAGGATCCTTTCGATTTGATTTTTTTTGGTAATAAG
graX E	GTTGTTATGCGATTCTGATACAAG
graX F	TGTTTCGATTGCACTATCCATAC
Reporter construction	
pSA14-Fw	TGGAATTGTGAGCGGATAAC
pSA14-Rv	CTCTTCGCTATTACGCCAG
mprFp-Fw (PstI)	CTGCTGCAGTATAGATAACCATATTGTTC
mprFp-Rv (BamHI)	GGAGGATCCTGATTCATTTTTTCACATCA
dltXp-Fw (PstI)	GGCTGCAGGCGCTGATGATAATTCAATAA
dltXp-Rv (BamHI)	CGGGATCCGATTTCATATTGCACCTCTTAAAG
sec4p-Fw (PstI)	GGCTGCAGGAGTGTGAATATATAAACAATG
sec4p-Rv (BamHI)	GCGGATCCTTATTCATTTTTATCTCCTTC
mprFp-GFP-Fw (SalI)	GTCGTCGACGTATAGATAACCATATTGTTC
mprFp-GFP-Rv (KpnI)	GGTGGTACCTGATTCATTTTTTCACATCA
Complementation plasmid	
graR Fw (KpnI)	GGGGTACCTCGAGAATGATATTGGGTGATATGG
graR Rv (EcoRI)	GGGAATTCCAAATTATTCATGAGCCATATA

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FUTURE PROSPECTS

Just as the scientific community predicted the possibility of a coronavirus pandemic many years ago (Morse et al., 2012), there is substantial evidence that antimicrobial resistance poses a great threat to animal and human health, which definitively can not be ignored any longer. Paradoxically, and with no intention to demonize them, pharmaceutical companies are curtailing anti-infective research programs. Why is this happening? A number of social and economical factors make antimicrobials less attractive from a business point of view these days. For instance, the aging of population has shifted drug discovery projects towards drugs for treating chronic conditions that mostly affect elderly such as hypercholesterolemia, hypertension or arthritis. These medications are normally long-term used, while antibiotics are commonly restricted to short-term treatments. In addition, the large number of commercial antibiotics already available and the global preconceived notion that antibiotics must be inexpensive result in a very high level of market competition. Medical community does not contribute to make antimicrobial-drug-discovery the “goose that lays golden eggs” for pharma companies either, since first-line use of newly developed antimicrobials is normally reserved for extreme resistance cases, thus negatively impacting sales.

We are confident that small spin-off and biotech companies might help to fill the gap in anti-infective research created by big pharma withdrawal and thus Recombina is committed to the innovation and technology that could put a tiny grain of sand in the AMR crisis.

Chapter I

With the previously mentioned objective in mind, we first attempted to find a novel molecule of marine origin that could enhance the negative effects, understood as toxic accumulation of intermediary metabolites or proteins, that over production of the major staphylococcal biofilm polysaccharide might entail. Though we have not yet been able to identify a singular compound capable of

exerting a PIA/PNAG-dependent antibiotic effect, we found that a subfraction obtained from the fermentation of a marine microbe, composed by Lumichrome, Soyasaponin and Malayamicin, specifically inhibits those bacteria producing PIA/PNAG. Preliminary studies aimed at the understanding of the molecular mechanisms underlaying the effect of sub-fraction TA-15-A-A112CHV-F.9/10.SF8 indicate that post transcriptional regulation of *ica* operon and *ica*-conditional repression of Protein A and other high molecular weight proteins could be crucial to exploit PIA/PNAG-associated fitness cost. Thus, next experimental steps that are currently being evaluated include the identification of those high molecular weight proteins that are missing from proteic profiles when bacteria are cultured in the presence of sub-inhibitory concentrations of SF8. Given the precedent work unveiling the negative effect of Lumichrome on sortase A (Chung et al, 2019), we are also planing to study the effect of SF8 in the strain *S. aureus* 15981Δ*srtA.* If this anchoring factor is indeed a target for SF8, mutant strain should show a lower susceptibility to this compound mixture. Additionally, we find quite attractive the previously formulated hypothesis that, since IcaC is normally the target for phase variation in PIA/PNAG production and its lack confers an advantage under poor nutrient conditions in comparison to the loss of the entire operon, other proteins encoded within the *ica* locus could have some other function (Brooks and Jefferson, 2014). Are IcaA or IcaD, for instance, involved in SF8 effect? To address this question, we have some experiments in mind, including the construction and analysis of individual complemented and epitope-tagged Ica-derivatives.

Apart from SrtA and Ica operon, and with the aim of having a global picture of the effect exerted by SF8, and at the same time going deeper into the mechanisms that could trigger lethality when PIA/PNAG is produced, we are considering the possibility of getting the resources to perform single-step and multi-step resistance studies. These approaches, which have recently been used to propose a new class of synthetic retinoids as effective antibiotics against

bacterial persisters (Kim et al., 2018), involve the formation of resistant mutant clones in one exposure or over many passages (subcultures) in the presence of the antimicrobial agent. We could then not only evaluate the frequency of resistant clones to SF8, but also find the type of mutations via whole genome sequencing, thus inferring the molecular basis underlaying PIA/PNAG associated fitness cost and, luckily, novel targets for antibiotic drug-discovery.

As an alternative to single and multi-step resistance experiments, it could also be of huge help to apply an inverse approach and perform an automated high-troughput staphylococcal killing assay. Using the Nebraska Transposon Mutant Library, to name an example, which is a collection of strains containing mutant derivatives of USA300 LAC in which individual genes have been disrupted by the insertion of the mariner Tn bursa aurealis, we could identify those mutants that are resistant to SF8 and therefore characterize its molecular targets.

Chapter II

Though the harsh truth is that only three clinical studies with repurposed drugs have been performed or are currently underway (Miró-Canturri et al., 2019), we continue being enthusiastic about the concept of drug repurposing. Thanks to a unique genetic tool like *S. aureus* ΔXV (Villanueva et al., 2018), we have found that Verteporfin, a drug that is normally prescribed for macular degeneration, is capable of blocking GraXRS Two-Component-System. Behaving like an anti-virulence compound, Verteporfin was really efficient in enhancing PMN-mediated bacterial killing, while topical administration of such drug in a murine model of surgical wound infection significantly reduced the bacterial load. In this regard, future experiments supporting the new novel antimicrobial therapeutical indication of Verteporfin must be undertaken. It would be very intriguing to study the effect of this drug using an animal model that did not show an intrinsic resistance to *S aureus,* mimicking human susceptibility to this pathogen. This could be case of a rabbit skin-infection model using the host-adapted ST121 strain (Viana et al., 2015), which was indeed the approach that revealed how

crucial the TCS GraXRS is when it comes to staphylococal virulence. Besides, since galenic formulation of drugs might have a great impact on their therapeutical effect, it would also be worth exploring new cream and ointment bases, or even its combination with topical antibiotics like mupirocin.

Could another porphyrin derivative display and improved GraXRS-blocking effect? We believe so. Porphyrins have already been clinically considered for antimicrobial photodynamic therapy due to their capacity to generate highly reactive radicals, but our study suggests that they might possess other biological properties that do not depend on light activation. Since these molecules show important pharmaceutical advantages like the relatively low *in vitro* and in vivo toxicity, a reasonable clearance time from the body, amphiphilicity and ability for numerous chemical modifications, a high binding affinity to cellular components (membranes, proteins, DNA) and a more than reasonable "therapeutic window" whereby they can kill bacteria but do not harm cultured human cells (Amos-Tautua et al., 2019), we are currently considering the possibility of analyzing the GraXRS-blocking potential of other porphyrins like Porfimer sodium, Bacteriocholorophyll A, N-Methylmesoporphyrin, Protoporphyrin, Siroheme or Ferrohem.

Experiments relying on the chemical mimicry existing between Verteporfin and heme group have suggested that redox active residue GraS C227 participates in the inhibition exerted by this FDA-approved drug. Though it is a matter of basic research, it could also be fascinating to understand how this residue mediates GraXRS activity. As described before, we found that replacement of cysteine by another residue had a negative impact on GraXRS activity, being such an outcome dependent on the polarity of the substitute amino acid. Thus, C227-A (non polar) GraS isoform led to a lower degree of transcriptional activity of *dlt*XP in comparison to that showed by the isoform in which C227 had been mutated to the polar amino acid serine. These results have prompted us to hypothesize that, in terms hydrogen-bonding potential, the

reduced form of cysteine could behave in a similar way to that showed by serine. Alanine, by contrast, lacks the hydrogen-bonding potential and thus has a higher impact on the transcriptional activity of the GraXRS-dependent promoter. Our reasoning is that serine retains the ability to form hydrogen-bonds independently of the redox status, leading to a constant state of activation, while alanine causes the opposite effect, being nonreactive (also independently of redox conditions) and simulating the oxidized constant off-mode. To certainly prove these hypotheses, we should perform i*n vitro* experiments in which the phosphorylation of the purified forms of GraS and its C227-A and C227-S derivatives in the presence of Verteporfin, oxidant and reducing agents would be assessed.

Finally, the same approach applied here to find GraXRS-targeting molecules might be used with any ΔXV derivative that had been restored with a single TCS. We are confident that such valuable *in vivo* tools could be an extraordinary complement to *in silico* and *in vitro* screens aimed at finding histidine kinase inhibitors.

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CONCLUSIONES

1. Recombina SL dispone de una plataforma que permite realizar cribados fenotípicos de forma sencilla y económica para la identificación tanto de compuestos anti-biofilm como de aquellos capaces de afectar específicamente a las cepas productoras del polisacárido PIA/PNAG.

2. Una subfracción (SF8) de un extracto procedente de la fermentación de un microorganismo marino inhibe específicamente el crecimiento de cepas de *S. aureus* productoras de PIA/PNAG, sin afectar a aquellas incapaces de producirlo. Este resultado valida la posibilidad de potenciar los efectos colaterales que conlleva la producción del polisacárido PIA/PNAG como potencial estrategia terapéutica antimicrobiana.

3. La sub-fracción 8 (SF8) contiene los principios activos Lumichrome, Malayamicina y Soyasaponina. Ninguno de ellos es individualmente capaz de mostrar la actividad que observamos en el extracto y sus correspondientes fracciones o subfracciones.

4. La sub-fracción 8 (SF8) inhibe la expresión de inhibe la expresión de IcaC, proteína A y otras proteínas de alto peso molecular, siendo este efecto dependiente de la funcionalidad del operón *ica*ADBC.

5. La cepa *S. aureus* MW2ΔXV y sus derivados restaurados en cada uno de los sistemas de dos componentes no esenciales representan un sistema *in vivo* idóneo para los cribados de compuestos capaces de bloquear sistemas de dos componentes como GraXRS.

6. El fármaco Verteporfina, prescrito hasta el momento para el tratamiento de la degeneración macular, es capaz de bloquear el sistema de dos componentes GraXRS.

7. La aplicación tópica de una formulación en base al fármaco Verteporfina es capaz de disminuir significativamente la carga bacteriana en un modelo murino de infección de sutura quirúrgica y reduce la capacidad de *Staphylococcus aureus* para evadir el efecto letal de los polimorfonucleares en la sangre.

8. El residuo redox- activo cisteína 227 (C227) del dominio catalítico del sensor GraS está implicado, al menos parcialmente, en el efecto inhibidor que ejerce el compuesto Verteporfin sobre el sistema GraXRS.

9. El medicamento Verteporfin podría incluirse en la lista de medicamentos cuya indicación terapéutica está siendo repropuesta para el tratamiento de infecciones asociadas al patógeno *S. aureus.*

CONCLUSIONS

1. Recombina SL has developed a platform that enables rapid and inexpensive phenotypic high throughput screenings aimed at the identification of anti-biofilm compounds and those that specifically inhibit PIA/PNAG producer strains.

2. A chemical sub-fraction proceeding from the fermentation of a marine microorganisms specifically inhibits PIA/PNAG producer strains but does not affect their PIA/PNAG negative derivatives. These results validate the hypothesis of considering the reinforcement of negative collateral effects entailed by PIA/PNAG production as a novel antimicrobial therapeutical approach.

3. Sub-fraction 8 (SF8) is composed by Lumichrome, Malayamicin and Soyaponin. Any of these molecules, when tested individually, is capable of reproducing the activity shown by the original extract or their fractions and sub-fractions.

4. Sub-fraction 8 (SF8) exerts a post transcriptional effect on *ica*ADBC operon, inhibiting IcaC protein translation, also inhibits the expression of protein A and other high-molecular-weight protein, being such an outcome dependent on *ica*ADBC functionality.

5. *S. aureus* ΔXV and its derivative strains in which a single nonessential Two Component System has been restored are highly valuable *in vivo* tools for high troughput screenings aimed at finding molecules capable of targeting Two Component Systems like GraXRS.

6. Verteporfin, a drug that has been prescribed for the treatment of macular degeneration up to date, blocks GraXRS Two Component System.

7. Topical application of a formulation based on the drug Verteporfin is capable of significantly reducing the bacterial load in a murine model of surgical infection and reduces the ability of *Staphylococcus aureus* to evade the lethal effect of polymorphonuclear cells in the blood.

8. The redox-active residue cysteine 227 (C227) located in the catalytic domain of GraS is involved, at least partially, in the GraXRS-blocking effect exerted by Verteporfin.

9. Verteporfin is a patent-free FDA-approved drug which could be considered as a novel candidate to be repurposed for anti-*S. aureus* therapeutical interventions.

ANEXO I

Supplementary Information

Table S1. Data obtained from the HTS

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Neomycin sulfate	Antibacterial	180,15	39,79
Promazine hydrochloride	Antipsychotic	-455,16	56,7
Econazole nitrate	Antifungal	173,73	50,6
Ascorbic acid	Antioxidant CNS Stimulant Hemostatic	132	18,77
Chlorphensin carbamate	Muscle relaxant	117,88	8,82
Hesperidin	Anti-haemorrhoids Antineoplastic Antioxidant	115,43	9,86
Nicergoline	Anti-ischemic vasodilator	108,5	49,62
Nalbuphine hydrochloride	analgesic	99,03	50,25
Acetylsalicylic acid	Analgesic Anti-inflammatory Antipyretic	89,91	8
Ornidazole	Antibacterial antiparasitic antiprotozoal	89,65	25,16
Tazobactam	Antibacterial	-383,54	60,19
Clomiphene citrate (Z,E)		85,25	53,63
Streptomycin sulfate	Antibacterial	83,29	29,18
Troglitazone	Antidiabetic anti-inflammatory	79,86	48,71
Daunorubicin hydrochloride	Antibacterial antineoplastic	70,01	30,82
Thioridazine hydrochloride	Antipsychotic	67,57	-26,73
Oxantel pamoate	Antihelmintic	66,59	6,44
Cefazolin sodium salt	Antibacterial	-1702,12	95,61
Triclabendazole	Antihelmintic	64,07	2,36
Scopolamin-N-oxide hydrobromide	Antispastic mydriatic	63,42	-1,86
Tiratricol, 3,3',5-triiodothyroacetic acid	Antihypothyroid hypocholesterolemic	60,48	34,92
Amidopyrine	Analgesic anti-inflammatory antipyretic	59,64	32,77
Aminocaproic acid	Antifibrionolytic Hemostatic	58,89	8,78
Trimethobenzamide hydrochloride	Antiemetic	55,32	-15,5
Azaguanine-8	Antineoplastic	53,86	4,14
Orphenadrine hydrochloride	Antihistaminic antiparkinsonian	47,19	-7,04
Sulindac	nalgesic anti-inflammatory antypyretic	45,8	-14,37
Busulfan	Antineoplastic	43,69	29,62
Flavoxate hydrochloride	Antispastic	43,25	2,87
Monensin sodium salt	Antibacterial	70,62	80,76
Hyoscyamine (L)	Antiemetic antispastic mydriatic	42,71	7,27

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Diacerein	Antiarthritic	42,06	15,85
Amisulpride	Antipsychotic	-310,8	55,94
Cefpodoxime proxetil	Antibacterial	-306,94	72,71
Demeclocycline hydrochloride	Antibacterial	-69,98	89,49
Amitryptiline hydrochloride	Antidepressant	41,03	-40,03
Piretanide	Antihypertensive Diuretic	39,78	-2,32
Amprolium hydrochloride	anticoccidial antiparasitic	38,28	-13,2
Ethosuximide	Anticonvulsant	37,08	26,96
Paclitaxel	Antineoplastic	36,91	38,02
Diazoxide	Antidiuretic antihypertensive vasodilator	36,44	37,36
Valproic acid	Anticonvulsant	36,39	5,2
Phenelzine sulfate	Antidepressant	36,16	33,49
Verteporfin		33,95	30,24
Imipramine hydrochloride	Antidepressant	32,51	-27,35
Clebopride maleate	Antiemetic Antispastic	31,2	30,25
Azacytidine-5	Antineoplastic	30,95	49,41
Adiphenine hydrochloride	Antispastic	30,73	-16,78
Sildenafil	Antihypertensive Erectile dysfunction treatment	30,69	20
Carprofen	Anti-inflammatory	30,38	24,96
Chlorpheniramine maleate	Antihistaminic antitussive sedative	29,77	50,15
Hydralazine hydrochloride	Antihypertensive	28,62	22,14
Prednisone	Anti-inflammatory antipruritic Immunosuppressant	27,07	-22,18
Acetazolamide	Anticonvulsant antiglaucoma diuretic	26,95	-8,17
Prednicarbate	Anti-inflammatory	26,56	-2,42
Clotrimazole	Antibacterial antifungal	26,55	15,35
Todralazine hydrochloride	Antihypertensive	25,89	-34,39
(-)-Emtricitabine	antiviral	-257,79	56,73
Alexidine dihydrochloride	Antibacterial	36,85	94,38
Riluzole hydrochloride	Antipastic Neuroprotectant	25,89	23,66
Chloropyramine hydrochloride	Antihistaminic	25,54	-4,78
Diphemanil methylsulfate	Antispastic antiulcer	25,17	-19,33
Isoflupredone acetate	Anti-inflammatory	23,83	-3,75
Ketoconazole	Antifungal	23,57	29,91
Diethylstilbestrol		23,25	35,06
Proglumide	Antiulcer	22,66	-0,42
Dicumarol	Anticoagulant	-523,22	74,71

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Desonide	Antipsoriatic	20,05	-0,61
Merbromin	Antibacterial	-3277,68	92,27
Verapamil hydrochloride	Antihypertensive	19,94	35,51
Acyclovir	Antiviral	19,72	28,55
Cisatracurium besylate	Muscle relaxant	19,72	-6,16
Disulfiram	Antabuse effect	19,14	46,42
Cefprozil	Antibacterial	18,36	7,81
Tolfenamic acid	Analgesic anti-inflammatory	18,25	33,52
Etomidate	Anesthetic Hypnotic	18,05	3,23
Tioconazole	Antifungal	17,7	54,75
Nitrofurantoin	Antibacterial	17,5	26,14
Pemetrexed disodium	Antienoplastic	16,84	-0,68
Carbinoxamine maleate salt	Antihistaminic	16,48	-2,82
Silodosin	Antihypertenisve	16,33	0,44
Sulconazole nitrate	Antifungal	82,06	72,66
Brompheniramine maleate	Antihistaminic antipruritic antitussive	15,57	26,91
Mitoxantrone dihydrochloride	Antineoplastic	15,07	23,47
Oxymetazoline hydrochloride	Nasal decongestant vasoconstrictor	14,94	39,45
Penciclovir	Antiviral	14,8	30,79
Dibucaine	Local anesthetic	14,78	-14,63
Spectinomycin dihydrochloride	Antibacterial	14,51	-1,78
Cytarabine	Antineoplastic	-241,17	58,32
Furazolidone		14	12,21
Moxalactam disodium salt	Antibacterial	-692,97	97,01
S-(+)-ibuprofen	Analgesic Anti-inflammatory	12,89	15,15
Auranofin	Analgesic	104,81	96,32
Indapamide	Antihypertensive diuretic	12,74	42,6
Hydrochlorothiazide	antihypertensive diuretic	12,24	-6,91
Metformin hydrochloride	anorectic antidiabetic antilipmic	11,91	5,61
Sulmazole	Cardiotonic	11,79	5,44
Oxybenzone		11,03	-5,77
Valsartan	Vasodilator Antihypetensive	10,84	-3,63
Repaglinide	Antidiabetic	10,38	4,34
Gallamine triethiodide	Muscle relaxant	10,12	21,75
Nefopam hydrochloride	Analgesic	9,68	40,78
Raltitrexed	Antineoplastic	9,63	-3,86
Suprofen	Analgesic Anti-inflammatory	9,36	13,93

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Thyroxine (L)	Antihypothyroid antilipemic hypocholesterolemic	9,33	13,73
Tacrine hydrochloride	CNS Stimulant	8,78	22,66
Sulbactam	Antibacterial	8,61	4,65
Edrophonium chloride	Anti-fatigue	8,56	24,75
Brimonidine L-Tartrate	Antiglaucoma	8,43	2,22
Guanethidine sulfate	Antihypertensive Local anesthetic	8,05	4,75
Tridihexethyl chloride	Antispastic	6,59	-1,83
Mefenamic acid	Analgesic anti-inflammatory antipyretic	6,5	17,94
Ethynylestradiol 3-methyl ether		6,39	14,47
Penbutolol sulfate	Antianginal Antiarrhythmic Antihyperensive	6,29	4,36
Lithocholic acid	Cholangogue Choleretic	6,06	25,71
Novobiocin sodium salt	Antibacterial	113,09	83,58
Antimycin A	Antibacterial antifungal	5,46	45,65
Clobutinol hydrochloride	Antitusive	-227,43	58,87
Dichlorphenamide	Antiglaucoma	5,35	-1,62
Zoxazolamine	Antigout Muscle relaxant Uricosuric	5,22	6
Clinafloxacin	Antibacterial	62,07	77,42
Trimipramine maleate salt	Antidepressant	5,03	-2,92
Piromidic acid	Antibactierial	4,87	1,47
Allantoin	Antipsoriatic Vilnerary	3,48	-5,66
Nafcillin sodium salt monohydrate	Antibacterial	-488,7	96,58
Hydroxyzine dihydrochloride	antiemetic antihitaminic antipruritic	3,28	46,1
Rofecoxib	Anti-inflammatory	2,98	8,5
Dipyridamole	Anticoagulant antiplatelet vasodilator	2,31	45,14
Phenylpropanolamine hydrochloride	Antihypotensive Nasal Decongestant Vasoconstrictor	2,3	17,48
Miconazole	Antifungal	2,1	53,1
Milnacipran hydrochloride	Antidepressant Analgesic	2,07	4,57
Dilazep dihydrochloride	Antiplatelet vasodilator	1,74	14,01
Amiloride hydrochloride dihydrate	antihypertensive diuretic	1,7	-4,42
Chlorzoxazone	Anticonvulsant Muscle relaxant	1,69	23,43
Canrenoic acid potassium salt	Antihypertensive diuretic	1,47	49,88
Roxithromycin	Antibacterial	107,08	81,98
Methazolamide	Antiglaucoma Diuretic	1,41	-2,55
Bisoprolol fumarate	Antianginal Antiarrhythmic Antihypertensive	1,11	10,04

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Atracurium besylate	Curarizing	0,78	-4
Sulfamethoxypyridazine	Antibacterial	0,78	11,18
Haloprogin	Antifungal	0,46	47,63
Tyloxapol	Mucolytic	0,15	-3,13
Oxyphenbutazone	Anti-inflammatory	-0,11	1,48
Troxipide	Antiulcer	-0,67	3,9
Gestrinone	Contraceptive	-0,95	-4,9
Methyldopa (L,-)	Antihypertensive	-1,5	23,67
Ubenimex	Antineoplastic Immunomodulator	-1,83	8,18
Indinavir sulfate	Antiviral	-2,1	12,26
Pyrithyldione	Hypnotic Sedative	-2,36	-0,49
Loracarbef	Antibacterilal	-208	64,86
Etoposide	Antineoplatic	-2,42	31,84
Gemfibrozil	Hypocholesterolemic lipid-lowering	-2,79	31,14
Testosterone propionate	Anabolic	-3,23	9,71
Albendazole	Antihelmintic antiparasitic	-4,56	12,37
Doxapram hydrochloride	Analeptic Eupneic	-4,89	5,08
Idebenone	Antineoplastic	-5,13	7,44
Amorolfine hydrochloride	Antifungal	-6,16	8,28
Meglumine	Antileishmanial Antiseptic Expectorant	-6,55	-2,24
Metronidazole	Antiamebic antibacterial antiprotozoal	-6,87	43,47
Flufenamic acid	Analgesic anti-inflammatory antipytetic	-7,92	35,14
Trifluoperazine dihydrochloride	Antiemetic antipsychotic	-8,18	12,76
Carbamazepine	Analgesic anticonvulsant antidiuretic	-9,02	15,35
Denatonium benzoate		-9,11	-2,3
Esmolol hydrochloride	Antiarrhythmic	-9,52	3,08
Carbachol	Antihypertenisve Vasodilator	-9,83	8,81
Pravastatin	Antilipemic	-10,01	17,7
Ketanserin tartrate hydrate	Antihypertensive Vasodilator	-10,37	0,68
Carbenoxolone disodium salt	Antiulcer	-10,37	31,75
Digoxigenin		-10,63	-1,84
Quinethazone	Antihypertensive Diuretic	-11	8,13
Rabeprazole Sodium salt	Antiulcer	-11,03	12,15
Amlexanox	Anti-inflammatory Immunomodulator	-11,48	6,07
Fulvestrant	Antineoplastic	-11,92	37,59
(+) -Levobunolol hydrochloride	Antiglaucoma	-11,94	12,79

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Morantel tartrate	Antihelmintic	-11,95	5,28
Fludarabine	Antineoplastic	-12	44,86
Phentolamine hydrochloride	Antihypertensive vasodilator	-12,39	33,97
Estradiol Valerate	Contraceptive	-12,79	39,05
Didanosine	Antiviral	-12,87	35,22
Rosiglitazone Hydrochloride	Antidiabetic	-13,02	22,84
Scopolamine hydrochloride	Antiemetic	-13,14	7,33
Lorglumide sodium salt	Antiulcer	-13,48	18,92
Etanidazole	Antineoplastic chemosensitizer	-13,95	5,37
Fenbufen	Analgesic anti-inflammatory antipytetic	-14,14	43,62
Hexetidine	Antifungal antiseptic	-14,41	3,16
Sulfaguanidine	Antibacterial	-14,59	-8,92
Pentobarbital	Anesthetic hypnotic sedative	-14,62	2,31
Enalapril maleate	Antihypertensive	-14,88	12,35
Dienestrol		-15,46	11,33
Nifedipine	Antianginal antihypertensive vasodilator	-15,96	20,06
Bacitracin	Antibacterial	-566,7	95,46
Gemifloxacin mesylate	Antibacterial	56,18	88,42
Lynestrenol	Contraceptive	-16,03	49,14
Mepivacaine hydrochloride	Local anesthetic	-16,23	15,16
Nomifensine maleate	Antidepressant	-16,29	54,22
Moroxidine hydrochloride	Antiviral	-16,63	33,73
Liranaftate	Antifungal	-17,29	-3,87
Prochlorperazine dimaleate	Antiemetic Antiphychotic	-17,54	31,07
Cladribine	Antineoplastic	-190,03	56,17
Iopromide	Contrastant	-17,55	11,66
Promethazine hydrochloride	Antihistaminic Sedative	-17,87	2,94
Iopamidol	Contrastant	-17,94	13,36
Fluphenazine dihydrochloride	Antipsychotic	-18	22,94
Azilsartan kamedoxomil	Antihypertensive	-18,15	52,25
Bemegride	CNS Stimulant	-18,76	-4,01
Clofilium tosylate	Antirrhythmic	-19,04	1,63
Thonzonium bromide	Antiseptic	-1906,98	97,79
Deoxycorticosterone	Anti-inflammatory	-19,28	15,63
Meprylcaine hydrochloride	Local anesthetic	-19,37	18,81
Iobenguane sulfate	Antineoplastic	-19,44	10,03
Danazol	Anabolic antigonadotropin	-19,49	48,35

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Crotamiton	Antipruritic	-20,88	5,88
Niacin	Antilipemic Vasodilator	-21,64	0,13
Canrenone	Diuretic	-21,66	-2,78
Zalcitabine	Antiviral	-21,74	17,05
Lamivudine	Antiviral	-184,31	57,07
Simvastatin	Antilipemic	-21,83	21,03
Ambroxol hydrochloride	Expectorant Mucolytic	-21,95	47,43
Anastrozole	Antineoplastic	-22,14	9,61
Xylometazoline hydrochloride	Nasal decongestant vasoconstrictor	-22,18	42,41
Pyrantel tartrate	Antihelmintic	-23,18	26,85
Benzthiazide	Antihypertensive Diuretic	-23,29	16,11
Piperacetazine	Antipsychotic	-23,5	4,93
Loperamide hydrochloride	Antidiarrheal	-24,09	46,02
Pergolide mesylate	Antiparkinsonian	-24,87	36,71
Sulfadimethoxine	Antibacterial	-24,87	10,97
Dehydroisoandosterone 3-acetate		-24,89	24,58
Cefaclor hydrate	Antibacterial	-25,06	20,33
Isoxsuprine hydrochloride	Vasodilator	-25,12	15,33
Algestone acetophenide	Contraceptive Anti-inflammatory	-25,44	16,35
Mephentermine hemisulfate	Antihypotensive Vasoconstrictor	-25,67	-0,93
Nevirapine	Antiviral	-25,98	2,05
Idoxuridine	Antiviral	-26	28,24
Paromomycin sulfate	Antiamebic Antibacterial	-26,04	38,02
Sotalol hydrochloride	Antianginal Antiarrhythmic Antihypertenisve	-26,15	0,64
Dosulepin hydrochloride	Antidepressant CNS stimulant	-26,53	6,63
Sulfadoxine	Antibacterial	-26,59	23,44
Azaperone	Antipsychotic Sedative	-26,7	11,73
Eprosartan mesylate	Antihypertenisve	-27,69	17,46
Raloxifene hydrochloride		-175,94	55,81
Bupropion hydrochloride	Antidepressant	-27,97	17,82
Entacapone	Antiparkinsonian	-28,06	10,82
Enilconazole	Antifungal	-28,16	7,65
Procarbazine hydrochloride	Antineoplastic	-28,47	14,3
Buspirone hydrochloride		-28,49	8,38
Methacycline hydrochloride	Antibacterial	91,59	88,74
Floxuridine	Antineoplastic Antiviral	119	73,49

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Amiprilose hydrochloride	Immunomodulator	-28,69	20,28
Propantheline bromide	Antispastic	-28,71	15,62
Indatraline hydrochloride	Antidepressant	-28,82	9,43
Zolmitriptan		-29,48	16,44
Fentiazac	Anti-inflammatory	-29,49	28,52
Sulfamethoxazole	Antibacterial	-29,88	50,57
Carbetapentane citrate	Antispastic Antitussive Local anesthetic	-29,89	35,12
Trimebutine	Antispastic	-30,3	8,05
Dimethadione	Anticonvulsant	-31,02	14,78
Reserpine	Antipsychotic	-31,21	25,06
Dolasetron mesilate	Antiemetic	-31,24	4,27
(R)-Propranolol hydrochloride	Antianginal Antiarrhythmic Antihyperensive	-31,26	20,01
Balsalazide Sodium	Anti-inflammatory	-31,32	13,93
Nelfinavir mesylate	Antiviral Antineoplastic	-31,57	8,27
Darifenacin hydrobromide		-31,57	2,75
Meclofenamic acid sodium salt monohydrate	Anti-inflammatory antipytetic	-31,62	37,53
Pepstatin A	Antiviral	-31,76	18,42
Decamethonium bromide	Muscle relaxant	-31,98	3,55
Nicotinamide		-32,25	12,17
Bezafibrate	Antilipemic Hypocholesterolemic Lipid-lowering	-32,54	34,48
Tolmetin sodium salt dihydrate	Anti-inflammatory	-33,03	29,83
Ciprofibrate	Hypocholesterolemic	-33,08	26,47
Lofepramine	Antidepressant Anxiolytic Sedative	-33,56	13,79
Ioversol	Contrastant	-33,78	13,59
Flurbiprofen	Analgesic Anti-inflammatory	-34,07	36,26
Delavirdine		-34,25	9,57
Althiazide	Antihypertensive	-34,36	3,74
Metolazone	Antihypertensive diuretic	-34,4	49,31
Nicorandil	Antianginal vasodilator	-34,45	50,28
Alfuzosin hydrochloride	Vasodilator	-34,51	8,73
Ranolazine	Antianginal	-34,64	18,65
Mesalamine	Anti-inflammatory	-35,03	17,32
(R)-(+)-Atenolol	Antianginal Antiarrhythmic Antihypertenisve	-35,64	15,89
Antipyrine	Analgesic anti-inflammatory antipyretic	-35,85	19,66
Benzylpenicillin sodium	Antibacterial	-161,88	57,51

Name	Therapeutic effect	% GraXRS inhibition	% Growth inhibition
Hydroflumethiazide	antihypertensive diuretic	-36,07	34,12
Sertaconazole nitrate	Antibacterial Antifungal	-36,3	28,45
Articaine hydrochloride	Anesthetic	-36,58	6,06
Clidinium bromide	Antispastic	-36,79	21,1
Baclofen (R,S)	Antispastic, muscle relaxant alcohol addiction treatment	-36,9	31,34
Megestrol acetate	Antineoplastic Contraceptive	-37,15	11,31
Sulfanilamide	Antibacterial	-37,2	1,39
Indoprofen	Analgesic Anti-inflammatory	-37,21	18,34
Donepezil hydrochloride	Anti-Alzheimer Antipsychotic CNS Stimulant	-37,59	20,14
Serotonin hydrochloride	CNS stimulant	-38,31	3,06
Flucloxacillin sodium	Antibacterial	-873,24	96,33
Norethynodrel	Contraceptive	-38,4	25,25
Mecamylamine hydrochloride	Antihypertensive	-38,43	18,72
Carbimazole	Antihyperthyroid	-158,16	60,16
Ethionamide	Antibacterial	-38,71	20,1
Droperidol	Antipsychotic	-39,45	30,66
Alprenolol hydrochloride	Antianginal antiarrhythmic antihypertensive	-39,6	20,27
Colistin sulfate	Antibacterial	-40,03	7,24
Rivastigmine		-40,28	18,34
Deflazacort	Anti-inflammatory Immunosuppressant	-40,63	30,15
Chicago sky blue 6B		-40,83	12,46
Mafenide hydrochloride	Antibacterial antiseptic	-41,09	25,49
Dorzolamide hydrochloride	Antiglaucoma Antihypertensive	-41,49	23,9
Clioquinol	Antiamebic Antifungal Antiseptic	-41,7	12,03
Vecuronium bromide	Muscle relaxant	-42,01	38,56
Levofloxacin	Antibacterial	-18,19	84,78
Rebamipide	Antiulcer	-42,04	-2,32
Mianserine hydrochloride	Antidepressant anxiolytic	-42,11	54,9
Fenoldopam	Antihypertenisve Vasodilator	-42,15	16,48
Nylidrin	Vasodilator	-42,16	19,62
Procaine hydrochloride	Local anesthetic	-42,26	24,61
Saquinavir mesylate	Antiviral	-42,73	18,75
Fluocinolone acetonide	Anti-inflammatory	-42,78	29,68
Vatalanib	Antineoplastic	-42,79	13,83
Itraconazole	Antifungal	-43,49	7,03
Dapsone	Antibacterial antimalarial	-43,51	27,04

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Toremifene	Antineoplastic	-43,53	15,8
Topiramate	Anticonvulsant Antimigraine	-43,96	24,78
Tribenoside		-43,97	27,84
Butoconazole nitrate	Antibacterial antifungal	-44,13	36,77
Phenethicillin potassium salt	Antibacterial	-44,54	26,1
Histamine dihydrochloride	Antineoplastic Analgesic	-44,94	17,03
Nomegestrol acetate	Contraceptive	-45,08	15,32
Isopyrin hydrochloride	Analgesic Anti-inflammatory antipyretic	-45,41	0,1
Pancuronium bromide	Muscle relaxant	-45,42	8,38
Glibenclamide	Antidiabetic	-45,58	19,29
Acetylsalicylsalicylic acid	Analgesic anticoagulant anti-inflammatory	-45,9	52,71
S(-)Eticlopride hydrochloride		-46,05	19,06
Fenbendazole	Antihelmintic	-46,46	42,38
Dropropizine (R,S)	Antitussive	-46,68	23,53
Nialamide	Antidepressant	-46,77	23,53
Oxalamine citrate salt	Anti-inflammatory Antispastic Antitussive	-47,48	24,45
Eserine hemisulfate salt	Antiglaucoma	-47,65	8,28
Benperidol	Antipsychotic	-47,77	4,37
(-)-Isoproterenol hydrochloride	Bronchodilator Vasodilator	-48,08	25,75
Pentylenetetrazole	Analeptic CNS stimulant	-48,36	29,53
Ethoxzolamide	Antiglaucoma Antiulcer Diuretic	-48,82	11,04
Nifenazone	Analgesic anti-inflammatory antipytetic	-48,87	41,43
Pheniramine maleate	Antihistaminic antitussive sedative	-49,13	21,16
Neostigmine bromide	Anti-fatigue	-49,28	41,43
Metyrapone		-49,42	16,42
Amphotericin B	Antibacterial antifungal	-49,42	17,85
Hexestrol	Antineoplastic	-49,77	34,27
Clocortolone pivalate	Anti-inflammatory	-49,88	15,29
Nefazodone hydrochloride	Antidepressant	-50,74	37,07
D-cycloserine	Antibacterial	-50,91	29,21
Ioxaglic acid	Contrastant	-50,93	27,16
Cilnidipine	Antihypertensive	-51,4	47,94
Procyclidine hydrochloride	Antiparkinsonian Muscle relaxant	-51,89	31,5
Dicyclomine hydrochloride	Antispastic	-52,29	30,98
Ethaverine hydrochloride	Antispastic	-52,31	27,27
Avermectin B1	Antihelmintic	-52,87	52,15

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Diclofenac sodium	Anti-inflammatory	-138,18	60,5
Ketoprofen	Analgesic anti-inflammatory antipytetic	-53,21	36,49
Bicalutamide	Antineoplastic	-53,59	22,13
Naftifine hydrochloride	Antifungal	-53,66	37,86
Mefexamide hydrochloride	CNS Stimulant	-53,78	48,05
Tazarotene	Antipsoriatic antiacne	-53,78	12,37
N-Acetyl-DL-homocysteine Thiolactone	Expectorant	-53,91	-2,78
Idazoxan hydrochloride	Antiparkinsonian Antipsychotic	-54	19,96
Itopride		-54,1	10,52
Ethambutol dihydrochloride	Antibacterial	-54,17	33,98
Loxapine succinate	Antipsychotic anxiolytic	-54,31	42,15
Ronidazole	Antibacterial Antiprotozoal Antitrichomonal	-54,42	26,93
Celecoxib	Anti-inflammatory	-55,09	35,82
Estrone		-55,22	27,77
Bethanechol chloride		-55,47	36,84
Iopanoic acid	Contrastant	-55,73	47,98
Panthenol (D)	Anti-alopecia	-55,74	26,13
Nafronyl oxalate	Anti-ischemic Antispastic Vasodilator	-55,77	41,61
Mexiletine hydrochloride	Antirrhythmic local anesthetic	-55,78	15,94
Levalbuterol hydrochloride	Antiasthmatic Bronchodilator	-56,2	34
Acarbose	Antidiabetic	-56,2	13,37
Glutethimide, para-amino	Antineoplastic	-56,24	13
Atorvastatin		-56,45	26,4
Enalaprilat dihydrate	Antihypertenisve	-56,69	17,43
Iodixanol	Contrastant	-56,77	26,9
Sarafloxacin	Antibacterial	50,98	78,47
Rifabutin	Antibacterial	-263,66	94,54
Apramycin	Antibacterial	-57,08	24,34
4-aminosalicylic acid	Antibacterial Antifungal	-57,38	32,03
Estramustine	Antineoplastic	-57,75	28,39
Perindopril	Antihypertenisve	-57,86	35,66
Quinapril hydrochloride	Antihypertensive	-58,34	37,86
Bufexamac	Analgesic anti-inflammatory antipyretic	-58,36	-0,36
Primaquine diphosphate	Antimalarial	-58,57	19,87
Mirtazapine	Antidepressant	-58,58	18,26
Benzoxiquine	Antiseptic	-58,6	26,12

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Acetaminophen	Analgesic Antipyretic	-58,62	30,78
Nadifloxacin	Antibacterial	19,1	91,49
Fluvoxamine maleate	Antidepressant CNS Stimulant	-58,62	20,82
Viomycin sulfate	Antibacterial	-58,64	21,3
Anthralin	Antipsoriatic	-58,7	35,17
Mevastatin	Hypocholesterolemic	-58,74	7,12
Triamterene	antihypertensive diuretic	-58,8	33,33
Atropine sulfate monohydrate	Antispastic mydriatic	-58,8	10,64
Stavudine	Antiviral	-59,02	42,59
Cefepime hydrochloride	Antibacterial	-627,3	96,49
Rifaximin	Antibacterial	-73,94	94,16
Fluvastatin sodium salt	Antilipemic	-59,17	21,35
Selegiline hydrochloride	Antiparkinsonian	-59,32	8,09
Valdecoxib	Antiarthritic Anti-inflammatory	-59,55	23,53
Doxycycline hydrochloride	Antibacterial	103,15	89,58
Carbadox	Antibacterial	-128,95	68,09
Fleroxacin	Antibacterial	71,83	75,89
Clavulanate potassium salt	Antibacterial	-182,94	69,76
Nalmefene hydrochloride		-59,61	29,23
Ethopropazine hydrochloride	Antiparkinsonian	-60,17	37,18
Perospirone	Antipsychotic	-60,17	23,07
Pyridostigmine iodide		-60,19	7,04
Levocabastine hydrochloride	Antihistaminic	-60,46	13,11
Adamantamine fumarate	Antiviral	-60,47	14,13
Homatropine hydrobromide (R,S)	Antispastic mydriatic	-60,49	23,02
Iocetamic acid	Contrastant	-60,5	31,59
Antipyrine, 4-hydroxy		-60,54	29,84
Acebutolol hydrochloride	Antianginal antiarrhythmic antihypertensive	-60,61	21,49
Benoxinate hydrochloride	Local anesthetic	-61,1	17,15
Urosiol		-61,13	18,36
Imatinib	Antineoplastic	-61,16	17,69
Oxethazaine	Local anesthetic	-61,6	27,72
Spironolactone	Diuretic	-62,18	47,47
Diphenhydramine hydrochloride	Antiemetic antihitaminic antitussive	-62,3	26,15
Cefdinir	Antibacterial	-543,28	96,98
Phenothiazine	Antipsychotic Antiemetic Antimigraine	-62,33	23,09
Nabumetone	Analgesic Anti-inflammatory	-62,68	15,39

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Formoterol fumarate	Antiasthmatic	-62,82	21,37
Montelukast	Antiasthmatic	-62,85	41,46
Butacaine	Anesthetic	-62,86	36,75
Etilefrine hydrochloride	Vasoconstrictor	-62,93	29,33
Nicardipine hydrochloride	Antianginal Antihypertensive	-62,98	36,03
Cyproterone acetate	Antineoplastic Contraceptive	-63,04	31,66
Flucytosine	Antifungal	-63,07	24,9
Ceftibuten	Antibacterial	-63,6	43,19
Captopril	Antihypertensive vasodilator	-63,65	32,62
Triclosan	Antibacterial Antifungal Antiseptic	66,77	92,54
Enoxacin	Antibacterial	59,74	75,6
Prothionamide	Antibacterial	-63,98	23,13
Latanoprost	Antiglaucoma	-64,27	35,04
Pramipexole dihydrochloride	Antiparkinsonian	-64,32	24,22
Flumethasone pivalate	Anti-inflammatory	-64,7	21,65
Telmisartan	Antihypertensive	-122,3	56,98
Trapidil	Vasodilator	-64,79	25,38
Ticarcillin sodium	Antibacterial	-65,01	39,96
Gliclazide	Anticoagulant antidiabetic	-65,1	20,94
2-Aminobenzenesulfonamide	Diuretic	-65,24	26,23
Sparfloxacin	Antibacterial	93,31	77,7
Palonosetron hydrochloride	Antiemetic	-65,67	24,35
Clarithromycin	Antibacterial	-78,27	78,85
Trimeprazine tartrate	Antihistaminic Antipruritic Sedative	-65,67	36,24
Ezetimibe	Hypocholesterolemic	-65,89	30,12
Sertraline	Antidepressant CNS Stimulant	-65,92	27,77
Pantoprazole sodium	Antiulcer	-65,93	25,01
Azithromycin	Antibacterial	47,23	76,8
Pioglitazone		-65,94	49,33
Iodipamide	Contrastant	-66	22,47
Trichlormethiazide	Antihypertensive Diuretic	-66,22	34,42
Bromperidol	Antipsychotic	-66,5	24,11
Chloramphenicol	Antibacterial	-66,59	31,82
Ritonavir	Antiviral	-66,76	23,43
Oxymetholone	Anabolic	-66,77	30,74
Gatifloxacin	Antibacterial	46,56	85,07
Prazosin hydrochloride	Antihyperensive	-66,79	41,03

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Gemcitabine	Antineoplastic	16,91	77,03
Equilin		-67,5	31,04
Anagrelide	Thrombolytic	-67,62	27,36
Amrinone		-67,72	23,98
Bucladesine sodium salt		-67,95	27,44
Sulfacetamide sodic hydrate	Antibacterial Antipsoriatic	-68,19	31,39
Chlorpromazine hydrochloride	Antiemetic antihypertensive antipsychotic	-68,2	21,73
Hexachlorophene	Antiseptic	-89,63	96,86
Ampicillin trihydrate	Antibacterial	-68,37	51,2
Moxifloxacin	Antibacterial	81,62	85,18
Pralidoxime chloride		-68,46	32,36
Rufloxacin	Antibacterial	185,42	78,28
Methocarbamol	Analgesic muscle relaxant	-68,77	37,24
Doxylamine succinate	Anti-anorectic antiemetic antihitaminic	-69,42	31,34
Nitrendipine	Antihypertenisve	-69,5	20,56
Pyrvinium pamoate		-69,57	47,75
Betazole hydrochloride	Diagnostic	-69,68	34,62
Ibudilast	Anti-inflammatory	-69,76	26,66
Fosfosal	Analgesic	-70,02	27,64
Ampiroxicam	Anti-inflammatory Analgesic	-70,56	20,67
Pinacidil	Antihypertensive vasodilator	-70,78	23,63
Guanabenz acetate	Antihypertensive	-70,81	49,39
Chlormadinone acetate	Antineoplastic	-70,86	22,71
Ifenprodil tartrate	Vasodilator	-71,29	-2,74
Flurandrenolide	Anti-inflammatory antipruritic	-71,6	7,16
Enrofloxacin	Antibacterial	57,64	79,49
Aceclofenac	Analgesic anti-inflammatory	-71,63	47,77
Pindolol	Antianginal antiarrhythmic antiglaucoma	-71,65	16,73
Piracetam	CNS stimulant	-71,74	32,16
Proparacaine hydrochloride	Anesthetic	-71,74	16,25
Butalbital	Hypnotic sedative	-71,9	30,64
Capecitabine	Antineoplastic	-72,51	36,58
Theophylline monohydrate	Bronchodilator CNS Stimulant Diuretic	-72,58	17,5
Carvedilol	Antihypertensive	-73,15	38,7
Tolcapone	Antiparkinsonian	-153,68	80,96
Naphazoline hydrochloride	Nasal Decongestant Vasoconstrictor	-73,21	31,21

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Vardenafil	Erectile dysfunction treatment	-73,5	30,48
Cromolyn disodium salt	Antiasthmatic Anti-inflammatory	-73,73	36,78
Pirenzepine dihydrochloride	Antiulcer	-73,78	53,32
Methiazole	Antihelmintic	-74,31	35,26
Astemizole	Antihistaminic	-74,53	45,44
Nitazoxanide	Antiprotozoal	-156,12	67,85
Levodopa	Antiparkinsonian	-74,56	32,69
Propoxycaine hydrochloride	Anesthetic	-74,69	35,08
Hexamethonium dibromide dihydrate	Antihypertensive	-74,88	38,01
Pipemidic acid	Antibacterial	-75	31,5
Pidotimod	Immunostimulant	-75,19	30,25
Retinoic acid	Keratolytic	-75,34	31,67
Meticrane	antihypertensive diuretic	-75,48	30,78
Diflunisal	Analgesic anti-inflammatory antipyretic	-75,59	36,34
Formestane	Antineoplastic	-75,81	37,55
Salmeterol	Bronchodilator	-76,08	35,89
Furaltadone hydrochloride	Antibacterial	-76,32	42,27
Benfluorex	Anorectic Antidiabetic CNS Stimulant	-76,34	48,19
(-)-Eseroline fumarate salt	Analgesic	-76,36	38,77
Tolazoline hydrochloride	Vasodilator	-76,63	29,47
Sulfaphenazole	Antibacterial	-76,71	33,25
Betaxolol hydrochloride	Antiglaucoma Antihypertensive	-77,05	33,04
Cefuroxime axetil	Antibacterial	-292,99	81,47
Doxazosin mesylate	Antihypertensive	-77,09	32,45
Thiocolchicoside	Antispastic muscle relaxant	-77,1	25,35
Deferoxamine mesylate	Chelating	-77,6	11,92
(-) -Levobunolol hydrochloride	Antiglaucoma	-77,83	32,28
Oxfendazol		-77,96	22,73
Dofetilide	Antiarrhythmic	-77,98	28,67
Isradipine	Antianginal Antihypertenisve	-78,27	28,94
Besifloxacin hydrochloride	Antibacterial	3,54	93,13
Ritodrine hydrochloride	Tocolytic	-78,27	33,08
GBR 12909 dihydrochloride	Antidepressant	-78,44	40,62
Benzydamine hydrochloride	Analgesic anti-inflammatory antipyretic	-78,45	21,55
Naltrexone hydrochloride dihydrate	Analgesic	-78,65	44,74
Methotrimeprazine maleat salt	Analgesic antiemetic sedative	-78,73	24,75

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Moxisylyte hydrochoride	Erectile dysfunction treatment vasodilator	-78,87	32,03
Tolbutamide	Antidiabetic	-79,31	42,04
Methicillin sodium		-108,81	61,23
Ethacrynic acid	Diuretic	-79,47	36,71
Minoxidil	Anti-alopecia antihypertensive vasodilator	-79,54	23,87
Trimethadione	Anticonvulsant antiepileptic	-79,69	26,49
Viloxazine hydrochloride	Antidepressant	-80,39	31,41
Beclomethasone dipropionate	Anti-inflammatory	-80,42	33,68
Methylhydantoin-5-(L)	Anticonvulsant	-80,74	32,02
Nandrolone	Antianemic	-80,83	25,62
Isotretinoin	Keratolytic	-80,84	39,59
Tolterodine tartrate	Muscle relaxant	-81,01	29,02
Isoxicam	Analgesic anti-inflammatory antipyretic	-81,11	34,02
Methylhydantoin-5-(D)		-81,23	39,9
Ramipril	Antihypertensive	-81,32	39,43
Nadolol	Antianginal Antihypertensive	-81,57	28,71
Darunavir		-81,92	27,33
Fenspiride hydrochloride	Antitussive bronchodilator	-82,49	35,5
Lamotrigine	Anticonvulsant	-82,56	34,85
Eszopiclone	Hypnotic	-82,74	30,69
Biperiden hydrochloride	Antiparkinsonian	-82,74	27,8
Amiodarone hydrochloride	Antianginal Antiarrhythmic	-82,98	39,24
Isosorbide mononitrate	Antianginal	-83,08	38,07
Racepinephrine hydrochloride	Bronchodilator Vasoconstrictor	-83,27	40,22
Hemicholinium bromide	Curarizing	-83,5	22,9
Cyclopenthiazide	Antihypertensive Diuretic	-83,52	30,86
Ganciclovir	Antiviral	-83,68	35,7
Theobromine	Bronchodilator Diiuretic	-83,79	18,3
6-Furfurylaminopurine		-83,79	30,55
Gabapentin	Anticonvulsant	-83,82	32,52
Valacyclovir hydrochloride	Antiviral	-83,86	29,66
Felbamate	Antiepileptic	-83,9	25,32
Acitretin	Antipsoriatic	-83,94	37,88
Methantheline bromide	Antispastic	-84,06	36,12
Aliskiren hemifumarate	Antihypertensive	-84,08	51,77
Abacavir Sulfate	Antiviral	-84,24	25,36
Dehydrocholic acid	Choleretic	-84,61	43,92

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Sulfadiazine	Antibacterial	-84,96	35,72
Aminophylline	Bronchodilator CNS Stimulant Diuretic	-85,43	33,71
Sulfamonomethoxine	Antibacterial	-85,49	34,48
Lacosamide	Analgesic	-85,65	42,45
Azacyclonol	Antipychotic	-85,74	49,8
Trifluridine	Antiviral	-85,96	40,26
Tranylcypromine hydrochloride	Antidepressant	-85,97	39,35
Oxaprozin	Analgesic Anti-inflammatory	-86,02	38,05
Chlorpropamide	Antidiabetic	-86,19	30,69
Aprepitant	Antiemetic	-86,3	25,16
Etodolac	Analgesic anti-inflammatory antiplatelet	-86,55	7,19
Remoxipride Hydrochloride	Antipsychotic	-86,75	33,14
(S)-Naproxen	Analgesic anti-inflammatory antipyretic	-86,89	27,44
Sibutramine hydrochloride		-86,95	27,39
Altrenogest	Progestogen	-87,03	26,53
Minaprine dihydrochloride	Anti-Alzheimer antidepressant	-87,05	23,96
Milrinone	Vasodilator	-87,12	36,15
Guanfacine hydrochloride	Antihypertensive	-87,5	27,84
Niclosamide	Antihelmintic	116,07	90,79
Raclopride		-87,61	19,36
Lidocaine hydrochloride	Antiarrhythmic local anesthetic	-87,63	28,02
Camptothecine (S,+)	Antineoplastic	-87,69	42,31
Isometheptene mucate	Antimigraine Vasoconstrictor	-87,95	32,75
Timolol maleate salt	Antianginal Antiarrhythmic Antiglaucoma	-87,98	34,73
Heptaminol hydrochloride	Analeptic Positive inotropic Vasodilator	-88,19	31,1
Zoledronic acid hydrate	Antiosteoporosis	-88,21	27,42
R(-) Apomorphine hydrochloride hemihydrate	Antiparkinsonian emetic	-88,26	45,85
Dexfenfluramine hydrochloride	Anorectic	-88,3	21,82
Pentetic acid	Chelating Radioprotectant	-88,51	36,08
Ciclesonide		-89,18	34,19
Nimesulide	Anti-inflammatory	-89,45	41,47
Xamoterol hemifumarate		-89,65	31,65
Procainamide hydrochloride	Antiarrhythmic Local anesthetic Vasodilator	-89,87	38,31
Avobenzone	Cytoprotectant	-89,94	22,53
Alcuronium chloride	Muscle relaxant	-90,3	21,55

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Flutamide	Antineoplastic	-90,34	35,22
Benzonatate	Antitussive Local anesthetic	-90,44	36,13
Mebendazole	Antihelmintic	-90,46	45,43
Piperidolate hydrochloride	Antispastic	-90,49	30,25
Mupirocin		697,49	90,55
Nocodazole	Antineoplastic	-90,59	38,69
Amcinonide	Anti-inflammatory	-90,6	37,23
Pregabalin	Anticonvulsant Anxiolytic	-91	26,29
Meclozine dihydrochloride	Antiemetic antihistaminic sedative	-91,05	40,88
Urapidil hydrochloride	Antihypertenisve Vasodilator	-91,15	32,12
(+)-Isoproterenol (+)-bitartrate salt	Antiasthmatic Bronchodilator Vasodilator	-91,3	37,05
Ethamivan	Analeptic CNS stimulant	-91,36	24,39
Alverine citrate salt	Antispastic	-91,4	43,1
Lansoprazole	Antiulcer	-91,51	40,13
Pentamidine isethionate	Antifungal antiparasitic antiprotozoal	-91,52	21,99
Nateglinide	Antidiabetic	-91,59	33,72
Luteolin	Expectorant	-91,67	33,09
Tropicamide	Mydriatic	-91,82	35,29
Cyclobenzaprine hydrochloride	Muscle relaxant	-92,37	48,86
Chenodiol	Cholagogue Choleretic	-92,42	31,17
Azatadine maleate	Antihistaminic	-92,53	27,1
Pivampicillin	Antibacterial	-92,59	35,73
Gefitinib	Antineoplastic	-92,64	1,49
Amyleine hydrochloride	Local anesthetic	-92,66	36,21
Lovastatin	Hypocholesterolemic	-92,78	28,42
Ceftazidime pentahydrate	Antibacterial	-92,88	34,64
Proguanil hydrochloride	Antimalarial	-93,07	37,02
Thiamphenicol	Antibacterial	-93,09	36,43
Nimodipine	Vasodilator	-93,27	34,19
Homoveratrylamine	Antihypertenisve	-93,71	30,75
Etifenin	Chemosensitizer	-93,83	27,42
Prenylamine lactate	Antianginal anxiolytic vasodilator	-94,3	30,27
Zonisamide	Anticonvulsant	-94,41	23,1
Domperidone	Antiemetic	-94,59	21,74
Benserazide hydrochloride	Antiparkinsonian	-94,79	37,55
Parbendazole		-94,9	49,73
Irbesartan	Antihypertensive	-95,26	37,24

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Terbinafine	Antifungal	-95,4	36,77
Nelarabine		-95,62	38,72
Actarit	Anti-inflammatory Immunomodulator	-95,89	28,16
Nisoxetine hydrochloride	Antidepressant	-95,97	34,42
Epirizole	Analgesic anti-inflammatory antipyretic	-96	37,42
Diltiazem hydrochloride	antianginal antiarrhythmic antihypertensive	-96,14	39,91
Methylatropine nitrate	Antispastic Mydriatic	-96,35	34,09
Dioxybenzone		-96,59	32,33
Paroxetine Hydrochloride	Antidepressant CNS Stimulant	-96,65	39,8
Olopatadine hydrochloride	Antihistaminic	-96,78	45,99
Salbutamol	Bronchodilator tocodytic	-97,15	40,99
Stanozolol		-97,38	45,23
Levamisole hydrochloride	Antihelmintic immunomodulator	-97,76	33,22
Metoclopramide monohydrochloride	Antiemetic	-97,78	38,72
Ciclopirox ethanolamine	Antibacterial antifungal	-97,82	31,43
Picotamide monohydrate	Anticoagulant antiplatelet thrombolytic	-97,92	52,93
Vorinostat	Antineoplastic	-97,99	38,43
Clorsulon	Antihelmintic	-98,09	30,75
Benidipine hydrochloride	Antihypertenisve	-98,21	29,82
Azlocillin sodium salt	Antibacterial	-98,29	52,04
Naftopidil dihydrochloride	Antihypertensive	-98,74	29,35
Fluocinonide	Anti-inflammatory	-98,74	26,58
Tolnaftate	Antifungal	-99,07	22,7
Diprophylline	Analeptic antispastic bronchodilator	-99,35	40,88
Lodoxamide	Antihistaminic	-99,4	33,28
Ziprasidone Hydrochloride	Antipsychotic	-99,56	24,78
Propafenone hydrochloride	Antiarrhythmic	-99,76	26,43
Cimetidine	Antiulcer	-99,96	36,06
Hymecromone	Muscle relaxant	-100,21	36,05
Ciprofloxacin hydrochloride monohydrate	Antibacterial antiprotozoal	318,88	75,9
Oxolinic acid	Antibacterial	-100,69	43,75
Oxprenolol hydrochloride	Antianginal Antiarrhythmic Antihyperensive	-100,83	35,25
Imiquimod	Antiviral	-100,93	36,27
Yohimbine hydrochloride	Erectile dysfunction treatment vasodilator	-100,93	29,93

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Sulfathiazole	Antibacterial	-101,05	34,61
Famotidine	Antiulcer	-101,11	44,08
Flunisolide	Anti-inflammatory	-101,16	4,99
Fexofenadine hydrochloride	Antihistaminic	-101,24	43,72
Estriol		-102,31	41,82
Fluticasone propionate	Anti-inflammatory Vasodilator	-102,34	39,35
Isocarboxazid	Antidepressant	-102,59	29,68
Iproniazide phosphate	Antidepressant antihypertensive	-102,6	46,73
Diflorasone Diacetate	Anti-inflammatory antipruritic antipsoriatic	-102,95	39,56
Isoconazole	Antibacterial antifungal	157,84	71,03
Terfenadine	Antihistaminic antipruritic	-103,02	47,57
Cefotaxime sodium salt	Antibacterial	904,42	93,91
Tetracycline hydrochloride	Antibacterial	507,2	89,84
Fluspirilen	Antipsychotic	-103,03	42,46
Allopurinol		-103,15	32,74
Imidurea	Antifungal	-103,25	40,84
Diloxanide furoate	Antiamebic	-103,3	32,57
Amfepramone hydrochloride		-103,32	48,19
(R)-Duloxetine hydrochloride		-103,38	39,02
Clindamycin hydrochloride	Antibacterial	821,87	91,69
Lymecycline	Antibacterial	-103,49	38,55
Protriptyline hydrochloride	Antidepressant	-103,57	38,31
Norgestimate		-103,62	49,57
Rasagiline	Antiparkinsonian	-103,68	31,41
Flubendazol		-103,69	30,94
Chlorhexidine	Antibacterial antiseptic	1668,32	97,77
Sertindole	Antipsychotic	-103,79	21,36
Chlortetracycline hydrochloride	Antiamecib antibacterial	512,98	92,51
Tamoxifen citrate	Antineoplastic	63,72	61,04
Clopidogrel	Antiplatelet	-104,24	36,31
Cefoxitin sodium salt	Antibacterial	-83,74	70,78
Dihydrostreptomycin sulfate	Antibacterial	277,27	85,53
Gentamicine sulfate	Antibacterial	440,04	76,9
Erythromycin	Antibacterial anti-inflammatory	723,07	91,66
Chloroxine		349,51	78,59
Phenprobamate	Muscle relaxant Sedative Anticonvulsant	-104,39	25,62
Josamycin	Antibacterial	557,7	93,87

Name	Therapeutic effect	% GraXRS inhibition	% Growth inhibition
Aminopurine, 6-benzyl		-105,16	38,6
Tiaprofenic acid	Analgesic anti-inflammatory antipyretic	-105,17	30,97
Diatrizoic acid dihydrate	Contrastant	-105,35	32,5
Tulobuterol	Bronchodilator	-105,41	38,81
Clemizole hydrochloride	Antibacterial antifungal antihistaminic	-105,59	51,25
Liothyronine		-105,65	37,42
Pyrimethamine	Antimalarial antiprotozoal	-105,74	42,48
Phenoxybenzamine hydrochloride	Antihypertensive	-106,01	37,37
Methenamine	Antibacterial	-106,04	43,48
Felodipine	Antianginal antihypertensive	-106,4	22,71
Acetohexamide	Antidiabetic	-106,41	32,62
Tolazamide	Antidiabetic	-106,51	21,64
Losartan	Antihypertensive	-107,01	49,19
Ethynodiol diacetate	Contraceptive	-107,42	39,56
Benztropine mesylate	Antiparkinsonian	-107,54	43,26
Pyrazinamide	Antibacterial	-107,58	31,43
Clozapine	Antiparkinsonian Antipsychotic	-107,6	30,24
Tocainide hydrochloride	Anesthetic Antiarrhythmic	-107,7	41,15
Oxandrolone		-107,76	40,12
(S)-propranolol hydrochloride	Antianginal Antiarrhythmic Antihyperensive	-107,94	42,55
Cephalosporanic acid, 7-amino	Antibacterial	-108,02	32,48
(+,-)-Synephrine	Vasoconstrictor	-108,06	36,68
Molindone hydrochloride	Antipsychotic	-108,12	19,77
(S)-(-)-Cycloserine	Antibacterial	-108,83	39,52
Pridinol methanesulfonate salt	Antiparkinsonian	-109,03	28,88
Pivmecillinam hydrochloride	Antibacterial	-109,08	39,11
Docetaxel	Antineoplastic	-109,08	48,65
Phensuximide	Anticonvulsant	-109,72	43,66
Phthalylsulfathiazole	Antibacterial	-109,99	34,43
Monobenzone		-110,36	36,89
Nilvadipine	Antianginal antihypertensive	-110,36	14,19
Cloxacillin sodium salt	Antibacterial	-3481,68	97,96
Carteolol hydrochloride	Antiglaucoma antihypertensive	-110,36	30,27
Propidium iodide	Antibacterial	-110,43	25,42
Spaglumic acid	Antiallergic Vasodilator	-110,68	47,2
Levopropoxyphene napsylate	Analgesic Antitussive	-110,78	41,3

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Meropenem	Antibacterial	-77,35	65,94
Tegaserod maleate	Gastreoprokinetic	-110,93	24,89
Sulfisoxazole	Antibacterial	-110,98	38,48
Phenindione	Anticoagulant	-111,13	36,21
Nifuroxazide	Antibacterial	-111,21	23,26
Bephenium hydroxynaphthoate		-111,71	33,83
Gliquidone	Antidiabetic	-111,74	38,57
Niflumic acid	Analgesic anti-inflammatory antipyretic	-111,81	33,92
Ropivacaine hydrochloride	Anesthetic	-112,36	30,12
Haloperidol	Antiemetic antipsychtotic	-112,51	45,81
Lomerizine hydrochloride	Antimigraine	-112,59	35,98
Amodiaquin dihydrochloride dihydrate	Anti-inflammatory antimalarial	-112,61	28,5
Fosinopril	Antihypertenisve	-112,67	44,87
Posaconazole	Antifungal	-112,7	32,09
Clonixin Lysinate	Analgesic Antifungal	-112,72	46,78
Perphenazine	Antiemetic antipsychotic	-112,76	45,76
Ticlopidine hydrochloride	Anticoagulant antiplatelet	-112,99	36,58
Ambrisentan	Antihypertensive	-113,24	37,48
Phenylbutazone	Anti-inflammatory	-113,26	38,76
Butylparaben	Antifungal	-113,53	30,11
Griseofulvin	Antifungal anti-inflammatory	-114,03	44,62
Phenformin hydrochloride	Antidiabetic	-114,06	41,65
Nilutamide	Antineoplastic	-114,32	32,66
Nystatine	Antifungal	-114,5	43,69
Glipizide	Antidiabetic	-114,93	40,32
Cyproheptadine hydrochloride	Antihistaminic antipruritic sedative	-115,61	49,45
Tripelennamine hydrochloride	Antihistaminic	-116	43,21
Risedronic acid monohydrate	Antiosteoporosis	-116,41	27,68
Adapalene	Keratolytic Anti-inflammatory	-116,43	43,29
Etoricoxib	Analgesic anti-inflammatory	-116,53	32,73
Norfloxacin	Antibacterial	85,65	78,14
Cloperastine hydrochloride	Antitussive	-116,69	25,72
Dopamine hydrochloride	Antihypertensive	-116,73	33,91
Trimetazidine dihydrochloride	Antianginal antischemic vasodilator	-116,78	31,21
Sulfasalazine	Antibacterial anti-inflammatory	-116,96	31,18
Ethoxyquin	Antifungal	-117,13	26,14
Clobetasol propionate	Anti-inflammatory	-117,17	30,31

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Homosalate	Radioprotectant	-117,23	38,83
Moxonidine	Antihypertenisve	-117,33	38,39
Toltrazuril	Anticoccidial	-117,4	44,7
Pentolinium bitartrate	Antihypertensive	-117,49	47,71
Amethopterin (R,S)	Anti-inflammatory Antineoplastic Immunosuppressant	-117,98	47,44
Olmesartan	Antihypertensive	-118,17	40,86
Trimethoprim	Antibacterial antimalarial	-118,56	42,83
Terbutaline hemisulfate	Antiasthmatic Bronchodilator Muscle relaxant	-118,57	8,47
Telenzepine dihydrochloride	Antiulcer	-118,62	32,14
Ofloxacin	Antibacterial	71,59	81,8
Lomefloxacin hydrochloride	Antibacterial	102,61	77,55
N6-methyladenosine	Antineoplastic	-118,68	33,69
Azapropazone	Analgesic Anti-inflammatory	-118,81	35,82
Flumequine	Antibacterial	-118,91	52,63
Propylthiouracil	Antihyperthyroid	-118,96	31,86
Aminacrine	Antiseptic	-118,97	36,7
N-Acetyl-L-leucine	Antivertigo	-118,98	34,65
Ipriflavone	Antiosteoporosis	-119,09	32,83
Mitotane	Antineoplatic	-119,35	41,41
Rizatriptan benzoate	Antimigraine Vasoconstrictor	-119,46	28,48
Felbinac	Analgesic Anti-inflammatory	-119,71	28,67
Halofantrine hydrochloride	Antimalarial	-119,74	45,21
Ketorolac tromethamine	Analgesic Anti-inflammatory Antipyretic	-119,81	34,37
Diclazuril		-119,9	49,72
Digitoxigenin	Cardiotonic	-119,97	35,71
Aceclidine Hydrochloride	Antiglaucoma	-120,09	36,35
Fluoxetine hydrochloride	Antidepressant	-120,33	30,18
Guaiacol	Expectorant	-120,38	38,02
Alosetron hydrochloride	Antidiarrheal	-120,4	39
Flunixin meglumine	Analgesic Anti-inflammatory antipyretic	-120,55	40,91
Adenosine 5'-monophosphate monohydrate	Antiarrhythmic	-120,6	42,89
Debrisoquin sulfate	Antihypertensive	-120,95	29,24
Terconazole	Antifungal	-120,95	28,43
Molsidomine	Antianginal anticoagulant antiplatelet	-121,21	31,94
Naloxone hydrochloride	Opioate antidote	-121,46	44,9

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Tomoxetine hydrochloride		-121,53	27,37
Trolox	Antioxidant	-121,9	32,46
Norcyclobenzaprine	Antiulcer	-122,49	36,27
Cilostazol	Anticoagulant	-122,51	27,06
Ethinylestradiol	Contraceptive	-122,84	36,57
Cyclopentolate hydrochloride		-122,89	48,17
Cefadroxil	Antibacterial	-122,95	43,38
Modafinil	CNS stimulant	-123,16	34,62
Olanzapine	Antipsychotic	-123,22	40,68
Trazodone hydrochloride	Antidepressant	-123,48	24,86
Acenocoumarol	anticoagulant	-123,53	37,99
Bepridil hydrochloride	Antianginal Antiarrhythmic Antihypotensive	-123,74	44,03
Meptazinol hydrochloride	Analgesic	-123,91	32,49
Tiabendazole	Antifungal Antihelmintic antiparasitic	-123,96	37,85
Bifonazole	Antifungal	-124,19	49
Chloroquine diphosphate	Anti-inflammatory antimalarial antiprotozoal	-124,37	34,34
Quinidine hydrochloride monohydrate	Antiarrhythmic antimalarial	-124,42	24,25
Diethylcarbamazine citrate	Antihelmintic	-124,57	37,81
Alprostadil	Erectile Dysfunction treatment Vasodilator	-124,66	39,34
Methyldopate hydrochloride	Antihypertensive	-124,75	24,35
Ozagrel hydrochloride	Antianginal	-124,78	31,72
Progesterone	Progestogen	-125,25	35,51
EPIA/PNAGndrosterone	Anabolic	-125,94	39,83
Camylofine chlorhydrate		-126,38	35,61
Aminohippuric acid		-126,73	27,79
Spiperone	Antipsychotic	54,85	69,33
Nifurtimox		-127	41,36
Triflupromazine hydrochloride	Antiemetic antipsychotic anxiolytic	-127,23	39,11
Melatonin	Anticonvulsant antioxidant immunostimulant	-127,29	42,79
Omeprazole	Antiulcer	-127,53	31,84
Zotepine	Antipsychotic	-128,12	30,6
Nizatidine	Antiulcer	-128,13	42,05
Budesonide	Anti-inflammatory	-128,25	34,65
(R) -Naproxen sodium salt	Anti-inflammatory	-128,4	23,73
(+,-)-Octopamine hydrochloride		-129,2	38,1
Artemisinin	Antimalarial	-129,28	32,52

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Alfadolone acetate	Anesthetic	-129,58	40,79
Prilocaine hydrochloride	Local anesthetic	-129,98	43,77
Moricizine hydrochloride	Antiarrhythmic	-130,23	40,5
Lincomycin hydrochloride	Antibacterial	193,73	91,33
Sulpiride	Antidepressant antiemetic antipsychotic	-130,36	35,25
Clofibrate	Antilipemic	-130,53	46,06
Fluconazole	Antifungal	-130,61	37,78
Ribavirin	Antiviral	-130,84	35,17
Oxytetracycline dihydrate	Antibacterial	-16,96	62,92
Bendroflumethiazide	Antihypertensive diuretic	-130,92	40,66
Bromocryptine mesylate	Antiparkinsonian	-130,94	45,21
Dibenzepine hydrochloride	Antidepressant	-131,21	25,23
Chlorothiazide	Antihypertensive diuretic	-131,94	30,24
Methoxamine hydrochloride	Antihypotensive vasoconstrictor	-132,01	39,04
Tinidazole	Antiamebic Antibacterial	-132,05	30,76
Corticosterone	Anti-inflammatory immunosuppressant	-132,28	41,04
Minocycline hydrochloride	Antibacterial	127,99	90,93
Adrenosterone		-132,47	31,12
Mifepristone	Abortifacient	-132,58	26,3
Loteprednol etabonate	Anti-inflammatory	-132,82	42,24
Candesartan	Antihypertensive	-132,93	39,9
Mephenytoin	Anticonvulsant	-133,02	40,9
Piperacillin sodium salt	Antibacterial	-133,23	48,29
Chlormezanone	Anxiolytic Muscle relaxant	-133,27	38,18
Fendiline hydrochloride	Antianginal	-133,84	26,42
Ondansetron Hydrochloride	Antianemic	-134,08	38,4
Piribedil hydrochloride	Antiparkinsonian Vasodilator	-134,34	34,7
Primidone	Anticonvulsant	-134,9	42,18
Cefoperazone dihydrate	Antibacterial	415,31	83,3
Memantine Hydrochloride	Anti-Alzheimer Antiparkinsonian Antispastic	-135	35,15
Carbidopa	Antiparkinsonian	-135,14	40,14
Cyclizine hydrochloride	Antiemetic antihistaminic antivertigo	-135,37	32,87
Alfaxalone	Anesthetic	-135,65	34,76
Lopinavir	Antiviral	-135,79	24,13
Aztreonam	Antibacterial	-135,83	48,79
Ethamsylate	Antiplatelet Hemostatic	-136,4	44,76

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Etofylline	Antispastic Bronchodilator Cardiotonic	-136,54	38,06
Phentermine hydrochloride		-136,67	36,62
Hycanthone	Antihelmintic Antiparasitic	-136,75	40,49
Tibolone		-137	48,6
Altretamine	Antineoplastic	-137,25	40,74
Triflusal	Anticoagulant antiplatelet	-137,66	38,19
Anethole-trithione	Choleretic	-137,71	42,82
Mefloquine hydrochloride	Antimalarial	-138,02	49,41
Meloxicam	Anti-inflammatory	-138,12	40,13
Bretylium tosylate	Anesthetic Antiarrhythmic Antihypertenisve	-138,12	44,37
Thiorphan	Antidiarrheal	-138,31	29,8
1,8-Dihydroxyanthraquinone	Laxative Antiemetic	-52,31	60,06
Thioperamide maleate	Antiemetic	-138,85	38,19
Aripiprazole	Antipsychotic	-138,9	43,34
Amoxapine	Antidepressant antipsychotic	-139,29	48,46
Pilocarpine nitrate	Antiglaucoma	-139,73	44,71
Dextromethorphan hydrobromide monohydrate	antitussive	-139,74	36,97
Mesoridazine besylate	Antipsychotic	-141,03	36,99
Acefylline	CNS stimulant	-141,37	23,22
Sulfamethizole	Antibacterial	-141,44	34,52
Vigabatrin hydrochloride	Anticonvulsant antiepileptic	-142,42	29,39
Propofol	Anesthetic Sedative	-142,44	43,98
Dihydroergotamine tartrate	Antimigraine	-142,53	42,19
Rimantadine Hydrochloride	Antiviral	-143,38	34,95
Lidoflazine	Antianginal Antiarrhythmic Vasodilator	-143,66	34,88
Alfacalcidol	Antiosteoporosis	-143,72	39,73
Sulfinpyrazone	Antiplatelet uricosuric	-143,9	28,41
Bromhexine hydrochloride	Expectorant	-144,22	39,94
Sulfaquinoxaline sodium salt	Antibacterial	-144,43	39,65
Dinoprost trometamol	Oxytocic	-144,43	36,91
Misoprostol	Antiulcer	-144,65	50,57
Dimaprit dihydrochloride		-144,8	41,03
Mebeverine hydrochloride	Antispastic	-144,87	28,48
Pirlindole mesylate	Antidepressant	-146,43	38,55
Zomepirac sodium salt	Anti-inflammatory	-147,45	33,24
Rimexolone	Anti-inflammatory	-147,58	40,8
Erlotinib	Antineoplastic	-147,91	35,07

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Asenapine maleate	Antipsychotic	-148,15	39,29
Metaproterenol sulfate, orciprenaline sulfate	Bronchodilator	-148,44	33,5
Niridazole	Antihelmintic antiparasitic antiprotozoal	-148,46	43,94
Iohexol	Contrastant	-148,59	34,99
Clofazimine	Antibacterial	273,41	81,3
Tolvaptan	Antihypertensive diuretic	-148,71	36,42
Mephenesin	Anticonvulsant local anesthetic muscle relaxant	-149,28	49,28
Fursultiamine Hydrochloride	Anti-Alzheimer	-149,3	45,09
Fusidic acid sodium salt	Antibacterial	361,06	91,91
Benzathine benzylpenicillin	Antibacterial	-149,39	51,81
Dexrazoxane hydrochloride	Chemoprotectant	-149,51	39,66
Methylergometrine maleate	Hemostatic Oxytocic	-150,28	37,45
Risperidone	Antipsychotic	-151,26	41,65
Lofexidine	Antihypertensive	-151,52	36,39
Gabexate mesilate	Anticoagulant	-151,66	36,7
Nitrocaramiphen hydrochloride		-151,96	41,55
Dequalinium dichloride	Antibacterial Antiseptic	-131,97	78,57
Pentoxifylline	Bronchodilator Vasodilator	-153,1	34,83
Thalidomide	Hypnotic Immunosuppressant	-153,59	40,23
Exemestane	Antineoplastic	-155,08	42,38
Letrozole	Antineoplatic	-155,32	40,66
Oxybutynin chloride	Antispastic	-155,46	32,53
Kanamycin A sulfate	Antibacterial	698,91	74,53
Amikacin hydrate	Antibacterial	1163,39	92,93
Atovaquone	Antimalarial antiprotozoal	-155,49	36,03
Butylscopolammonium (n-) bromide	Antispastic	-155,52	38,85
Metoprolol-(+,-) (+)-tartrate salt	Antiarrhythmic antihypertensive	-156,17	40,62
Flunarizine dihydrochloride	Anticonvulsant vasodilator	-156,22	31,21
Cortisol acetate	Anti-inflammatory	-156,38	32,87
Metaraminol bitartrate	Antihypotensive vasoconstrictor	-156,42	50,72
Oxiconazole Nitrate	Antifungal	-156,94	39,13
Tenoxicam	Analgesic anti-inflammatory antipyretic	-157,27	37,85
Glimepiride	Antidiabetic	-157,36	37,38
Tigecycline		-157,71	30,04
Amifostine		-157,96	42,07

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Pizotifen malate	Antihistaminic Antimigraine Sedative	-158,08	40,75
Metixene hydrochloride	Antiparkinsonian antispastic	-158,81	27,38
Nifekalant	Antiarrhythmic	-159,2	42,17
Khellin	Antispastic antitussive vasodilator	-159,36	48,51
Dipivefrin hydrochloride	Antiglaucoma	-159,69	31,15
Pargyline hydrochloride	Antidepressant antihypertensive	-160,04	47,24
Caffeine	CNS Stimulant	-160,13	43,44
Paliperidone	Antipsychotic	-160,21	44,17
Brinzolamide	Antiglaucoma Diuretic	-160,6	40,06
Chlorprothixene hydrochloride	Antiemetic Antipsychotic	-160,78	38,82
Etofenamate	Anti-inflammatory	-161,47	48,49
Pimozide	Antipsychotic	-36,39	66,56
Pronethalol hydrochloride	Antianginal Antiarrhythmic Antihypertenisve	-161,74	36,26
Moclobemide	Antidepressant	-161,82	39,05
Dantrolene sodium salt	Muscle relaxant	-162,15	31,34
Fluorometholone	Anti-inflammatory	-162,37	27,17
Vinpocetine	CNS Stimulant Neuroprotectant Vasodilator	-35,51	67,13
Lisinopril	Antihypertensive vasodilator	-162,45	32,05
Calcipotriene	Antipsoriatic	-162,61	54,39
Fomepizole		-162,62	38,87
Probucol	Antilipermic Hypocholesterolemic	-163,46	37,5
Zuclopenthixol dihydrochloride	Antipsychotic Antiviral Sedative	-163,49	42,58
Zardaverine	Bronchodilator	-163,57	40,74
Levonordefrin	Vasoconstrictor	-164,58	35,06
Pefloxacine	Antibacterial	40,67	68,21
Praziquantel	Antihelmintic	-165,02	25,44
Torsemide	Antihypertensive Diuretic	-165,09	44,22
Granisetron	Antiemetic	-166,09	52,05
Maprotiline hydrochloride	Antidepressant Anxiolytic	-166,35	41,55
Loratadine	Antihistaminic	-167,11	36,6
Azathioprine	Antineoplastic immunosuppressant	-167,21	50,85
Cyanocobalamin	Analgesic	-167,36	45,44
Flecainide acetate	Antiarrhythmic	-168,09	38,99
Dicloxacillin sodium salt hydrate	Antibacterial	119,67	81,21
Famciclovir	Antiviral	-168,18	48,74
Miglitol	Antidiabetic	-168,28	40,92

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Deptropine citrate	Antihistaminic Bronchodilator Vasodilator	-168,93	49,42
Cyclosporin A	Immunosuppressant	-169,41	39,86
Antazoline hydrochloride	Antihistaminic sedative	-31,51	68,68
5-fluorouracil	Antineoplastic	-31,36	70,27
Tracazolate hydrochloride	Anticonvulsant Sedative	-169,77	39,01
Cinoxacin	Antibacterial	-170,3	39,83
Nebivolol hydrochloride	Antihypertensive	-170,57	47,84
Medrysone	Anti-inflammatory	-170,79	41,82
Ibandronate sodium	Antiosteoporosis	-171,09	36,43
Bupivacaine hydrochloride	Local anesthetic	-171,16	36,61
Carbarsone	Antiamebicantiprotozoal	-171,23	41,43
Linezolid	Antibacterial	93,91	62,09
Fenoterol hydrobromide	Bronchodilator tocolytic	-171,75	36,57
Meclocycline sulfosalicylate	Antibacterial	87,48	77,44
Chlorthalidone	Antihypertensive Diuretic	-172,61	39,4
Melengestrol acetate		-173	46,59
Pipenzolate bromide	Antispastic	-173,25	41,84
Ceforanide	Antibacterial	-20,07	69,45
Oxcarbazepine	Anticonvulsant	-173,28	44,74
Cefixime	Antibacterial	-63,52	67,49
Pirenperone		-173,75	38,83
Mebhydroline 1,5-naphtalenedisulfonate	Antihistaminic	-173,84	47,01
Tosufloxacin hydrochloride	Antibacterial	-59,88	78,57
Methylprednisolone, 6-alpha	Anti-inflammatory Immunosuppressant	-173,92	38,12
Rifapentine	Antibacterial	101,55	78,07
Zimelidine dihydrochloride monohydrate	Antidepressant	-28,25	55,63
Mometasone furoate	Anti-inflammatory	-174,08	38,79
Closantel	Antihelmintic antiparasitic	141,43	75,63
Bisacodyl	Laxative	-174,76	38,82
Tegafur	Antineoplastic	-175,65	46,24
Trimetozine	Sedative	-176,09	37,52
Streptozotocin	Antineoplastic	-176,31	40,9
Glycopyrrolate	Antispastic	-176,37	41,35
Triprolidine hydrochloride	Antihistaminic sedative	-177,47	29,17
Cinnarizine	Antihistaminic antivertigo sedative	-26,35	69,62
Indomethacin	Analgesic anti-inflammatory antipyretic	-177,56	35,46

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Zaleplon	Hypnotic sedative	-178,45	46,15
Isosorbide dinitrate	Antianginal	-178,87	26,98
Halcinonide	Anti-inflammatory antipruritic	-179,07	35,06
Cefotiam hydrochloride	Antibacterial	-1007,95	95,38
Betahistine mesylate	Vasodilator	-179,43	36,15
Azelastine hydrochloride	Antihistaminic	-180,09	41,22
Ribostamycin sulfate salt	Antibacterial	-180,56	47,77
Dobutamine hydrochloride	Analeptic Cardiotonic Positive inotropic	-180,61	38,91
Folinic acid calcium salt	Antianemic	-180,72	40,09
Bosentan	Vasodilator	-180,86	53,39
Diperodon hydrochloride	Local anesthetic	-180,87	34,04
Alendronate sodium	Antiosteoporosis	-180,91	29,58
Ibutilide fumarate	Antiarrhythmic	-181,04	27,87
Acetylcysteine	Mucolytic	-181,64	45,19
Levetiracetam	Anticonvulsant	-182,71	24,87
Desloratadine	Antihistaminic	-22,83	62
Cetirizine dihydrochloride	Antihistaminic antipruritic	-182,79	37,78
Amlodipine	Antihypertensive	-183,03	40,87
Vancomycin hydrochloride	Antibacterial	-820,38	95,15
Clofibric acid	Antilipemic	-183,34	42,53
Finasteride	Anti-alopecia antineoplastic	-184,05	32,97
Phenacetin	Analgesic antipyretic	-185,28	39,03
Cisapride	Gastroprokinetic	-185,94	40,33
Betamethasone	Anti-inflammatory Antipruritic Immunosuppressant	-186,53	39,25
Clorgyline hydrochloride	Antidepressant	-187,19	43,51
Dacarbazine	Antineoplastic	-187,32	45,14
Metrizamide	Contrastant	-188,45	44,17
Zaprinast	Erectil dysfunction treatment	-188,96	39,8
Lacidipine	Antihypertensive	-188,97	43,85
Quetiapine hemifumarate	Antipsychotic	-189,06	54,02
Imipenem	Antibacterial	-1799,83	97,05
Methiothepin maleate	Antipsychotic	-189,16	48,36
Pramoxine hydrochloride	Local anesthetic	-189,79	40,71
Biotin		-189,81	40,82
Clenbuterol hydrochloride	Antiasthmatic Bronchodilator Tocolytic	-189,88	44,23
Diphenidol hydrochloride	Antiemetic antivertigo	-189,94	30,46
Chlorotrianisene	Antineoplastic	-190	43,91

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Tetramisole hydrochloride	Antihelmintic antiparasitic immunomodulator	-190,8	37,06
Ropinirole hydrochloride	Antiparkinsonian	-190,86	42,74
Dronedarone hydrochloride	Antiarrhythmic	-191	41,63
Galanthamine hydrobromide	Analgesic anti-alzheimer anti-fatigue	-191,3	44,44
Mesna	Chemoprotectant	-191,31	47,78
Roxatidine Acetate hydrochloride	Antiulcer	-191,43	43,96
Thiostrepton	Antibacterial	10,1	91,31
Nitrofural	Antibacterial	-191,59	38,05
Fenofibrate	Hypocholesterolemic Lipid-lowering Uricosuric	-191,6	35,52
Rifampicin	Antibacterial	-360,18	92,86
THIP Hydrochloride	sedative	-191,79	38,76
Clomipramine hydrochloride	Antidepressant	-191,8	32,4
Tropisetron hydrochloride	Antiemetic	-192,25	36,97
(S)-(-)-Atenolol	Antianginal antiarrhythmic antihypertensive	-192,32	39,47
Thiethylperazine dimalate	Antiemetic Antivertigo	-192,36	50,1
Flumethasone	Anti-inflammatory	-192,38	45,47
Grepafloxacin		82,7	82,91
Furosemide	Antihypertensive Diuretic	-192,43	35,68
D,L-Penicillamine	Analgesic	-193,14	31,31
Fenoprofen calcium salt dihydrate	Anti-inflammatory	-193,33	41,23
Clemastine fumarate	Antiemetic antihistaminic sedative	-193,36	38,14
Zidovudine, AZT	Antiviral	-193,49	36,62
Vidarabine	Antiviral	-193,53	46,46
Cefotetan	Antibacterial	-194,65	49,78
Pimethixene maleate	Antihistaminci antitussive Bronchodilator	-195,39	29,95
Carisoprodol	Analgesic antipyretic muscle relaxant	-195,66	47,09
Probenecid	Antigout uricosuric	-195,72	35,28
Benzocaine	Local anesthetic	-195,88	15,71
Tobramycin	Antibacterial	-485,01	87,09
Isoetharine mesylate salt	Bronchodilator	-196,04	36,65
Tiapride hydrochloride	Antiemetic antipsychotic anxiolytic	-196,81	40,91
Pemirolast potassium	Anti-inflammatory Antipruritic Antihistaminic	-197,24	41,37
Isopropamide iodide	Antiulcer	-197,74	43,01
Amprenavir	Antiviral	-198,04	40,69

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Pyrilamine maleate	Antihistaminic antipruritic antitussive	-198,32	40,25
Bacampicillin hydrochloride	Antibacterial	-199,63	43,09
Fenipentol	Choleretic	-200,02	42,45
Acamprosate calcium		-200,52	47,87
Methapyrilene hydrochloride	Antihistaminic Sedative	-201,74	36,89
Sisomicin sulfate	Antibacterial	-8,89	57,15
Ipsapirone		-202,19	49,37
Tenatoprazole	Antiulcer	-202,4	40,53
Carmofur	Antineoplastic	-7,88	58,75
Cefpiramide	Antibacterial	-7,51	60,64
Topotecan	Antineoplastic	-203,08	53,57
Sulfabenzamide	Antibacterial	-203,71	29,17
Voriconazole	Antifungal	-203,78	47,27
Cefsulodin sodium salt	Antibacterial	-5,82	65,88
Nisoldipine	Antianginal antihypertensive	-205,35	35,05
Metergoline	Antiprolactin	-206,31	46,57
Pregnenolone	Anabolic anti-inflammatory	-207,03	39,31
Suloctidil	Antiplatelet vasodilator	-207,41	41,52
Alizapride hydrochloride	Antiemetic	-207,57	44,25
Clopamide	Antihypertensive Diuretic	-207,72	47,01
Leflunomide	Immunosuppressant	-208,18	32,32
Thioguanosine	Antineoplastic	-208,21	41,13
Methacholine chloride		-208,69	42,28
Aniracetam	Anti-alzheimer	-209,7	48,9
Cortisone	Anti-inflammatory Immunosuppressant	-210,96	40,33
Beta-Escin	Antineoplastic diuretic	-211,18	42,73
Butenafine Hydrochloride	Antifungal	-211,75	53,9
Norethindrone	Contraceptive	-212,05	39,33
Sulfachloropyridazine	Antibacterial	-212,2	34,6
Androsterone	Anabolic	-212,35	37,25
Acetopromazine maleate salt	Antiemetic antipsychotic antitussive	-212,97	41,38
Bambuterol hydrochloride	Bronchodilator Tocolytic	-213,37	36,56
Prednisolone	Anti-inflammatory Immunosuppressant	-214,55	40,16
Bimatoprost	Antiglaucoma	-214,63	40,86
Practolol	Antianginal Antihypertensive	-215,33	35,43
Dyclonine hydrochloride	Local anesthetic	-215,84	37,99
Etretinate	Antipsoriatic	-217,14	46,43

Name	Therapeutic effect	% GraXRS inhibition	% Growth inhibition
Buflomedil hydrochloride	Vasodilator	-217,14	43,52
Guaifenesin	Bronchodilator expectorant	-218,14	46,77
Amoxicillin	Antibacterial	-218,73	48,36
Ethisterone	Contraceptive	-218,88	35,33
Glafenine hydrochloride	Analgesic	-219,16	38,27
Efavirenz	Antiviral	-219,95	48,65
Fludrocortisone acetate	Anti-inflammatory antipruritic	-220,48	37,57
Argatroban	Anticoagulant	-220,79	44,69
Pitavastatin calcium	Hypocholesterolemic	-221,75	47,2
Hydroxychloroquine sulfate	Antimalarial	-222,05	48,01
Papaverine hydrochloride	Antispastic antitussive erectile dysfunction treatment	-222,84	46,71
Proadifen hydrochloride	Local anesthetic	-224,52	45,55
Spiramycin	Antibacterial	6,02	65,82
Escitalopram oxalate	Antidepressant	-224,95	39,74
Benzamil hydrochloride	Antihypertensive diuretic	-225,18	35,27
Estradiol-17 beta	Antigonadotropin	-225,5	45,32
Zafirlukast	Antiasthmatic	97,7	76,25
Zopiclone	Hypnotic sedative	-225,91	47,08
Pranlukast	Antiasthmatic	-227,03	38,21
Clonidine hydrochloride	Analgesic antihypotensive sedative	7,96	58,79
Labetalol hydrochloride	Antihypotensive	-227,54	39,22
Hydrocortisone base	Anti-inflammatory	-228,11	42,38
Guanadrel sulfate	Antihypertenisve	-228,59	45,51
Dydrogesterone	Progestogen	-228,84	45,82
Cycloheximide	Antibacterial	-230,46	42,63
Isoniazid	Antibacterial	-231,26	25,29
Tirofiban hydrochloride	Antiplatelet	-231,86	49,29
Oxibendazol		-233,13	52,06
Mizolastine		-235,15	47,3
Tetracaïne hydrochloride		-235,34	39,39
Picrotoxinin	Analeptic	-237	40,42
Dimenhydrinate	Antiemetic antihistaminic antivertigo	-239,52	40,87
Terazosin hydrochloride	Antihypertensive	-239,59	44,48
Vincamine	CNS Stimulant Vasodilator	-239,9	34,95
Meclofenoxate hydrochloride	CNS Stimulant	-239,98	45,96
Xylazine	Analgesic sedative	-240,09	54,4
Dexamethasone acetate	Anti-inflammatory immunosuppressant	-240,22	42,34

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Doxepin hydrochloride	Anticonvulsant antidepressant antipruritic	-240,65	37,76
Disopyramide	Antiarrhythmic	-240,7	34,42
Doxorubicin hydrochloride	Antibacterial antineoplastic immunosuppressant	14,31	61,8
Tizanidine hydrochloride	Muscle relaxant	-241,2	51,82
Butamben	Anesthetic	-241,28	48,68
Sulfamerazine	Antibacterial	-241,28	39,67
Podophyllotoxin	Antiviral	-241,42	47,82
Bumetanide	Diuretic	-241,45	36,21
Trioxsalen		-242,77	42,13
Florfenicol	Antibacterial	15,77	66,02
Fipexide hydrochloride	Anti-fatigue CNS stimulant	16,02	62,49
Digoxin	Cardiotonic	-243,64	47,02
Sulfamethazine sodium salt	Antibacterial	-243,86	46,28
Methimazole		-244,16	48,79
Norgestrel-(-)-D	Contraceptive	-244,58	48,39
Acemetacin	Anti-inflammatory	-245,1	39,07
Pinaverium bromide	Antipastic	-745,12	93,96
Nortriptyline hydrochloride	Antidepressant CNS stimulant	-245,81	36,4
Benfotiamine		-246,03	40,17
Benazepril hydrochloride	Antihypertensive	-246,13	52,78
Diosmin		-247,64	54,83
Hexylcaine hydrochloride	Anesthetic	-248,37	45,8
Temozolomide	Antineoplastic	-249,74	48,89
Celiprolol hydrochloride	Antianginal antihypertensive	-249,89	51,28
Dimethisoquin hydrochloride	Antipruritic lical anesthetic	-252,01	53,39
Drofenine hydrochloride	Antispastic	-252,06	43,32
Emedastine	Antihistaminic	-252,44	51,39
Sulfameter	Antibacterial	-253,12	43,16
Desipramine hydrochloride	Antidepressant CNS Stimulant	-253,36	45,93
Ketotifen fumarate	Antihistaminic	-254,34	35,52
Acipimox	Antilipemic	-256,89	53,12
Colchicine	Antigout Anti-inflammatory	-257,58	44,38
Folic acid		-259,46	51,71
Citalopram Hydrobromide	Antidepressant	-260,3	35,94
Pranoprofen	Anti-inflammatory	-260,41	47,6
Demecarium bromide	Antiglaucoma	-265,08	53,76
Pyridoxine hydrochloride		-266,05	53,65

Name	Therapeutic effect	% GraXRS inhibition	% Growth inhibition
Thioproperazine dimesylate	Antiemetic antipsychotic	27,55	56,28
Lanatoside C	Cardiotonic	-269,8	39,07
Mercaptopurine	Immunosuppressant	-272,06	53,63
Reboxetine mesylate	Antidepressant	-272,43	50,71
Mepenzolate bromide	Antispastic antiulcer	-274,45	40,21
Nalidixic acid sodium salt	Antibacterial	-274,99	41,26
Suxibuzone	Analgesic anti-inflammatory antipyretic	-275,31	40,27
Racecadotril	Antidiarrheal	-275,67	54,96
Dirithromycin	Antibacterial	32,47	69,13
Triamcinolone	Anti-inflammatory immunosuppressant	-276,79	43,59
Venlafaxine	Antidepressant	-278,74	49,73
Phenazopyridine hydrochloride	Analgesic	-279,32	49,57
Estropipate		-286,23	39,89
Tetraethylenepentamine pentahydrochloride	Antilipemic	-290,81	41,19
Oxacillin sodium	Antibacterial	-2146,59	96,8
Tranilast	Antiallergic	-291,5	52,04
Trihexyphenidyl-D,L Hydrochloride	Antiparkinsonian	-298,84	42,47
Alclometasone dipropionate	Anti-inflammatory	-305,4	45,83
Dipyrone	Analgesic antiasthmatic antipyretic	-306,55	33,48
Homochlorcyclizine dihydrochloride	Antihistaminic sedative	-306,56	39,22
Benzethonium chloride	Antibacterial antiseptic	-316,26	49,84
Mirabegron		-320,08	45,79
Sumatriptan succinate	Antimigraine	-323,76	46,63
Doxofylline	Bronchodilator	-325,87	46,56
Perhexiline maleate	Antianginal	-326,3	39,93
Sulfapyridine	Antibacterial	-330,79	48,84
Tetrahydrozoline hydrochloride	Nasal decongestant vasoconstrictor	-337,86	44,24
Piroxicam	Analgesic anticoagulant anti-inflammatory	-337,96	36,5
Zileuton	Antiasthmatic	-341,85	44,19
Cefmetazole sodium salt	Antibacterial	-2413,38	97,12
Tylosin	Antibacterial	45,13	85,17
Quinacrine dihydrochloride hydrate	Antihelmintic antileishmanial antimalarial	59,1	62,81
Thiamine hydrochloride	Immunostimulant	-346,43	43,69
Bromopride	Antiemetic	-349,22	49,43
Ethotoin	Anticonvulsant	-350,77	50,03

Name	*Therapeutic effect*	*% GraXRS inhibition*	*% Growth inhibition*
Warfarin	Anticoagulant	-352,58	45,34
Famprofazone	Analgesic antipyretic	-353	46,62
3-alpha-Hydroxy-5-beta-androstan-17-one		-353,82	40,87
Irsogladine maleate	Antiulcer	-354,28	40,84
Opipramol dihydrochloride	Antidepressant antipsychotic	-355,1	44,38
Ivermectin	Antihelmintic antiparasitic	81,94	68,9
Epirubicin hydrochloride	Antineoplastic	83,01	60,93
Succinylsulfathiazole	Antibacterial	-362,6	46,99
Pempidine	Antihypotenisve vasodilator	-371,17	46,07
Methyl benzethonium chloride	Antibacterial	-1322,89	96,66
Irinotecan hydrochloride trihydrate	Antineoplastic	-387,09	42,15
Midodrine hydrochloride	Antihypertensive	-392,31	37,84
Tramadol hydrochloride	Analgesic	-410,34	49,33
Cephalothin sodium salt	Antibacterial	-972,09	94,62
Cefuroxime sodium salt	Antibacterial	-837,14	87,29
Ranitidine hydrochloride	Antiulcer	-413,31	35,38
Ampyrone	Analgesic anticoagulant anti-inflammatory	-418,54	39,74
Secnidazole	Antiamebic	-418,93	48,42
Benzbromarone	Antianginal Antigout Antispastic	131,47	56,37
Tranexamic acid	Hemostatic	-428,99	37,53
Chlorcyclizine hydrochloride	Antiemetic antihistaminic sedative	-434,53	54,63
Diphenylpyraline hydrochloride	Antihistaminic antipruritic sedative	-488,99	50,6
Troleandomycin	Antibacterial	289,19	76,05