Hello everyone. My name is Marina Varela. I am currently a trainee at WCAIR in the University of Dundee. In this presentation, we'll cover a few concepts and considerations regarding Pan Assay Interference Compounds, also known as PAINS. And to start off, I wanted to give an overview of what a PAIN compound is. Now, like I said, they are Pan Assay Interference Compounds and that means that they are molecular entities with intrinsic reactivity that are capable of interfering with one or more types of assays and give out false positive results. And to illustrate this, in 2014, Nature published a commentary article introducing PAINS as chemical con artists. And this is in reference to the misleading characteristic that these molecules may possess. But what is the actual problem with PAINS? Well, if you happen to find an active compound from your screening, but this result is actually false positive, meaning it's not coming exclusively from a specific interaction with the target, but rather from the compound causing interference with your assay. This molecule is non-progressable. This means even if you modify the structure, if the particular portion that is causing interference is still present, you might still get a false positive result and therefore not fully explore the structure activity relationship. What do PAIN compounds look like? Well from that same Nature article, this cartoon illustrates a few know PAINS and their respective forms of interference that they cause are described in the black boxes below. Structure wise, PAINS can be whole reactive molecules like toxoflavin or just small substructures can be problematic. You might also find in the same structure, different PAIN alerts causing different types of interference, which is the case of curcumin. And as you can see from the black boxes, each of these compounds causes specific assay interferences. But instead of going over all of them individually, I will highlight a few of the most common ones. For instance, compounds that are redox cyclers can interfere with redox dependent assays. And one common feature for this type of compound is the presence of phenol or quinone rings. Compounds with intrinsic reactivity can covalently binds to proteins in a nonspecific, non-drug like way and therefore cause a false signal. In the case of curcumin, this is due to the presence of this unsaturated ketone moiety, also known as a Michael acceptor. Compounds that are unstable under the assay conditions can also cause interference by breaking apart and releasing reactive molecules. Or how well do this characteristics correspond to Pan Assay interference. And to understand this, Capuzzi's group searched PubChem for compounds tested in multiple assays and categorized them into frequent hitters and infrequent hitters for the ones tested in protein-protein interaction assays and using the same technology. Random compounds correspond to those that were tested in at least 25 different assays. And lastly, known drugs that were tested in other bioassays such as for drug repurposing. Then all of those compounds were scanned for PAIN alerts. And the majority of them, as you can see, turned out to be non-PAINS. Then they looked at the activity calls for each category in three specific assays, different detection technologies. So luciferase, beta-lactamase, and fluorescence, as well as activity calls irrespective of the detection method in all assays. Analyzing the data, one can observe that PAINS were more consistently active in all assays. Irrespective of the detection technology used, the highest percentages of actives is observed for frequent hitters and drugs for both PAINS and non-PAINS compounds. And this suggests that one must consider the molecular entity as a whole before trying to derive any assay promiscuity from the dat. To illustrate this behavior I have here, the case curcumin. Curcumin is a natural product with a simple structure that has been vastly studied over the last few decades. Most studies in vitro show excellent promise of this compound and derivatives for many different activities. This fact raises the question, well, is this a PAIN compound? Is it solid gold in reference to its bright yellow color? Is it "Curecumin" due to all sorts of activity detected in vitro. In fact, there is even a database dedicated the curcumin research with over 100 analogs published in over 10 thousand papers describing different biological studies. As well as over 500 patents deposited for hominoids. This is probably a great propagation of a misleading data and inadequately assessing compounds. And most likely the reason why there is no clinical use to grow prominoids to date and not the observed results might actually

be due to the fact that curcumin can cause all known types of interference, such as covalently binding to proteins like I mentioned, due to the presence of the Michael acceptor moieties, it can collect metals, it can form aggregates. It can enter the redox cycle because of the presence of this two phenol rings, it can cause membrane disruption and also interfere in fluorescence based assays. And even with the use of orthogonal assays, one may still find problems with different sorts of interference. But that is not to say that all compounds with pain alerts are bad or misleading. In fact, 5% of FDA approved drugs possess some pain alerts within their structure. And to exemplify this, here I have the structure of four known and useful drugs with clear PAIN alerts in their structure. The first one is doxorubicin, an anti-tumor oral drug. And you can see here the presence of two fused quinone rings that may have redox activity. Sulfasalazine has this azyl group marked as an alert. But this is in fact a bond that makes this a prodrug. And when broken, it releases the antimicrobial sulfonamides and the anti-inflammatory salicylates. Thirdly, we have an alkyl aniline moiety present in ketoconazole, which was the first systemic antifungal agent in the market. And lastly, we have carbidopa that has the catechol ring moiety that can be further oxidized and form the quinone. Now, all of those drugs are discovered prior to the development of PAINS filters, and are good reminder that even if a molecule has an alert, that does not necessarily mean it will be bad, promiscuous, or even certainly cause interference in the assay. These are the alerts are something to be mindful of when designing and assessing activity, but not a death sentence for the compound straight away. Now, I have been talking about compounds causing interference, but how does the actually come about? And that mainly depends on three things. The compound being tested, the assay, and the detection technology. I have two examples in the next couple of slides that can illustrate how the compounds can cause interference. The first one covers the interference in color metric assays, such as the ones based on resazurin and MTT. And these assays are highly used in high-throughput screening to determine cell viability. And that is because when the cell is alive and functioning, it has an active metabolism that produces NADPH. NADPH will act as a reducing agent capable of converting resazurin, which is blue and of low fluorescence, into it's highly fluorescent reduced form resorufin. Now if a test compound can also chemically reduce one to the other, it can cause interference. Another thing to bear in mind is that if a test compound contain either fluorophores or chromophores that emits signals in the same range of wavelength as the probe, that will cause a falsel readout as well. Neufeld's group published a study showing just how much particular compounds can reduce resazurin to resorufin in the absence of any cells. And as an example, one of the compounds tested was glutathione. And looking at the mass spectra here, you can see that after just one hour, over 75 % of the resazurin had been converted. And this trend continues in all time points to a point where almost all of resazurin has been converted. Comparing with other data from the experiment, they associated a higher reductive capacity of glutathione to the presence of thiol and carboxylic acid groups. And these are commonly found in tests compounds and show just how much interference a chemical reaction can cause. Another example, quite a specific actually is the interference of salicylic containing molecules with TR-FRET assays. This is a particular case where the interference did not come from a PAIN alert. Hanley's group were looking for compounds that could interact with an allosteric pocket in a particular protein, PD-1, and modulate its interaction with a different protein, PD L2. And after running the screening, Salicylate-containing molecules showed to be the most promising and were chosen for in-vitro analysis. However, these molecules are fragments, so they are quite small and high concentrations would be needed in the assay to elicit a response. And in high concentrations, even weak flurophores or chromophores can cause interference in calorimetric assays. So therefore, they chose TR-FRET assay instead. As a form of assessment because this assay is not particularly affected by this sort of interference. The assay works by energy transfer from this Europium atom to an acceptor dye after energy has been given to the system in a distant dependent manner. So if the proteins interact, it changes the conformation of the complex and the energy can be transferred from the

Europium to the acceptor dye, which will then emit a signal that can be quantified and associated with activity. When the first results came in, they show what the scientists are expecting. An increase in emission much higher than that observed for the control. However, somewhere along the way they came across the fact that so salicylate moieties can actually interact with the Europium
atom and this interaction causes a long-lasting fluorescence to be admitted and therefore transferred to the acceptor dye. And to check for the occurrence of this phenomenon, they removed PD L2 from the assay to exclude the protein-protein interaction factor. And therefore, any changes observed would be only due to the interference. The results shown by the red bars confirm the higher emissions than the vehicle or obtained irrespective of the presence of PD L2. To then properly assess the activity of the fragments, they ran an orthogonal assay based on SPR technology. And notice that none of the compounds previously deemed less active had a significantly different result than the vehicle. And this shows the importance of understanding the assay and possible interferences you may be susceptible to, regardless of being from PAINs or not. As well as using orthogonal assays to confirm or refute the activity observed. And to finish off, I would like to summarize all of the concepts covered in this presentation. Starting off by the definition of PAINS as being structural features that can cause assay interference and give out false positive results. Now this does not necessarily result in promiscuity. As promiscuous molecules are more associated with non-specific interaction with several targets. Whereas for PAINS, this is not always the case and you might not even interact with the protein at all. Many filters have been developed and are available to search for PAIN alerts in structures. But having an alert does not mean that the comparable certainly have PAIN behavior. And to check the reliability of the results from a PAIN compound, orthogonal assays are needed using different technologie.s And to finalize I'd like to remind you all that just like Lipinski's rule of five, these are guiding filters and not rules set in stone to be followed. They are quite helpful in industry when a huge chemical libraries are screened and filters are needed to help with hit selection. But be careful not to blindly use this filters without looking at the whole picture. Sometimes what I say is not giving you a true answer and you need to find it elsewhere and be able to critically analyze and interpret the data. These are the main references I used for this presentation. And in the parenthesis you can see the overall theme of each one of them and read more about what has been discussed. Thank you all for the time and attention.