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In Vivo PAkt Detection and in Silico Molecular Docking to Determine the Potential of BYL as an PI3K Inhibitor to Block Growth Signal Cascade and in Breast Cancer MCF Cell Line

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Abstract

Comparison of genome between normal and breast cancer cell line shows that there are mutations across PI3Ka that probably cause breast cancer. PI3Ka is a kinase that plays a significant role in cell signalling transduction by phosphorylation cascade and produce an effect on cell. SH2 domain on PI3Ka binds to activated phosphor-tyrosine amino acids in growth factors, activating PI3Ka p110 catalytic domain, which recruits PIP2 to membrane and phosphorylates it to PIP3. This leads to cell signalling outcomes, including activation of PDK which phosphorylates Akt, cell proliferation and death. Protein/lipid phosphorylation levels are tightly controlled by phosphorylases and kinases; therefore, transduction levels can be turned on/off via altering kinases or phosphorylases and PI3K can be a target for treating breast cancer. However, we need find out which PI3K inhibitor (A66, TGX and unknown inhibitor BYL) shows that greatest potency and efficacy against PI3Ka with respect to pAkt in MCF-7 cell line. Here we show BYL has the potential to be a PI3Ka inhibitor and all of them inhibit pAkt. Using in silico technique of molecular docking, we explain that BYL shows the same amount of four hydrogen bonds as A66. By carrying out an experiment that generate a concentration-response curve, we demonstrate that BYL has the highest potency and efficacy with respect to pAkt level among the three inhibitors, greater than A66 which is known previously to be a potent and efficacy PI3Ka inhibitor. Our results demonstrate BYL and A66 show higher potency and efficacy than TGX; TGX with less hydrogen bonds between drug-protein interactions reveals lower potency and efficacy. Our assay accessed the inhibition of pAkt caused by three PI3Ka inhibitors (A66, TGX and BYL) in breast cancer cell line MCF-7 and found out that tumour derived stimulation of pAkt were responsive to all three inhibitors with BYL exhibits the greatest inhibition, followed by A66 and TGX in vivo due to different drug-protein interaction. Furthermore, PI3Ka inhibition has the potential to block growth signalling cascade in breast cancer and is a major target of anti-breast cancer development.

Keywords: BYL, PI3K, breast cancer, antitumor, growth

1. Data Analysis

To study whether the drug-protein interactions be used to discover new inhibitors of a PI3Ka signal transduction pathway in cells, we use in silico technique of Molecular Docking via Pymol software. We put three unknown inhibitors into the active site of PI3Ka which is ATP binding site. A66 is a known PI3K inhibitor and mimic the same hydrogen bond as ATP to the ATP binding site in PI3K (four hydrogen bonds); polar groups are also involved in drug-protein interaction to help binding. Using A66 as a template, TGX, unknown inhibitors of compound 1 and 2 have two hydrogen bonds each while compound 3 has four hydrogen bonds. Therefore, compound 3 to be the potential PIK3a inhibitor.

With raw alphascreen data (duplicates) that were conducted in workshop 4 with a duplicate of two inhibitors

(TGX and BYL). Firstly, to test the statistic significant between two groups/inhibitors, we carry out a t-test to get a p-value in excel using function/formula of TTEST with tails of 2 and type of 2. I get a p-value of 0.836000634 which is greater than 0.05 so there is not statistically significant between Veh and Veh+Ins. There is probably something wrong with my experiment.

Using the raw data to present the effect of TGX and BYL, we must convert the data, generating a concentration-response curve and find their potency and efficacy (EC50 and Emax respectively).

When look at the data, there are some problems with my raw data. The pAkt levels in Veh alone and Veh+Ins seem to be quite similar which should not because A-D1 there is no inhibitor or insulin and the pAkt level should be low which is present in my data, however, with B-D 2 there are insulin present and there should be a huge increase in pAkt level as insulin can activate PIK3a that phosphorylates Akt. This matches with my p-value of greater than 0.05.

In addition, response does not look consistent with a low to high concentration because different concentrations of each inhibitor show a similar pAkt compared to basal levels, there is hardly any inhibition for two drugs; for group B3-8, there is an increase in pAkt level which is opposite to what is expected. This may be because cells did not stimulate properly; I did not aspirate all the media/drug mix so there is still inhibition reaction going on when detect pAkt levels later.

However, the data looks consistent between duplicates as between the same inhibitor, the data looks like each other for each well.

Firstly, convert the raw data to percentage of Insulin+Veh values, that is, (A3/A2)*100, (A4/A2)*100,…, (B3/B2)*100,…, (C3/C2)*100,…, (D3/D2)*100,…, (D8/D2)*100.

Secondly, convert the inhibitor concentration from uM to M using scientific notation.

Then, add the extra data set as in experiment I only did two inhibitors (TGX and BYL), therefore, I require the third data set of A66.

Next, reformat the data (transpose to vertical) that are required as indicated in on-line EC50 tool (https://www.aatbio.com/tools/ic50-calculator). That is, enter the different concentration in M in the first column from low to high concentration and for the second and third column, enter the corresponding percentage. For the other two inhibitors, add two more new data forms and reformat the data. Then this online tool will generate the concentration-response curve, EC50 and Emax for each of the three inhibitors.

Emax is not calculated automatically, it is extracted from the span of the data which is the difference between Max and Min value in the equation.

Finally, add the EC50 and Emax value to the data set and make n=10, the other 9 sets come from class. From the data set, we are able to see if there are differences in efficacy and potency between the three inhibitors.

Based on my data, the EC50 online tool generate an odd curve for concentration-response of TGX and BYL but still formulates a EC50 value of 9.4235e-7 for TGX and 7.8071e-10 for BYL; Emax of 13.6087 for TGX and 27.6666 for BYL so they should be excluded.

Class data has an average EC50 of 3.32E-07 and standard deviation (SD) of 9.99289E-08 for TGX while my EC50 for TGX is 9.4235e-7 which is not within 2 SD of mean; average Emax of 59.05 and SD of 3.807726 which my result does not fit within 2SD of mean. Similarly, class data has an average EC50 of 2.49E-07 and SD of 9.35883E-08 for BYL while my EC50 for BYL is not within 2SD of mean; average Emax of 93.71333 and SD of 3.277594 which individual data does not fit. The value for EC50 and Emax does not fit within two SD of the mean, it does not show adequate trend. Therefore, I cannot use my own data to analysis BYL and TGX response.

From the data set, we are able to see if there are differences in efficacy and potency between the three inhibitors. By checking that, we use ANOVA which tells if one or more treatments are significantly different via giving a F-statistic value. Then use Tukey to test which means are significantly different from each other.

With my own individual data, ANOVA and Tukey does not show one or more treatments are significantly different and there is no mean that is significantly different from each other so my data should not be included.

However, using three sets of class data, both ANOVA and Turkey show significantly different and are statistically significant.

2. Results

Table 1. Decision table for selecting ligands for biochemical testing

COMPOUND	Val795 BB[a]NH	Val795 BB[a]CO	Gln803 SC[b]NH2	Gln803 SC[b]CO	Total num of Hydrogen bonds	Test Y/n?
A66	Y	Y	Y	Y	4	Y
TGX221	Y	N	N	Y	2	Y
1	Y	N	N	N	1	N
2	Y	N	Y	N	2	N
3	Y	Y	Y	Y	4	Y

a BB=back bone; b SC=side chain

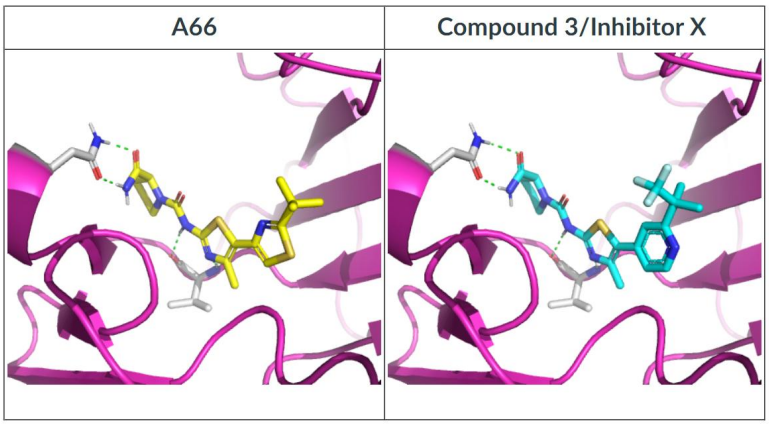


Figure 1. Predicted drug binding modes for a PI3Ka selective A66 and a potential PI3Ka inhibitor compound 3 (BYL) using Pymol

Jamieson used A66 as a potent and selective PI3Ka inhibitor model and found that carboxamide of the ligand forms polar hydrogen bond with carboxamide of the protein is essential in the inhibition reaction in the active site of PI3Ka. A66 as a selective PI3Ka inhibitor and TGX as a non-PI3Ka selective inhibitor in experiment. We used molecular docking Pymol to discover a potential PI3Ka inhibitor that shows the most similar features to A66, such as sum of hydrogen bonds and polar interactions with PI3Ka active site. Four drugs except compound 1 have at least one hydrogen bond interaction with both Val795 and Gln803 (Table 1). One polar hydrogen atom in TGX is not involved in hydrogen bonds; the interaction TGX with Val NH is not a predicted hydrogen bond. Overall, compound 3 shows the same hydrogen bonds as A66.

The predicted modes of A66 and compound 3 (Figure 1) show they have a similar core but different interaction with hydrophobic: A66 has five members in the ring with a sulphur while compound 3 has six with a nitrogen and additional halides attached to carbon. They make the same predicted hydrogen bonds with PI3Ka. Therefore, we select compound 3 as a potential PI3Ka inhibitor because it best mimic the pharmacophore of A66.

Effect of three inhibitors at multiple concentrations on pAkt level on insulin treatments. Insulin is at 500nM, and inhibitors are ranging from 1.28-4000nM from column 3 to 8. Preincubation with inhibitor is 15 mins. Stimulation is 20 mins. P-value generated from TTEST for column 1 (Veh) and 2 (Veh+Ins) smaller than 0.05.

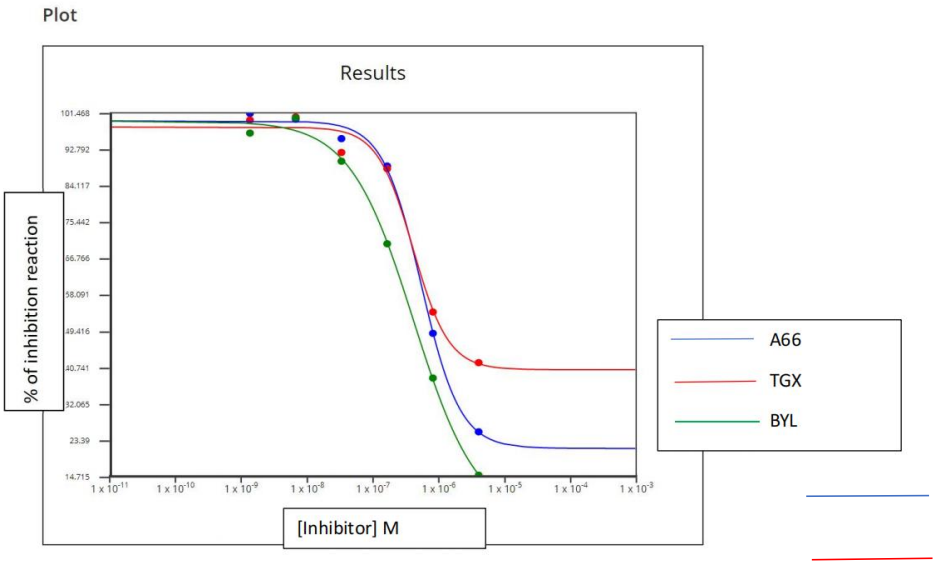
Table 2. Raw alphascreen data (duplicates) generated in MCF-7

	1	2	3	4	5	6	7	8	
A	4064	54340	53984	53416	48400	44010	26610	13789	**A66**
B	3456	51250	53090	52215	52160	49560	24850	12739	**A66**
C	4465	53980	54767	53842	51260	48840	27441	21935	**TGX**
D	4008	50590	49730	51331	45150	43480	28758	21631	**TGX**
E	4980	66520	63830	67417	58782	45660	23020	9331	**BYL**

F	3415	64440	62859	64037	59058	46250	26680	9925	**BYL**
	4.27731E-09								

In our experiment, Veh only (Figure 2) have extreme low pAkt as there is no stimulation and are our basal cell levels. Insulin as an agonist cause a huge increase in pAkt level (from thousands to millions) when compare Veh only to Veh+Ins. T-test comparing between Veh and Veh+Ins strengthen it: p-value is smaller than 0.05 so there is significant difference between them; MCF-7 responds to insulin by activating PI3Ka and increasing pAkt level.

All three inhibitors decrease pAkt level at its lowest concentration compared to Veh+Ins, therefore, they all inhibit PI3Ka at different concentrations. From lowest to highest concentration, they show a decrease trend in pAkt with BYL decrease pAkt the most (average of 63344 to 9628), followed by A66, with TGX decrease the least. CRC (Figure 3) illustrates BYL has the greatest efficacy due to highest Emax (95.75) compared to A66's 78.52 and TGX's 58.19 using MCF-7 cells and pAkt alphascreen. BYL is the second most potent inhibitor against PI3Ka whose EC50 is 4.06E-07, smaller than A66 but greater than TGX. Based on individual data, there are differences between the potency and efficacy for three inhibitors with respect to pAkt.



	EC50	Emax
A66	5.31E-07	78.52
TGX	3.96E-07	58.19
BYL	4.06E-07	95.75

Figure 3. CRC of pAkt in insulin treatment in MCF-7 varying three inhibitors (A66, TGX and BYL) at different concentrations

Different colours indicate different inhibitors treated into cell. The curve illustrates potency and efficacy of inhibitors; EC50 and Emax are indicated in the table. Increased efficacy (Emax) from TGX to BYL; increased potency and decreased EC50 from A66 to TGX.

Table 3. A table of ten data sets with different EC50 and Emax. The first nine data sets represent class data, and the tenth is individual data

EC_{50}（M）			Emax		
A66	**TGX**	**BYL**	**A66**	**TGX**	**BYL**
4.85E-07	2.76E-07	3.32E-07	87.44	59.92	95.72
4.12E-07	3.96E-07	1.14E-07	79.72	56.31	90.37

4.95E-07	3.01E-07	3.42E-07	76.11	57.23	92.21
4.04E-07	4.86E-07	3.15E-07	78.79	61.6	89.72
3.13E-07	3.45E-07	3.95E-07	82.18	58.14	97.65
2.03E-07	1.09E-07	2.36E-07	70.42	65.11	91.17
3.26E-07	3.20E-07	1.78E-07	81.86	63.04	94.92
2.11E-07	4.21E-07	1.59E-07	82.53	51.38	99.76
4.61E-07	3.31E-07	1.71E-07	71.34	58.72	91.9
5.31E-07	3.96E-07	4.06E-07	78.52	58.19	95.75

Looking at a larger sample, BYL shows the highest Emax for all ten data sets and most efficacious inhibitor. However, three inhibitors show a similar EC50 with any one of them can be the most potent.

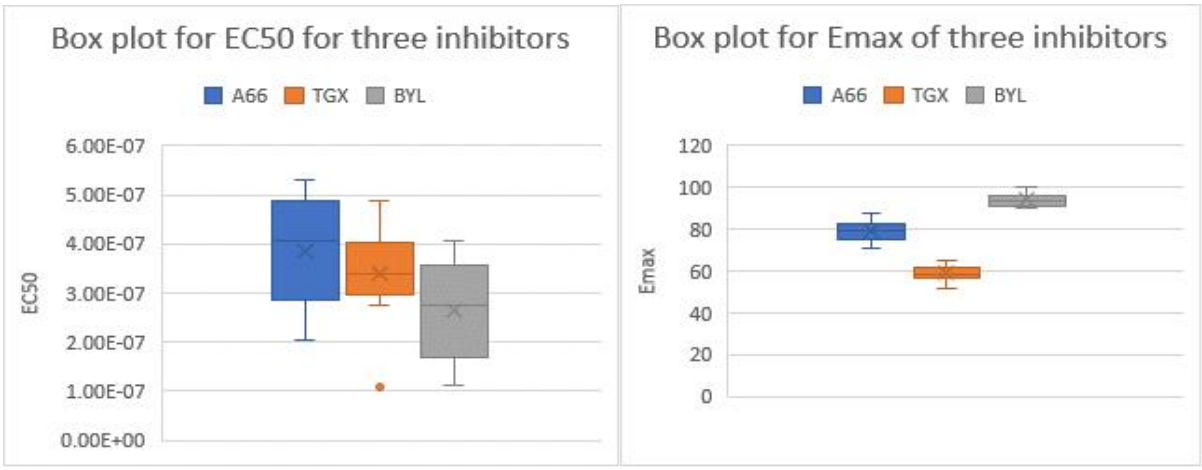


Figure 4. Two box and whiskers representing EC50 and Emax of three inhibitors (n=10)

The cross represent median of Emax or EC50. Whiskers extend to the largest and smallest values. For EC50 of TGX, there is a potential outliner and marked separately. BYL has the lowest median EC50 and highest median Emax, showing it has the highest potency and efficacy among the three.

Using ANOVA with Tukey, p-value of 0.0622 in EC50, greater than 0.05, indicating no significant difference between the three.

P-value of Emax is 3.3307e-16: lower than 0.05 so there is significantly difference. Tukey indicates Emax for A66 vs TGX, A66 vs BYL and TGX vs BYL are significantly different from each other.

In conclusion, based on individual and larger sample data, there is significant difference in efficacy between the three inhibitors but not potency.

3. Discussion

In this study we showed that BYL can be a potential PIK3a inhibitor due to it best mimic the pharmacophore of A66 which a potent and selective PI3Ka inhibitor. Secondly, MCF-7 respond to agonist insulin by increasing in pAkt level which is an outcome of activating PI3Ka. In addition, BYL decreases pAkt level in insulin treatment; the higher the concentration of BYL, the more inhibition reaction of pAkt and PI3Ka. BYL also shows a higher potency and efficacy than A66. Among the three inhibitors, there are statistically significant different between efficacy of them as shown by the ANOVA and Tukey test of Emax; but for potency, there is not enough evidence to state that there are significantly difference between EC50 of them.

From the predicted binding model of A66 to PI3Ka, we selected BYL as a potential one, we found Val795 and Gln803 are involved in drug-protein interaction. To see if they are the reasons of A66 being a selective PI3Ka inhibitor, therefore, support BYL as a PI3Ka selective inhibitor, we illustrate what happens if we change the ligand A66 or protein PI3Ka. When change the structure of A66, A66 S is more selective and more potent against p110a compared to A66 R because IC50 increases from 32nM in A66 S to greater than 5000nM in A66

(Jamieson et al., 2011). A66 R does not form a hydrogen bond with Val on p110a backbone and does not present a similar pose to A66 S, indicating the interactions involved in A66 S but not in A66 R which is Val is essential for its high selectivity and potency to PI3Ka. Similarly, stimulating A66 S ligand into PI3Ka proteins that undergo in vitro mutagenesis, mutated Q/Gln amino acid caused the smallest inhibition percentage (almost 0% inhibition) when A66 S inhibits 40% WT PI3Ka (Jamieson et al., 2011): without WT Gln residue, A66 S is unable to inhibit pAkt, therefore, Gln in PI3Ka is essential; A66 S can only inhibit pAkt if it has an interaction with Gln on PI3Ka.

Pymol produced a predicted binding model for BYL and Furet's crystal structure of BYL bound to PI3Ka provides the observed structural data (Furet et al., 2013). When compare the two models there are four hydrogen bonds involving in drug-protein interactions: two hydrogen bonds with Q859 and two with V851 which Val795 and Gln803 are analogue of them and matches with predicted Pymol binding.

In conclusion, both Val and Gln is important in A66's ability of being a potent and selective PI3Ka inhibitor. Thus, we can use these interactions to determine whether BYL is a potent PI3Ka inhibitor; our predicted BYL binding model indicates it mimic pharmacophore of A66 (four hydrogen bonds) and presents a similar interaction with the observed crystal structure of BYL bound to PI3Ka.

To seek the biochemical evidence of BYL as a potent PI3Ka inhibitor, in vitro assay of purified enzyme experiment conducted with recombinant PI3K protein is carried out, Fritsch Table 1 shows BYL is most selective and potent to PI3Ka rather than PI3Kb or other recombinants. This is because IC50 of PI3Kb is at least 200 folds increase compared to PI3Ka and at least 50 folds increases compare IC50 of other PI3K tested to PI3Ka (Fritsch et al., 2014).

Jamieson and Zheng both determine the IC50 for A66 and we compare the relative difference in IC50 between PI3Ka and PI3Kb. From Table 1 of Jemieson et al, A66 S has an IC50 folds increase change of 390 (12500/32=390) in alpha compared to beta and PIK74 has an increase in fold change of 13.3 (80/6=13.3) in a to b; from Zheng Table 1, A66 S has a 224-increase fold change of IC50 in PI3Ka contrast to PI3Kb. Thus, purified enzyme assays indicate A66 S is an absolute potent and selective PI3Ka inhibitor since the relative difference of IC50 between PI3Ka and PI3Kb show similar folds change in similar experiments; PIK74 is considered as a PIK3a inhibitor as well in Jamieson. Comparing with individual data, BYL is more potent than A66 S and is possibly a PI3Ka selective inhibitor.

However, there are limitations with making this comparison with assay cell types and assay condition. Using different cell lines, the same drug at the same concentration can lead to different pAkt response due to drugs have different selectivity in different cell lines.

Based on my data using MCF-7 cell, TGX has the smallest EC50, followed by BYL and A66: TGX is the most potent drug, followed by BYL and A66; BYL has the greatest Emax, followed by A66 and BYL, so are their efficacy.

From Jamieson Figure 3, they used a variety of different cell lines and used Western Blot to express A66's effect on pAkt levels in those cells. We see different cell lines respond differently to different concentrations of A66. A66 is more sensitive in HCT, SK-OV3 and T47D cell lines as they require less A66 to cause a decrease in pAkt; the Western Blot shows the same trend as own data: increase A66 concentration decreases pAkt in these three cells. On the other hand, MCF does not show much sensitivity and the concentration response does not show any evidence that A66 is sensitive to PI3Ka: at highest concentration of 10uM, it hardly decreases any pAkt (Jamieson et al., 2011). However, those three cell lines that A66 is sensitive to all harboured H1057R mutation in PI3Ka protein while MCF-7 did not and A66 is resistant to it. Jamieson Figure 4 further proves PI3Ka/p110a sensitive inhibitors are sensitive in H1057R cells but not in cells contain E545K mutations (MCF-7) and PTEN and concludes that the sensitivity of PI3Ka inhibitors is a direct consequence from H1057R mutations (Jamieson et al., 2011). Since H1057R increases intrinsic sensitivity of p110a inhibitor, it may not be a good idea to use it as a cell line.

Jamieson Figure 4 uses Western Blot to see the effect of isoform selective inhibitors (TGX and PIK-75) on activation of Akt/PKB using only one concentration. PIK-75 in this is considered to be an alternative PI3Ka inhibitor. In MCF7 cell line, TGX does not inhibit pAkt level as much as PIK-75 at the lowest concentration of each drug they could make (100nM) (Jamieson et al., 2011). Therefore, with the same basal pAkt level, TGX has a smaller span across the maximum and minimum pAkt it can inhibit and has a lower Emax compared to PIK-75 and a lower efficacy which shows a similar trend in Emax in my experiment (PI3Ka inhibitor has a higher Emax than PI3Kb inhibitor). When combine TGX and PIK-75, there is inhibition reaction which is higher than TGX alone but similar to PIK alone: this is consistent with the conclusion that PIK is more selective to PI3Ka than TGX. In other cell types in 4B, TGX does not inhibit the activation of pAkt at all at the concentration that it would be inhibit PI3Kb effectively (Jamieson et al., 2011). As a result, TGX is more selective to PI3Ka in

MCF-7 compared to other cell lines although still not as sensitive as to PI3Kb; TGX produce a decline in pAkt/PI3Ka only in MCF-7 and we can use it to estimate if an inhibitor is not PI3Ka sensitive using MCF-7 cell line because there will be a visualisable Emax/EC50. Using other cell lines, TGX does not decrease pAkt at all and it is hard to generate Emax/EC50 for us to demonstrate if BYL is PI3Ka or PI3Kb selective.

Elkabets Figure 1c presents molecular assay demonstrates that in five tested cell types, BYL block PI3Ka activity as seen by the inhibition of pAkt. However, this does not translate into whole cell assays investigating cell viability shown in Figure 1a. For example, BYL causes repression of pAkt and decrease cell viability in MCF-7 cell but in HCC1954, BYL inhibits pAkt but not decrease cell viability, it is resistant in HCC1954 (Elkabets et al., 2013). There is no relationship between if BYL block PI3Ka/pAkt activity and cell viability response. Therefore, maybe PI3K signalling pathway is not available as a drug target. Figure 1a also expresses that BYL decrease cell viability more in mutant cell lines than in wild type. In the wild type cell types, they are resistant to BYL and cell viability does not decrease. This is ideal for BYL being a therapeutic drug treatment for cancer as BYL does not alter survival of normal cells but cause the death of some PIK3CA mutated breast cancer cell lines (Elkabets et al., 2013). In addition, BYL as a therapeutic treatment may induce therapeutic effect in some cancer cell lines harbour PIK3CA mutations such as MCF-7 but not in others (HCC1954). This matches with my data that BYL is the most efficacious inhibitors in inhibiting pAkt among the three tested in MCF-3; since I did a molecular readout, whether BYL causes decrease in cell viability in MCF-7 is unknown and need further experiments.

Fritsch massively increases number of cell lines and cell viability assays (Figure 3b) shows cancer cell lines carry PIK3CA mutant are more responsive than PIK2CA WT. With a larger number of cell lines used, Fritsch supports Elkabets's conclusion BYL is responsive to some mutant cell lines. With 440 cancer cell lines express PIK3CA WT, differently from Elkabets who conclude WT are all resistant to BYL, Fritsch states some WT is responsive to BYL but fewer than PIK3CA-mut (22.7% WT vs 64.7% mut). Also, Fritsch confirms the data in Jamieson that same drug in different cell lines have different response trend: A66 is more selective in HCT than MCF. BYL selectively inhibits PIK3CA mutant cancer cells and from individual data, it is in good therapeutic efficacy for inhibiting PI3Ka/pAkt in cancer cells that express PIK3CA-mut but not strongly in PIK3CA-WT (Fritsch et al., 2014). This is a similar to what is concluded in my experiment: MCF-7, harbouring PIK3CA-mut is sensitive to BYL with respect to pAkt. However, I only used one mut and no WT cells, based on these literacy BYL is a PI3Ka inhibitor that can lead to decrease cell viability in MCF-7.

Based on the results obtained so far, BYL is an efficacious, potent and selective PI3Ka inhibitor in MCF-7 cell with good pharmacophore property mimic PI3Ka inhibitor A66; BYL is most selective and sensitive to PIK3CA mutation that present in MCF-7, indicating BYL is a good PI3Ka inhibitor that inhibits pAkt. Further studies will be required to determine whether BYL leads to the death of MCF-7 cancer cells and tumour repression.

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Experimental Protocol/Lab Notes

Making drug solutions and buffer:

Buffer/Vehicle needed to carry both AGG/TGX and Unknown inhibitor X:

7 mLs @ 0.2% DMSO

Pipette 14 uL of DMSO into 6986 uL of SFM (serum free media)

Make Insulin @ 2 uM, (2500 uLs)

Pipette 3.33 uL of insulin stock (1.5 mM in water) and add 2496.67 uL of SFM

Make A66/TGX @ 8 uM, (500 uL)

Pipette 1 uL of A66/TGX (4mM in DMSO) and add 499 uL of SFM

Make Unknown Inhibitor X @ 8 uM, (500 uL)

Pipette 1 uL of Unknown Inhibitor X stock (4mM in DMSO) and add 499 uL of SFM

To make 500 uL of each inhibitor (TGX/A66 and Unknown Inhibitor X) concentrations ranging from 0.128 to 4000 nM:

1.6 uM: pipette 100uL of 8uM inhibitor into 400uL of Vehicle

320 nM: pipette 100uL of 1.6uM inhibitor into 400uL of Vehicle

64 nM: pipette 100uL of 320nM inhibitor into 400uL of Vehicle

12.8 nM: pipette 100uL of 64nM inhibitor into 400uL of Vehicle

2.56 nM: pipette 100uL of 12.8nM inhibitor into 400uL of Vehicle

Aliquot Drugs into Strip Tubes for Pre-incubation

Pipette 150 uL of each inhibitor (TGX/A66) conc (6 different concentrations) or Veh into its appropriate strip tube and warm for 5 minutes (see figure 1 for strip tube layout)

With one more series of 8 strip tubes, pipette 150 uL of each Unknown Inhibitor X conc or Veh into its appropriate strip tube and warm for 5 minutes (see figure 1 for strip tube layout)

Preincubation (15 minutes)

Using a multichannel, pipette 50 uL of each TGX/A66 concentration (6 different concentrations) or vehicle from strip tubes into B2-B9 and into C2-C9.

Using a multichannel, pipette 50 uL of each Unknown Inhibitor X concentration or vehicle from strip tubes into D2-D9 and into E2-E9.

Pre-incubation lasts for 15 minutes with inhibitor.

Aliquot Drugs into Strip Tubes for Stimulation

Pipette 150 uL of each inhibitor (TGX/A66) conc or Veh into its appropriate strip tube and warm for 5 minutes (see figure 1 for strip tube layout)

With one more series of 8 strip tubes, pipette 150 uL of each Unknown Inhibitor X conc or Veh into its appropriate strip tube and warm for 5 minutes (see figure 1 for strip tube layout).

Pipette 150 uL of 2 uM insulin into the appropriate strip tubes of the two series of strip tubes (that is, pipette except for the one with Vehicle only)

For the Vehicle strip tube, pipette 150 uL of SFM and 150 uL of buffer into the strip tube.

Stimulation (20 minutes):

Using a multichannel, pipette 100 uL of each TGX/A66 concentration with insulin or insulin alone or vehicle alone from strip tubes into B2-B9 and 50uL into C2-C9.

Using a multichannel, pipette 100 uL of each Unknown Inhibitor X concentration with insulin or insulin alone or vehicle alone from strip tubes into D2-D9 and 50uL into E2-E9.

Stimulation lasts for 20 minutes with inhibitor and the agonist insulin.

Put the plate on ice and aspirate all the media/drug mix, then using a multichannel pipette to add30 uL of ice cold lysis buffer into each well.

Put the plate on plate rocker for at least 10 minutes

Using a micro-multichannel pipette, pipette 8 uL of lysate containing samples into a white ½-area 96 well plate.

pAKt detection (using a Perkin Elmer AlphaScreen Sure Fire (Ser473) kit)

Add 8 uL of acceptor mix to each well containing sample, tap and rock the plate. Cover the plate with foil and stay at room temperature for two hours.

Add 4 uL of donor mix to each well containing sample under low light, tap and rock the plate. Seal and leave at room temperature for two hours.

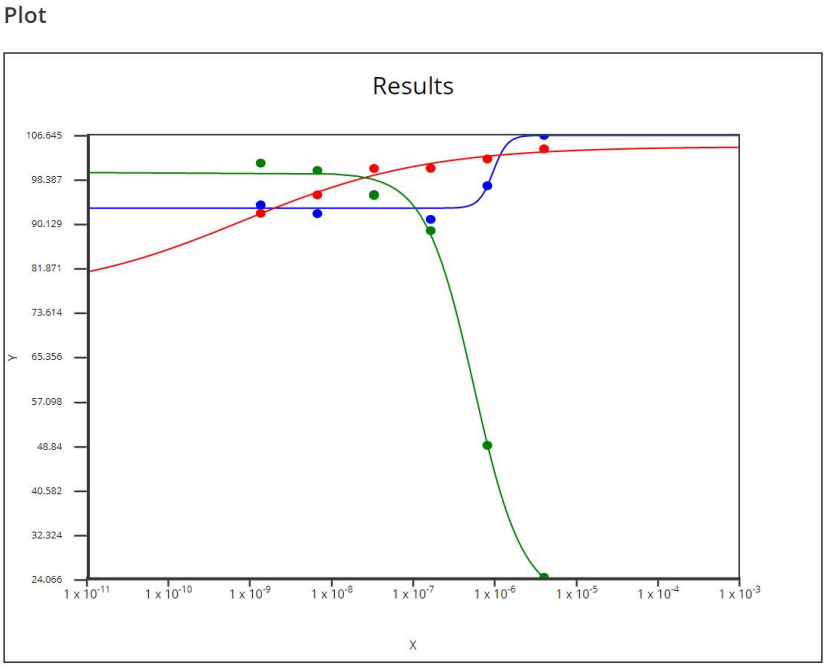
Appendix

Appendix 1. Raw Alphascreen Data (duplicates) Generated in MCF-7

5482	5779	5307	4940	5045	4615	4876	4613
4631	4739	4525	4671	4920	4836	5217	6325
9570	9366	8476	9183	9022	8891	8994	9916
9392	10870	10180	10110	11370	11530	11790	11130
0.836001							

Effect of three inhibitors at multiple concentrations on pAkt level on insulin treatments. Insulin is at 500nM, and inhibitors are ranging from 1.28-4000nM from column 3 to 8. Preincubation with inhibitor is 15 mins. Stimulation is 20 mins. P-value generated from TTEST for column 1 (Veh) and 2 (Veh+Ins) greater than 0.05 and no statistical difference.

Appendix 2. CRC of pAkt in Insulin Treatment in MCF-7 Varying Three Inhibitors at Different Concentrations



Different colours indicate different inhibitors treated into cell: TGX in blue, BYL inred and A66 in green. The curve illustrates potency and efficacy of inhibitors

Appendix 3. EC50 and Emax for Three Inhibitors Found in CRC Curve Increased Efficacy (Emax) from TGX to A66; Increased Potency and Decreased EC50 from TGX to BYL

	EC50	Emax
A66	5.31e-07	78.52
TGX	9.32e-07	13.61
BYL	7.81e-10	27.67