Cardiovascular research with HTRF assays

Abstract

Building upon the proven convenience and reliability of HTRF®, Revvity scientists have developed an ample portfolio of kits and reagents catering to researchers studying hypertension, nitric oxide donors, atherosclerosis, cardiac hypertrophy and arrhythmia.

cGMP, cAMP, IP-1, PCSK9, Phospho-proteins, Phospholamban, Apolipoproteins, and cytokines are only the start of the Revvity's all homogenous solutions for cardiovascular research. This note gathers publications that exemplify various applications of using HTRF to advance cardiovascular research and show how other researchers have harnessed the power of HTRF to advance their cardiovascular research.

Therapy Oriented Research – Study of the Nitric Oxide Pathway

Nitric Oxide is a key effector protein that - among other roles - is involved in cardiovascular hemostasis and vascular relaxation. Its major receptor is Soluble Guanylyl Cyclase (sGC), a receptor that is expressed in various cellular types but whose activity is best known in vascular smooth muscles.

In the event of a vascular stress, NO is synthesized at its location. It binds to sGC on the environing tissues, triggering the downstream decrease of Ca2+ and increase of cGMP, which in turn alters the activity of a collection of protein kinase G, cyclic nucleotide-gated ion channels and phosphodiesterases, leading to the vasodilatation of nearby vessels, blood flow increase and lowered tension.

It is suggested by risk factor and genetic studies that long-term low efficacy of this ligand/receptor couple may be key in the pathogenesis of hypertension, heart-failure symptoms, incidence of myocardial infarction, stroke and death.

The NO-sGC signaling pathway is therefore highly investigated for therapeutics that could restore deficient functioning

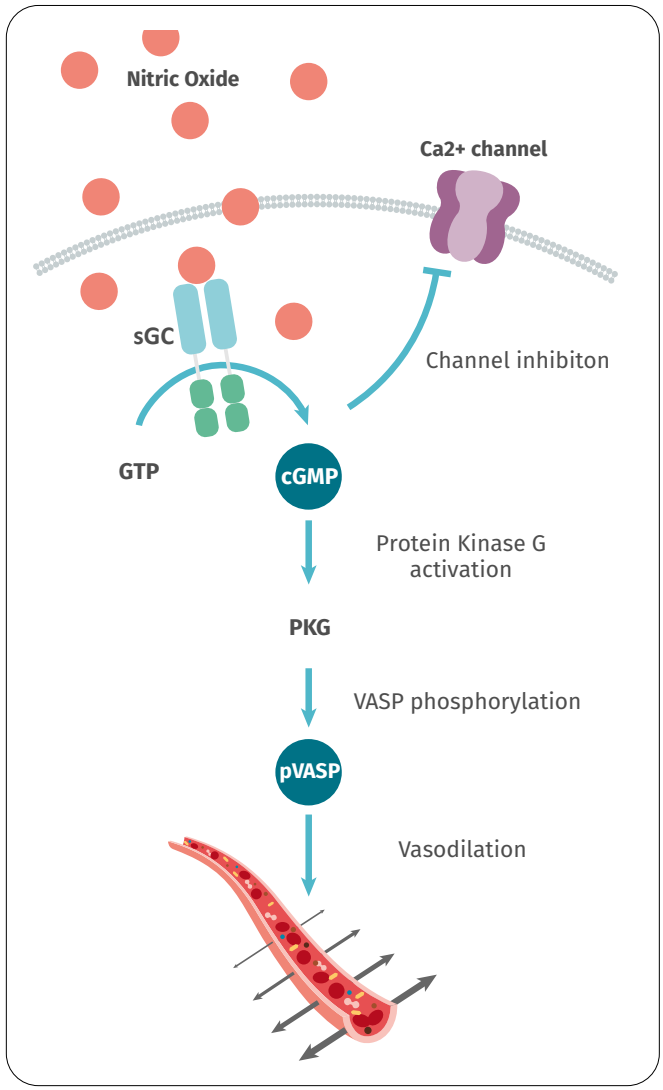


Figure 1: Nitric Oxide simplified signaling pathway.

Investigate an Inhibitor Mechanism of Action

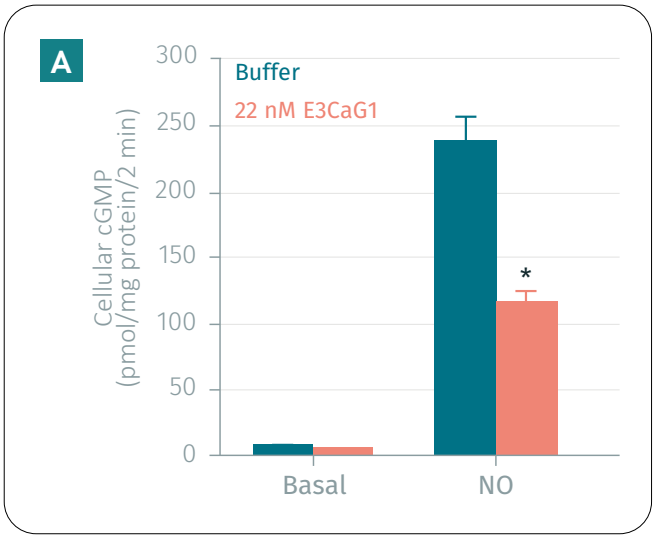
Ramanathan S, et al. (2011) Biochemistry 50(36): 7787-7799

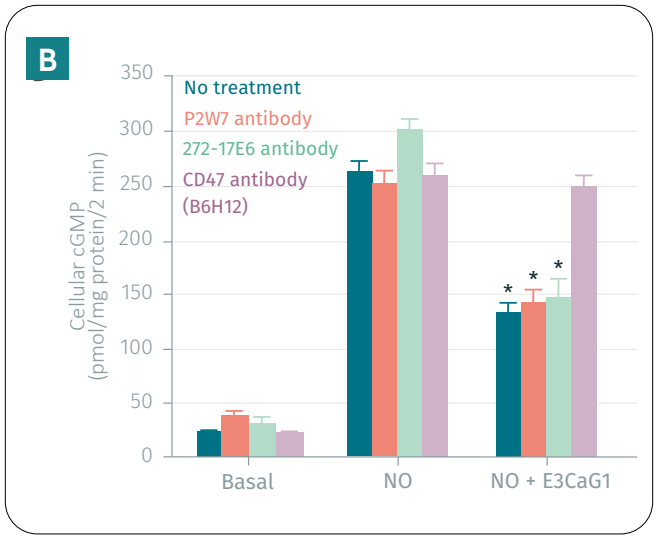
In 2011, Ramanathan *et al.* aimed to investigate and better describe the newly identified sCG inhibitor Thrombospondin-1 (TH-1), a multi-domain protein that binds to several membrane receptors (including CD47) and whose inhibitory potency on NO-sGC activity was assessed yet not explained.

They performed a collection of HTRF cGMP assays on live cells and cell lysates to assess the effects of various situations on sGC activity in the presence of NO.

The results allowed them to identify the E3CaG1 fragment of TH-1 as the carrier of the NO-sGC signaling inhibitory potency when bound to CD47 (fig 2.A). This finding was further confirmed using CD47 antibodies that restored the NO-sGC signaling in presence of E3CaG1 (Fig 2.B).

Additional, experiments including calcium imaging allowed them to determine that E3CaG1:CD47 binding results in an increase of intracellular calcium, which was confirmed as being the NO-sGC inhibitory trigger in another cGMP assay (Fig 2.C). They added to this finding by postulating and confirming the inhibitory potency of angiotensin II on NO-sGC activity since it triggers an increase in calcium when binding to its receptor AT1.





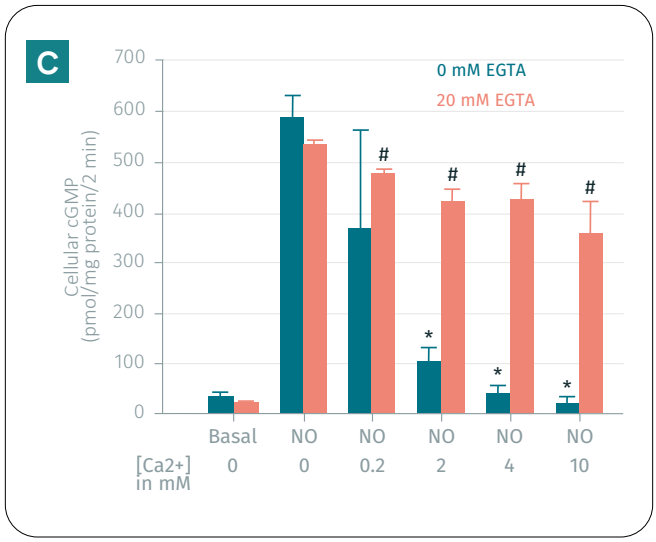


Figure 2: Cellular cGMP in Jurkat cells in presence or absence of NO stimulation. (A) Cellular cGMP is decreased when cells were incubated with the E3CaG1 fragment of TH-1. (B) Addition of anti-integrin Ab (P2W7 and 272-17E6 antibodies) has no effect on E3CaG1-induced inhibition. Addition of anti-CD47 antibody restores NO-induced sGO signaling. (C) Inomycin-induced Ca2+ increases inhibit NO-induced sGC signaling

The same cGMP assay was performed to evaluate the previously described lack of influence of phosphodiestererases in NO-sGC activity and confirmed those findings.

Develop Drug Candidates for Hypertension and Vascular Disorders

Tobin J.V, et al. (2018) JPET 365(3):664-675

A research approach to treat vascular disorders consists in identifying and developing inhibitors or stimulators of the NO-sGC activity in order to restore a deficient signaling to a healthy level.

In 2018, Tobin *et al.* sought to pharmacologically characterize IW-1973, a newly engineered sGC stimulator in clinical development (J.V. Tobin, 2018).

As the immediate product of NO-sCG signaling, cGMP was monitored in an HTRF assay following incubation with IW-1973. The authors also used the total VASP and phospho VASP Ser 239 HTRF assays to further evaluate the potency of their stimulator, as one of the end results of NO-induced sGC activity is the increased phosphorylation of the vasodilator-stimulated VASP into pVASP Ser 239 (Fig 3).

The parallel use of these two assays allowed to assess both the sGC binding and activity stimulation of the inhibitor (cGMP), and the transduction of this signal into an effective vasodilation (vasodilator-stimulated phosphorylation of VASP).

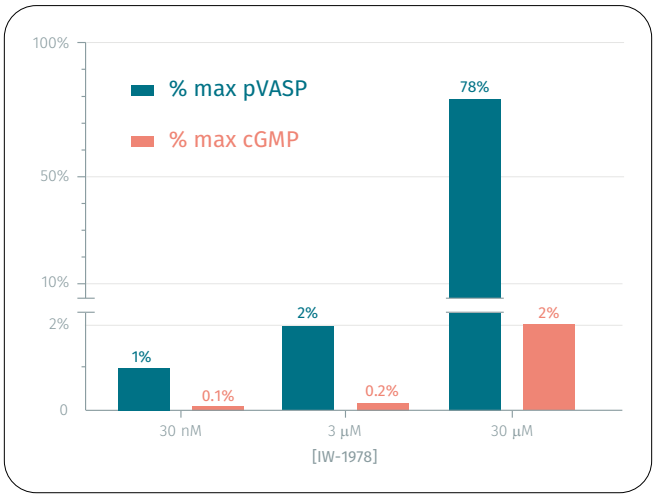


Figure 3: Dose-response analysis of IW-1973 over cGMP and pVASP Ser 239 % of maximum concentrations in HEK-293 GloSensor™ 40F cGMP cells without NO donor.

The results determined that IW-1973 stimulates sGC with an EC50 of 267 nM in the presence of 30 mM of NO donor. The co-measurement of cGMP and pVASP led the authors to conclude that effects on VASP phosphorylation almost reached completion (78% of maximum phosphorylation) when effects on cGMP were very low (2% of maximum [cGMP]).

High-Throuput Screening for Drug Candidates

Inhibitors of Apolipoprotein CIII Secretion

Jun Lee S & Mahankali M, et al (2017) Sci Rep 7: 5824

High triglyceride levels are known to correlate with increased risks of cardiovascular diseases. One of their key regulators and logical target for therapeutic application is Apolipoprotein CIII (ApoC-III) (roles in lipolysis and lipid synthesis).

In 2017, Jun Lee & Mahankali *et al.* aimed to identify small molecule inhibitors of ApoC-III that could potentially exhibit therapeutic effects. Using an HTRF ApoC-III assay, they screened over 950,000 compounds and qualified those exhibiting an inhibition higher than 50% as hits.

Further investigation of those hits was performed in dose-response analysis (fig. 4).

The overall hits hinted that trans-retinoic acids (atRA) might be especially potent inhibitors. Knowing that such molecules interact with two receptor families (RARs and RXRs), the authors further used the HTRF apolipoprotein assay to determine that atRA effects on ApoC-III were mediated through RARs only.

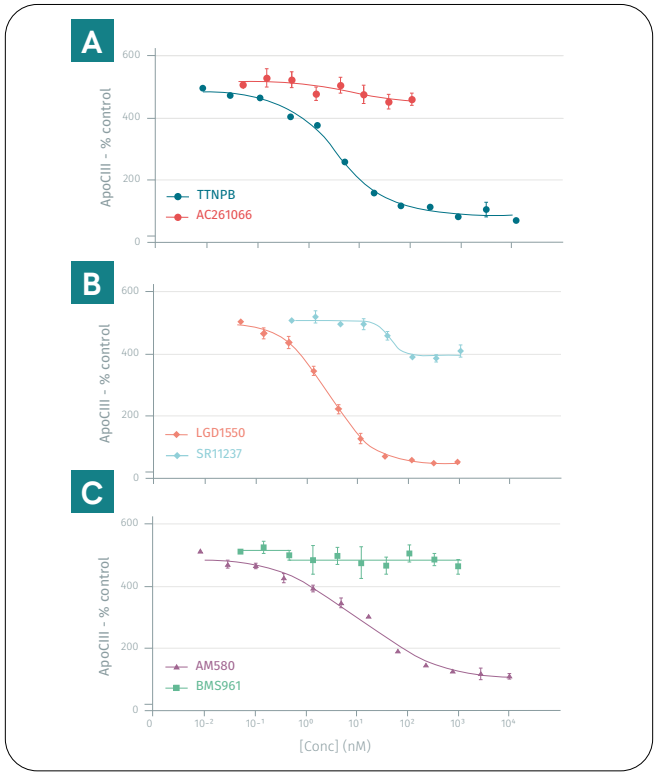


Figure 4: Effects of specific RARa activation on ApoC-III secretion. (a) TTNPB, RAR pan-agonist & AC21066, RARβ agonist (b) LGD1550, RAR pan-agonist & SR11237, RXR agonist (c) AM580, RARα agonist. & BMS961, RARγ agonist.

Overall, this study allowed for the identification of RAR receptors agonists of various potencies on ApoC-III secretion. AM580, the most potent of them was shown to decrease ApoC-III secretion by 80% and effectively improved body weight, hepatic condition, heart condition and triglyceride levels in high-fat diet mice.

"These studies suggest that an RARα specific agonist may afford a new strategy for lipid-lowering and CVD risk reduction" the authors concluded.

Characterizing Ligands

Case of Newly Developed Beta-Arestin Receptor Ligands

Mitronova G.Y, et al (2017) Sci Rep 7(1):12319

β-Arestin is a multifunctional signaling protein that mainly acts upon GPCR desensitization and which activation of EGFR/ERK signaling via the β-1-adrenergic receptor has been observed and suggested to be cardioprotective in heart-failure models.

To better and directly observe physiological and pathological functions of cells and tissues expressing β-Arestin Receptor (bAR), Mitronova *et al.* (2017) worked to develop fluorescent ligands of such receptors. They based their work on the known bAR antagonist carazolol and agonist BI-167107, which were bound to a collection of fluorophores.

Upon developing the fluorescent βAR ligands, Mitronova's team performed characterization steps to assess the impact of fluorophore binding on ligands' binding properties and bioactivity.

These included a FRET-based cAMP assay to evaluate the new ligands' potency and the HTRF Tag-lite β1AR or β2AR assays to determine their respective dissociation constants (Kd) and evaluate the impact of fluorophore binding on ligands' properties. This approach allowed them to conclude on the respective suitability of their fluorescent ligands for further studies of βAR-expressing cells and tissues, as well as provide recommendation regarding the experimental conditions in which each of them performs best.

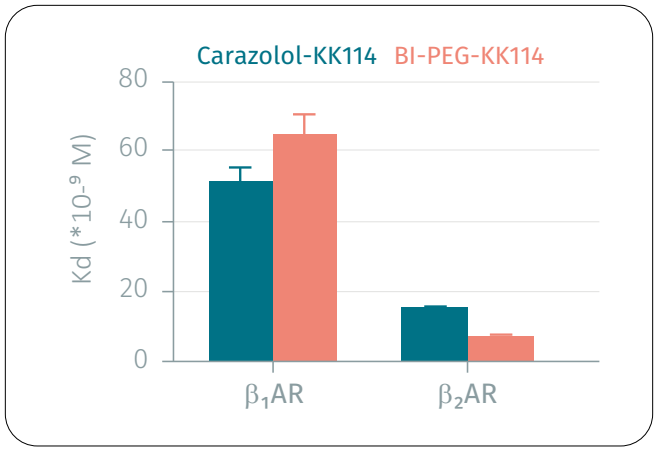


Figure 5: Kd app values of fluorescent ligands BI-PEG-KK114 and carazolol-KK114 obtained in HTRF assay.

Conclusion

The publications gathered in this Note show how HTRF assays have been a relevant tool for cardiovascular related research for years and has proved to be successful at handling the range of applications and experiments necessary to advance the knowledge of this area, including:.

- Thorough investigations of signaling pathways
- Pharmacological characterization of drug candidates
- Study of ligand/receptor interactions
- Robust screening of considerable amounts of compounds for potential molecules of interest

Works cited

- Tobin J.V, *et al.* (2018). Pharmacological characterization of IW-1973, a novel soluble guanylate cyclase stimulator with extensive tissue distribution, anti-hypertensive, anti-inflammatory, and anti-fibrotic effects in preclinical models of disease. J Pharmacol Exp Ther 365(3):664-675
- Mitronova G.Y, *et al.* (2017). High-Affinity Functional Fluorescent Ligands for Human β-Adrenoceptors. Scientific Reports 7(1):12319
- Ramanathan S, *et al.* (2011). Thrombospondin-1 and angiotensin II inhibit soluble guanylyl cyclase through an increase in intracellular calcium concentration. Biochemistry 50(36): 7787–7799
- Jun Lee S, *et al.* (2017). A novel role for RAR agonists as apolipoprotein CIII inhibitors identified from hight throughput screening. Scientific Reports 7: 5824

Related HTRF assays

Product	Tests	Cat. No.#
cGMP kit	500	62GM2PEG
Human Apolopoprotein A1 kit	500	64APAPEG
Human Apolopoprotein B kit	500	64APBPEG
Human Apolipoprotein C3 kit	500	63ADK001PEG
Human Apolipoprotein E kit	500	63ADK004PEG
Adrenoceptor Beta 1 labeled cells	200	C1TT1BETA1
Adrenoceptor Beta 2 labeled cells	200	C1TT1BETA2
Adrenergic beta2R stably expressing cells		C1SU1BETA2
Beta 1 adrenergic receptor green antagonist	5000	L0023GRE
Beta 2 adrenergic receptor green antagonist	5000	L0011GRE

Revvity, Inc
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com