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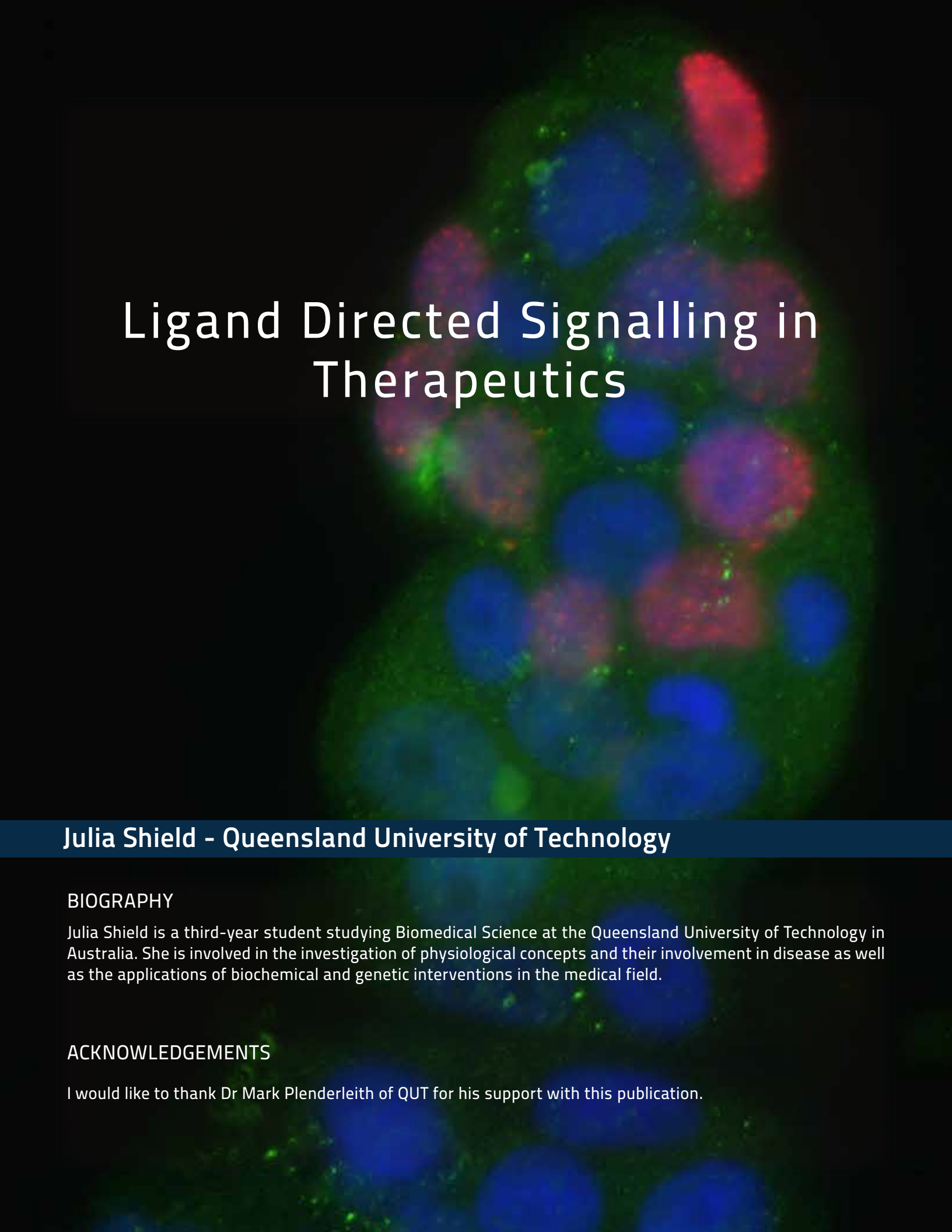

Ligand Directed Signalling in Therapeutics

Julia Shield - Queensland University of Technology

BIOGRAPHY

Julia Shield is a third-year student studying Biomedical Science at the Queensland University of Technology in Australia. She is involved in the investigation of physiological concepts and their involvement in disease as well as the applications of biochemical and genetic interventions in the medical field.

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Abstract

Research investigating ligand directed signalling or biased agonism has uncovered new opportunities for therapeutics targeting multiple diseases. Unlike balanced agonists which stimulate multiple pathways, a biased agonist binds to specific active-state receptors to stimulate a single cellular pathway. The ligand stabilizes receptor conformation with selective binding determined by the unique chemical structure of both receptor and agonist. G protein coupled receptors (GPCRs) enable a diverse range of ligand interactions through recruitment of various intracellular proteins. Activation of either G-protein or β-arrestin mediated pathways influence signalling bias following specific ligand binding.

> "The future of biased agonism research is promising as investigations continue for many diseases using advanced biotechnology."

Biased agonism has widespread therapeutic potential as certain signalling pathways can be stimulated for a desired biological effect while avoiding unwanted side effects. Several biased ligands are under investigation with therapeutic areas across cardiovascular, renal, and respiratory conditions. β-adrenoceptors are key targets for biased agonism based treatments. Deleterious cellular outcomes are avoided by inhibiting G-protein signalling. A number of challenges limit the validity of current research, such as unexpected propagation bias, production of false positive results in quantitative analysis, and

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differing results between animal and human trials. However, the future of biased agonism research is promising as investigations continue for many diseases using advanced biotechnology.

Basic Signaling Mechanisms

Since its discovery in 1998, biased agonism has described the action of a ligand specifically activating one of multiple downstream signalling pathways in a cell. This differs from balanced agonists which bind to the same receptors and activate multiple signalling pathways as illustrated in Figure 1 (Michel et al., 2018; Conibear, 2019). Biased agonists stabilize receptor conformation to selectively stimulate a single pathway, allowing targeted adjustment of cellular function. Biased selectivity is determined by specific structures of the binding ligand as they interact with residues in the receptor, producing conformational change to yield unique functional outputs (Franco et al., 2018).

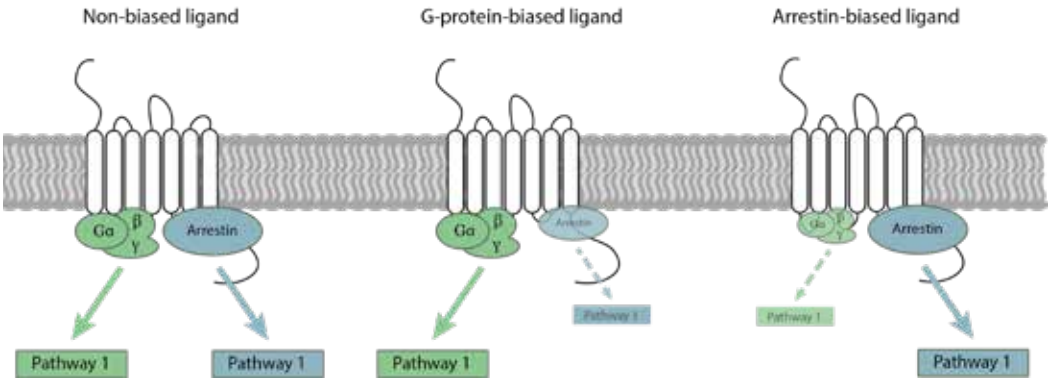

Figure 1. Activation of downstream pathways via balanced and biased agonists. Balanced agonists stimulate multiple pathways following receptor binding whereas G-protein or Arrestin-biased ligands only activate a single pathway (Adapted from Conibear, 2019).

Factors essential to the process of biased agonism include a receptor-active state and its interaction with cytosolic signalling peptides. Stimulation of specific pathways in the cell requires agonist specific receptor conformations. Numerous studies have validated this theory by demonstrating the stabilization of distinct receptor conformations after binding of a ligand (Palanche et al., 2001; Swaminath et al., 2004). As individual receptor regions modify conformation independently, coupling of signalling proteins to the unique receptor segments will produce differential activation of signalling pathways in the cell. Variability in signalling pathway stimulation via the formation of specific receptor conformations is extensively described in current scientific literature. Seven transmembrane receptors express pleiotropic effects with the signalling proteins they are coupled to. With the formation of multiple receptor conformations, ligands stabilise these conformations to selectively enable activated signalling pathways (Kenakin, 2009). This is supported by a study done by Michel et al. (2018) that describes the role of ligand binding stabilising a specific receptor conformation through the use of nuclear magnetic resonance (NMR). Therefore, ligand signalling bias results from the stabilization of specific receptor conformations which independently activate particular signalling pathways and molecules such as G-proteins and β-arrestins (Gundry et al., 2017).

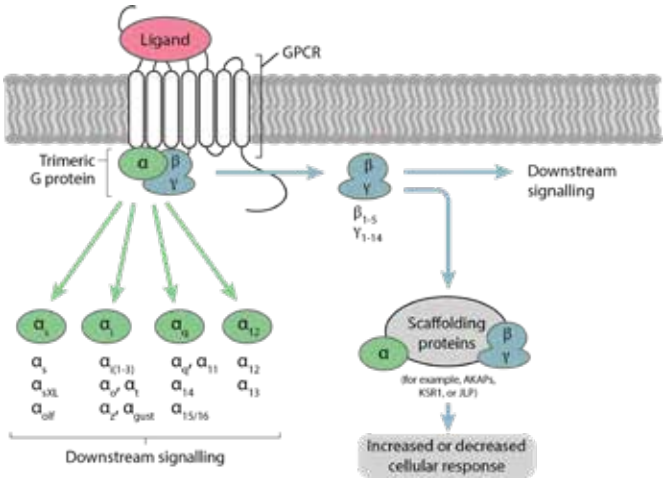

Figure 2. GPCR signalling via downstream proteins for diversification of signalling responses. Gα, Gβ, and Gγ proteins activate downstream signalling after receptor-binding. Gα proteins produce various signals across four families. Gβγ and Gα bind to scaffolding proteins which modulates their signalling (Adapted from Wootten et al., 2018).

G-Protein Coupled Receptors

Biased agonism involves GPCRs. These receptors implement various mechanisms which can modify ligand-receptor interaction and subsequent function. Diverse GPCR interactions with various intracellular proteins enable a response to multiple ligands. As illustrated in Figure 2, GPCR-ligand binding activates G-protein subunits Gα, Gβ, and Gγ which initiate different downstream signalling molecules for diversified responses (Wootten et al., 2018).

Signalling bias is observed following activation of effector proteins G-proteins or β-arrestins after specific ligand-receptor binding. This is because G-proteins activate second messengers, whereas β-arrestins activate MAP kinases. Agonists differentially activate these proteins with opposing susceptibility to internalization, phosphorylation, and desensitisation (Michel et al., 2018). β-arrestins facilitate receptor-trafficking and function as scaffolding molecules to help initiate signalling cascades. Altered receptor conformations following ligand binding induced conformational changes in scaffolding proteins to promote activation of downstream signalling pathways. Ligand-receptor binding produces conformational change in G-proteins which alters the guanosine triphosphate-guanosine diphosphate (GTP–GDP) exchange rate. Ligands inducing a fast rate of GTP association produce more downstream signalling per unit of time compared to ligands with a slow rate as seen in Figure 3 (Wootten et al., 2018). Chemical differences amongst ligands activate distinct signalling pathways due to distinct amino acids compositions within the receptor that couple to stimulatory G proteins (Michel et al., 2007).
As biased signalling can be altered by the location of ligand-receptor binding, the occurrence of

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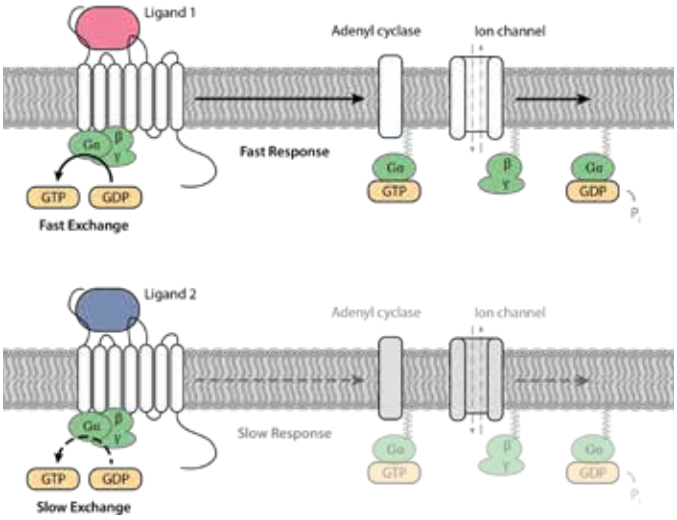

Figure 3. Ligands inducing different conformations within G proteins to produce different GTP–GDP exchange rates. Ligands inducing a faster exchange rate produces more G-protein and therefore downstream signalling events (Adapted from Wootten-et-al., 2018).

allosteric binding can produce a conformational change for the receptor and lead to biased agonism. Allosteric ligands can work as biased ligands to initiate specific receptor-mediated signalling. Furthermore, an allosteric ligand can activate specific downstream signalling by interacting with the orthosteric site ligand (Hodavance et al., 2016).

Therapeutic Use

Biased agonism has extensive therapeutic potential with drug development by stimulating specific signalling cascades for desirable biological effects while avoiding harmful outcomes as illustrated in Figure 4 (Franco et al., 2018).

For the development of therapeutic drugs with biased agonists, the physiological effects across multiple receptors must be thoroughly determined. Each pathway becomes unique depending on the disease state and bodily systems involved. The seven-transmembrane receptor (7TMR) and GPCR families are key targets for drug therapy. Numerous biased ligands specifically enable β-arrestins and G-proteins via 7TMRs as seen in Table 1 (Whalen et al., 2010; Bologna et al., 2017).

Opioid treatments

Opioid agonists producing analgesic effects are a well-known example of biased agonism in therapeutics. Early research established analgesic effects of β-arrestin2 following opioid receptor stimulation in mice. It indicates μ opioid receptor agonists are biased for G protein activation, becoming beneficial in pain management (Whalen et al., 2010). Findings enabled the development of Oliceridine, an opioid which activates G-protein signalling pathways with an efficacy comparable to morphine. The drug also averts respiratory depression and constipation by avoiding the β-arrestin pathway. A clinical study in 2016 confirming Oliceridine as a success demonstrates the therapeutic potential of biased agonism (Bologna et al., 2017).

Most opioids in clinical use selectively target the μ opioid receptor (μOR). Patients with chronic pain disorders commonly face undesirable side effects such as constipation, respiratory depression, and drug resistance and dependence. Side effects of μOR-based drugs were linked to β-arrestin2 pathway signalling as preclinical trials in mice demonstrated β-arrestin2 knockout produced a reduction in respiratory depression and morphine-tolerance (Mores et al., 2019). These findings have influenced the production of G protein-biased μOR agonists that minimize β-arrestin2 signalling. Therefore, opioids that selectively activate the G-protein pathway such as TRV130 have an improved therapeutic potential for moderate to severe pain.

Ligand	Receptor	Effector Bias	Therapeutic Area
Alprenolol, carvedilol	β1-adrenergic	β-Arrestin	Cardiovascular
MM07	Apelin	G-protein	Cardiovascular
SII, TRV120027	Angiotensin I	β-Arrestin	Release of inflammatory mediators
Fenoterol, carvedilol	β2-adrenergic	G-protein & β-Arrestin	Cardiovascular & renal

Figure 4: Biased agonist initiating a desired therapeutic effect on a receptor while avoiding unwanted side effects, compared to a typical agonist (Franco et al., 2018).

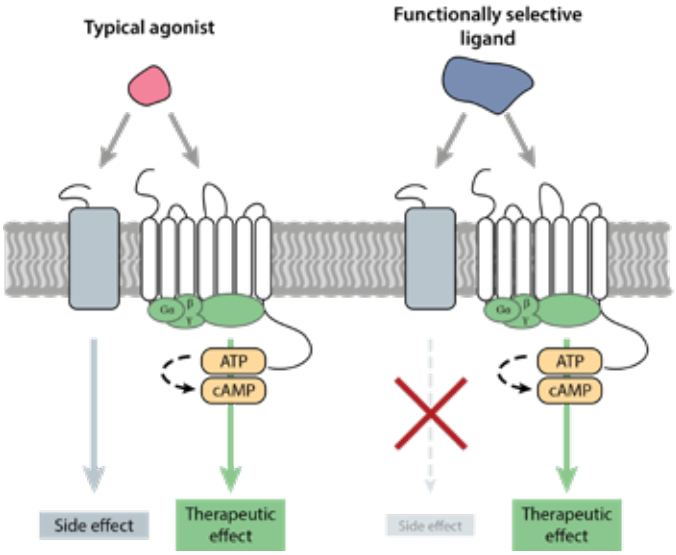

Figure 4: Biased agonist initiating a desired therapeutic effect on a receptor while avoiding unwanted side effects, compared to a typical agonist (Adapted from Franco et al., 2018).

The influence of β-arrestins on opioid analgesia has been the subject of many studies. Mice with β-Arrestin2 knockout experienced prolonged morphine-induced analgesia and an increased hypothermic response compared to wildtype mice. In addition to morphine, heroin produces enhanced analgesic efficacy in the β-arrestin2 knockout mice whereas other μ-OR agonists did not. Together, these studies show that the analgesic efficacy of μ-OR agonists are differentially regulated by β-arrestin2 *in vivo* (Mores et al., 2019). β-arrestin2 knockout mice were resistant to chronic morphine-induced tolerance, which correlates with preserved G protein signalling. This suggests that a G protein-biased ligand provides prolonged morphine-induced analgesia and a reduced tolerance to morphine, resulting in better patient treatment.

ß-adrenoceptor treatments

Therapeutic action of carvedilol through biased signalling mechanisms has been closely observed. Carvedilol is a β-adrenergic receptor antagonist used in the treatment of heart failure, myocardial infarction, and angina pectoris. Its cardioprotective mechanism works to decrease vascular resistance by inducing a vasodilation response, involving the relaxation of smooth muscle in blood vessels, causing an increase in diameter (Carr et al., 2016). The drug acts as a biased ligand on β1 and

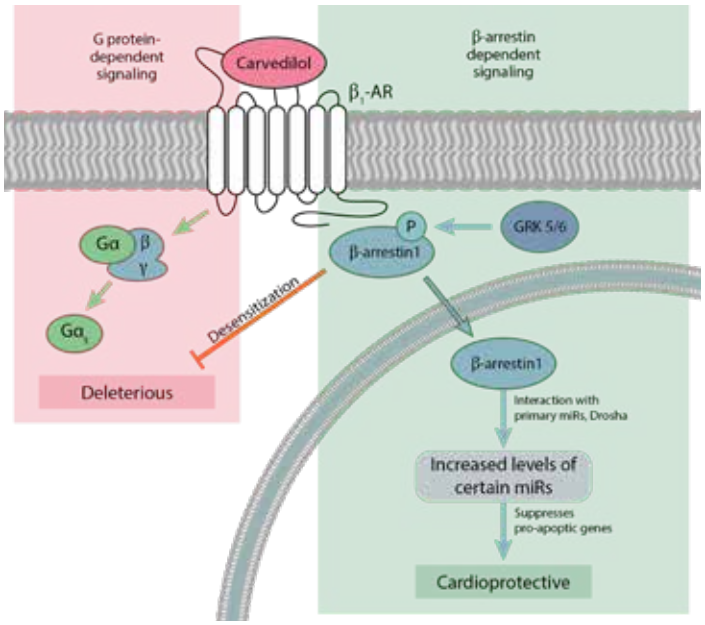

Figure 5: Carvedilol-initiated β1-adrenoceptor signalling. Biased activation of β-arrestin in cardiomyocytes stimulates miR subsets and represses pro-apoptotic genes to produce cardioprotective measures. G-protein activation is not induced (Adapted from Bologna et al., 2017).

> "Despite findings indicating therapeutic potential, several studies have identified challenges to biased agonism."

β2-adrenoreceptors to specifically stimulate β-arrestin dependent signalling as depicted in Figure 5 (Bologna-et-al., 2017). After binding to β1-adrenoreceptors, GRK5/6 phosphorylation of the receptor allows β-arrestin1 to activate five-miR compounds and the Drosha microprocessor complex in the nucleus. GRK5/6 is a G protein-coupled receptor kinase. This leads to initiation of RNA-helicase-miR processing which suppresses pro-apoptotic genes in cardiomyocytes to control myocyte contractility (Bologna et al., 2017). Pro-apoptotic genes encode proteins which have an essential role in the cell death pathway. This process occurs without the activation of G-protein signalling, avoiding deleterious outcomes in the cell.

In the pulmonary system β2-adrenoceptors help regulate movement of the smooth muscle cells in the airways. Therefore, β-agonists are often taken

to alleviate respiratory diseases such as chronic obstructive pulmonary disease and asthma as they induce a bronchodilation response, involving the relaxation of the smooth muscle of the bronchi, thus increasing the diameter and reducing airway resistance. Tachyphylaxis, which occurs when prolonged drug exposure reduces effectiveness, is a major issue in asthma treatment via β-arrestin mediated desensitization (Ippolito & Benovic, 2021). The efficacy of common asthma treatments such as Salbutamol are reduced, producing a decrease in the magnitude of bronchodilation. A reduced response causes asthma patients to become more reliant on β-agonists which increases the risk of damage associated with elevated sympathetic nervous system activity. Tachyphylaxis from β2-adrenoceptor activation occurs due to increased downregulation and desensitization of receptors following agonist binding (Matera et al., 2018). This is facilitated by β-arrestins, preventing interaction between the receptor and G-proteins. Phosphodiesterases reduce cAMP produced by G-proteins and block communication between G-protein and receptor, allowing β-arrestin to control desensitization in the cell (Ippolito & Benovic, 2021). Research findings show that β-arrestin2 removal in experimental mice produces a reduction in inflammation when treated with β-agonists relative to an unmodified subject. A reduced inflammatory phenotype could eliminate other factors that contribute to the poor outcomes of asthma patients, such as airway remodelling. It is therefore hypothesized that a biased ligand activating G-protein signalling pathway that avoids β-arrestin activation would not produce tachyphylaxis and act with a higher efficacy (Matera et al., 2018). This has become a growing area of interest in therapeutic research as G-protein mediated pathways in asthma treatment are explored.

Neurological disorder treatments

Biased agonism in drug development has significance in the treatment of nervous system disorders. Specifically, the receptors D_2 Dopamine and μ-opioid (μOR) are major targets in therapeutic design. D_2 Dopamine receptors were originally used to manage patients with schizophrenia by inhibiting adenylyl cyclase via $G_{\alpha i}/G_{\alpha 0}$ which is a G protein alpha subunit.. Current research has found that β-arrestin2 can regulate the AKT-GSK3 pathway via D2 dopamine receptors (Gundry et al., 2017). This causes dephosphorylation of AKT, protein kinase B, and alleviates behavioral effects. Lithium drugs are commonly used to target this complex in the management of mental health conditions. β-arrestin2 knockout in mice during experimental trials resulted in behavioral defects as the effects of lithium treatment were lost. A developed β-arrestin biased D_2 dopamine receptor agonist has a greater effect than balanced agonists in the treatment of schizophrenia in mice which suggests the necessity of biased agonists when managing such conditions.

As previously discussed, the μOR is a target for exogenous opioid analgesic agonists like morphine to reduce pain sensation. Morphine is biased for the G protein-mediated signalling pathway. β-arrestin2 removal in mice has resulted in amplified morphine-induced analgesia compared to unmodified mice. It was found that β-arrestin2 removal in mice protects them from unwanted side effects of analgesic medications including constipation and respiratory depression (Gundry et al., 2017). G protein-biased μOR agonists have been created to provide analgesia in animal models without such side effects which is promising for future human clinical trials (Gundry et al., 2017).

Cancer cell treatments

β-arrestins participate in multiple cancer-related signalling pathways via several receptor types. The protein β-arrestin1 has a known role in nicotine-induced replication of lung cancer cells using a process with a β-arrestin1-scaffolded complex and nicotinic acetylcholine receptor. Similarly, in cancerous ovarian cells β-arrestins mediate endothelin type A receptor-transactivation which is involved in metastasis activation (Michel & Charlton, 2018). Overexpression of β-arrestin1 in mice promotes tumour angiogenesis and development. As well as initiating proliferative signal pathways in cancer, β-arrestins are also involved in tumour suppression pathways. Transforming growth factor-β inhibits epithelial cell migration via β-arrestin2-mediated activation of the cell division cycle. β-Arrestin2 knockout in mice leads to increased tumour growth

and metastasis mediated by inflammation and angiogenesis compared to wildtype mice (Song et al., 2018). This research demonstrates that the β-arrestins are involved in a variety of cancer related signalling through biased agonism and can provide insight into potential future targets for chemotherapies.

Future Directions

Despite findings indicating therapeutic potential, several studies have identified challenges to biased agonism. These limitations complicate the identification of biased agonists and include an unexpected propagation of bias in data analysis and the need to clarify complicated physiological signalling pathways (Gundry et al., 2017). Techniques for quantitative analysis of bias and suitable screening assays can produce false positive results which skew the validity of obtained results (Michel et al., 2018). The belief that signalling bias is constant across other species implies a potential risk when investigating treatment options, such that animal models may prove problematic for biased agonist discovery. For example, histamine H4 receptor antagonist JNJ7777120 has a recognised role in reduction of inflammatory processes via the recruitment of β-arrestin in humans. This conflicted with trials on mice and dogs where JNJ7777120 acted as a partial agonist in the $G_{\alpha i}$-protein pathway, producing a completely different response (Michel et al., 2018). Different functions of the ligand across species has led to increased concern for drug toxicity studies conducted with mice and other animals rather than human samples.

Experimentation with biased agonism continues to evolve. Using a range of technologies, differential activation of G-protein and β-arrestin signalling molecules across multiple receptors continue to be discovered. Most ligands developed are synthetic but these can also be sourced from natural systems. Unique biased agonist profiles continue to be researched to test the effects of agonists and antagonists for many diseases (Kenakin, 2009).

Conclusion

The understanding of biased agonism mechanisms has caused the potential for therapeutic development across a number of diseases to be increasingly promising. Biased agonists are capable of binding specific active-state receptors in order to initiate cellular signalling of a single pathway, inducing a precise response. Activating intracellular proteins such as G-proteins or β-arrestins helps to influence cell signalling bias. As mechanisms involving specific pathway activation become better understood, its therapeutic potential is recognized as a subject for continued investigation. Biased agonism has introduced the ability to produce desired biological effects whilst avoiding undesirable outcomes.

As previously discussed, biased agonism is currently used in many common treatments. The drug carvedilol acts to decrease vascular resistance and help manage heart failure by binding to β1 and β2-adrenoreceptors to specifically stimulate β-arrestin dependent signalling. Similarly, in the respiratory system, treatments such as Salbutamol bind to β2-adrenoreceptors to produce a bronchodilation response to manage asthma. Biased agonism has additionally been applied to treatments for neurological disorders and several types of cancer.

However, challenges including false positive results, tachyphylaxis, and lack of clinical trials threaten the validity of current research. As scientific investigation continues into the signalling pathways of β-arrestins and G-proteins via GPCR activation, our understanding will continue to advance with biased agonism becoming an area of interest in the biotechnology field.

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